Normal human fibroblasts have been shown to undergo a p16Ink4a-associated senescence-like growth arrest in response to sustained activation of the Ras/Raf/MEK/ERK pathway. We noted a similar p16Ink4a-associated, senescence-like arrest in normal human astrocytes in response to expression of a conditional form of Raf-1. While HPV16 E7-mediated functional inactivation of the p16Ink4a/pRb pathway in astrocytes blocked the p16Ink4a-associated growth arrest in response to activation of Raf-1, it also revealed a second p21\(^{Cip1}\)-associated, senescence-associated, \(\beta\)-galactosidase-independent growth arrest pathway. Importantly, the p21\(^{Cip1}\)-associated pathway was present not only in normal astrocytes but also in p53-, p14\(^{ARF}\), and p16Ink4a/pRb-deficient high grade glioma cells that lacked the p16Ink4a-dependent arrest mechanism. These results suggest that normal human cells have redundant arrest pathways, which can be activated by Raf-1, and that even tumors that have dismantled p16Ink4a-dependent growth arrest pathways are potentially regulated by a second p21\(^{Cip1}\)-dependent growth arrest pathway.

Cell proliferation is dependent on the appropriate transmission of growth signals from the cell membrane to the cell nucleus. Key in regulating this process is the Ras family of proteins. Ras and Ras-related proteins are membrane-bound, receptor-associated GTPases (1, 2). These proteins are activated by ligand-bound growth factor receptors (1, 2) or other engaged pro-proliferation molecules (3) and in turn activate a series of downstream effector proteins including Raf, phosphatidylinositol 3-kinase, and RalGDS (4). While activation of RalGDS and phosphatidylinositol 3-kinase undoubtedly play a role in Ras function, activation of the Raf pathway appears to play a critical role in cellular proliferation (5–7). Raf is recruited to the cellular membrane by GTP-bound activated Ras, which results in Raf activation by a variety of means, including phosphorylation (8). Activated Raf then phosphorylates mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK), which in turn phosphorylates and activates p42/p44 mitogen-activated protein kinases (ERK1 and ERK2) (9). ERK1/2 activation and nuclear translocation in turn leads to phosphorylation of a variety of substrates, including transcription factors, which ultimately contribute to proliferation (2, 10, 11).

The importance of the Ras/Raf/MEK/ERK pathway in growth is further supported by its alteration in many human tumors. Approximately 50% of human colon carcinomas contain mutant, constitutively active Ras (12), an alteration that is believed to contribute to the proliferative signaling necessary for tumor growth. Tumors that typically lack Ras mutations such as brain tumors (gliomas) also have alterations in the Ras/Raf/MEK/ERK pathway. Approximately 30% of high grade human gliomas have an amplified, rearranged, and constitutively activated epidermal growth factor receptor (13, 14), a receptor known to signal through the Ras pathway (15). Additionally, Ras-GTP levels are elevated in a high percentage of high grade human gliomas (16), presumably as a result of signaling from a number of different growth factor receptors. Disregulation of the Ras/Raf/MEK/ERK pathway is therefore a common means by which tumors maintain and amplify mitogenic stimuli and thereby sustain proliferation.

While Ras/Raf/MEK/ERK pathway activation can promote cellular proliferation, it can also paradoxically provoke cell cycle arrest. Sustained activation of oncogenic Ras in normal human fibroblasts (NHF) leads not to proliferation but to permanent arrest in association with elevated levels of p53 and of the cyclin-dependent kinase inhibitors p16Ink4a and p21\(^{Cip1}\) (17). This irreversible arrest is also associated with expression of senescence-associated \(\beta\)-galactosidase (SA-\(\beta\)-gal) activity, suggesting that in response to high levels of activated Ras, normal human cells undergo a senescence-like growth arrest. The ability of oncogenic Ras to induce a senescence-like growth arrest in NHF appears to be closely linked to activation of Raf but not to other downstream targets of Ras, such as phosphatidylinositol 3-kinase or RalGDS, since only mutant forms of Ras that selectively activate the Raf pathway induced growth arrest comparable with that induced by constitutively expressed oncogenic Ras (17). This point is further supported by the finding that sustained activation of a conditional form of Raf-1, or overexpression of activated MEK1, in NHF leads to senescence-like arrest comparable with that induced by Ras (17, 18). Although the mechanism by which Ras or Raf induces a senescence-like arrest in NHF remains to be fully defined, NHF expressing HPV16 E6 and therefore lacking functional p53 (and p53-dependent induction of p21\(^{Cip1}\)) still undergo a p16Ink4a-associated, senescence-like arrest in response to Raf activation (18). This senescence-like, Raf-induced arrest could...
also be blocked by the MEK inhibitor PD98059 (18), further suggesting a link between Raf activation, p16\(^{ink4a}\) induction, and senescence-like arrest. As a whole, these results suggest that the Ras/Raf/MEK/ERK pathway, in addition to playing a key role in cellular proliferation, can also trigger a senescence-like, p16\(^{ink4a}\)-associated growth arrest that prevents proliferation of normal cells.

While normal human cells are programmed to undergo a senescence-like growth arrest in response to excessive stimulation of the Ras/Raf/MEK/ERK pathway, tumor cells could gain a growth advantage by inactivating this tumor-suppressive response. Functional inactivation of the pRb/p16\(^{ink4a}\) pathway is a common feature of many if not most human tumors, including human gliomas (13, 19). Given the role of p16\(^{ink4a}\) in mediating Ras/Raf-induced senescence, it seems likely that most human tumors lack important growth arrest programs linked to excessive Ras/Raf/MEK/ERK pathway activation. The cellular response to Ras/Raf/MEK/ERK activation in the absence of a functional p16\(^{ink4a}\)/pRb pathway, however, remains poorly defined. In NHF rendered unresponsive to p16\(^{ink4a}\), mediated cell cycle arrest by expression of cyclin D1 and a mutant Cdk4, expression of oncogenic Ras resulted in growth arrest and alterations in cell morphology similar to those noted in parental cells (20). It is unclear, however, if the arrest pathway activated in the absence of p16\(^{ink4a}\) was the senescence-like arrest pathway activated in p16\(^{ink4a}\)-proficient cells and, if so, what proteins were involved. Similarly, Raf activation in pRb-deficient small cell lung carcinoma cells resulted in cell cycle arrest, although again it was unclear if this arrest was associated with markers of senescence or involved either p16\(^{ink4a}\) or p21\(^{Cip1}\) (21). To more clearly define Raf-induced growth arrest pathways and, in particular, potential p16\(^{ink4a}\), independent alternate growth arrest pathways, we characterized the response of normal human glial cells to activation of a conditional form of Raf-1 and assessed whether this response was similar in pRb-deficient glioblastoma cells and in gliomas lacking p16\(^{ink4a}\) and/or pRb. The results of these studies show that rather than having a single p16\(^{ink4a}\)-dependent senescence-associated arrest pathway, astrocytes have a second distinct, SA-\(\beta\)-gal-independent, p21\(^{Cip1}\)-associated arrest pathway activated in response to Raf activation. This second pathway is present not only in normal astrocytes but also in glioma cells that have lost the function of several key cell cycle regulatory proteins including p16\(^{ink4a}\), p53, pRb, and p14\(^{ARF}\). These results suggest that normal cells (in this case astrocytes) have both primary and secondary mechanisms that can be used to prevent proliferation in the face of sustained Raf/MEK/ERK signaling and that even highly malignant tumors retain at least one pathway in a functional yet dormant state.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Normal human astrocytes (NHA), derived from fetal tissue, were purchased from Clonetics (Walkersville, MD). NHA were grown in phenol red-free astrocyte medium (Clonetics) containing 5% charcoal dextran-treated, estrogen-free fetal bovine serum (Omega Scientific, Inc., Tarzana, CA).

U251, SF126, U87, and U87 + E6 glioma cell lines were grown in Dubecco’s modified Eagle’s medium containing 20 mM HEPES, penicillin/streptomycin, and 10% charcoal dextran-treated, estrogen-free fetal bovine serum.

**Retrovirus Production and Infection**—To create cells that express conditional Raf-1, Phoenix A cells were transfected using LipofectAMINE (Life Technologies, Inc.) with EGFP::Raf-1::ER retroviral DNA. The pWZLhbla3 EGFP::Raf-1::ER retroviral vector encodes a protein that is a chimera of the catalytic domain of human Raf-1, the hormone binding domain of the human estrogen receptor, and an enhanced version of green fluorescent protein (EGFP). The vector also encodes blasticidin resistance. A similar retroviral vector for the expression of GFP::Raf-1::ER has been described previously (22). Infected cells show little Raf kinase activity until the chimeric protein is activated by the addition of 17β-estradiol (E\(_2\)) or the estrogen analog 4-hydroxytamoxifen (4HT) (23). As a control, cells were infected with pBabeurop3::Raf-1::ER retroviral DNA. The pBabeurop3::Raf-1::ER retroviral vector encodes a protein that is a chimera of a kinase-inactive version of the catalytic domain of Raf-1 and the hormone-binding domain of the estrogen receptor. The vector also encodes for puromycin resistance.

24 and 48 h after transfection of Phoenix A cells, the medium containing the packaged virus was placed on NHA. Infected NHA were selected for resistance to blasticidin S (25 μg/ml for 4–5 days) (ICN, Costa Mesa, CA) or puromycin (1 μg/ml for 4–5 days) (Sigma-Aldrich), and positive colonies were picked and expanded for further studies. To ensure successful infection, relevant expanded populations were analyzed by fluorescence-activated cell sorting to verify expression of EGFP::Raf-1::ER. Pooled populations of infected NHA were used for all experiments, since the limited life span of astrocytes in culture does not allow for development of clonal populations.

To create pRb-deficient cells with conditional activation of Raf-1, NHA first were infected with HPV16 E7 retroviral DNA packaged in Phoenix A cells, as described above. The pLNCX retroviral vector allows for constitutive expression of the HPV E7 protein (pRb inactivation) and neomycin resistance. Cells expressing E7 were selected for resistance to G418 (0.5 mg/ml for 6–7 days) (ICN). Infected cells that survived drug selection were then infected with the EGFP::Raf-1::ER retroviral DNA, as described. Cells were selected for resistance to blasticidin/G418 and were pooled and expanded for further studies. U251, SF126, U87, and U87 + E6 glioma cell lines were infected with EGFP::Raf-1::ER retroviral DNA as described and selected for resistance to blasticidin. U251 also were infected with pBabeurop3::Raf-1::ER retroviral DNA as described and selected for resistance to puromycin. Following blasticidin selection, cells infected with EGFP::Raf-1::ER were sorted using a Becton-Dickinson FACSVantage SE Cell Sorter, and the cells expressing the highest level of EGFP (upper 5% of expressers) were collected and expanded. All experiments were performed using this population of high expressing cells. These cells were periodically reanalyzed by fluorescence-activated cell sorting to ensure maintenance of EGFP expression level.

**Raf Activation and Inactivation**—EGFP::Raf-1::ER was activated by incubating cells for 4 days with either 1 μM E\(_2\) (Sigma-Aldrich) or 100 nM 4HT (Research Biochemicals International, Natick, MA). 1 μM stocks of E\(_2\) and 4HT were made in ethanol. For all studies, both control and E\(_2\) or 4HT-containing media were changed every 48 h. For studies done to assess the reversibility of the Raf-induced growth arrest, cells were incubated for 4 days with 1 μM E\(_2\). Cells then were grown extensively with PBS, grown in control medium for an additional 4 days, and analyzed.

**\(^{3}H\)Thymidine Incorporation**—The extent of proliferation was assessed by monitoring incorporation of [\(^{3}H\)thymidine (PerkinElmer Life Sciences). Cells were seeded into at least quadruplicate in 96-well plates, at a density of 16,000 cells/ml (NHA, NHA/E6, NHA/E7, and SF126 cells) or 12,000 cells/ml (U251 and U87 cells). Cells were grown for 4 days and then incubated with 20 μCi/ml of [\(^{3}H\)thymidine for 16 h. Incorporation was measured with a Tomtec Micro 96-cell plate harvester and a Betaplate 1205 liquid scintillation counter.

**Analysis of Senescence-associated β-Galactosidase Activity**—SA-β-gal activity was determined by the method of Dimri et al. (24). Cells were fixed in 2% formaldehyde, 0.2% glutaraldehyde and incubated with a 1 mg/ml X-gal solution (40 mM citric acid/sodium phosphate buffer (pH 6), 5 mM potassium ferrocyanide, 5 mM ferricyanide, 150 mM NaCl, 2 mM MgCl\(_2\), 1 mg/ml X-gal in dimethylformamide) for 16–24 h. Cells were then rinsed with PBS and methanol and air-dried. The percentage of SA-β-gal-positive cells was assessed by counting the number of blue cells in a ×10 microscopic field and dividing that number by the total number of cells in the field. The percentage of positively stained cells in each plate was the average of four fields per plate.

**Western Blot Analysis**—For Western blot analysis, cells were lysed in 0.5% SDS lysis buffer (100 mM NaCl, 50 mM Tris-Cl, pH 8.1, 5 mM EDTA, pH 8.0, 0.2% Na\(_2\)DODES). Equal amounts of cellular lysates were electrophoresed on 7.5–15% SDS-polyacrylamide gels and transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore). Western blots were probed with antibodies against the estrogen receptor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho-p44/p42 mitogen-activated protein kinase (Cell Signaling Technology, Beverly, MA), p16\(^{ink4a}\) (Oncogene Research Products, Boston, MA), p21\(^{Waf1}\) (Oncogene Research Products), p53 (Santa Cruz Biotechnology), and pRb (Santa Cruz Biotechnology). Anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibod-
ies were used (Santa Cruz Biotechnology), and Western blots were developed with an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech). Equal loading was determined by staining the membrane with Ponceau S (Sigma-Aldrich). Quantitative analysis was done on an AlphaImager 2200, using AlphaEase Software (Alpha Innotech Corp., San Leandro, CA). Autoradiographs are representative of 2–4 experiments.

**RESULTS**

**Raf Activation Results in a Phenotype Resembling Senescence in NHA**—4HT- or E2-mediated activation of Raf in proliferating NHA retrovirally infected with EGFPΔRaf-1:ER (NHA/RafER) led to elevated expression of the chimeric Raf:ER protein. Western blot analysis showed that E2 incubation led to a sustained high level of EGFP ΔRaf-1:ER expression in NHA/RafER cells (Fig. 1a), consistent with increased stability of the Raf-1:ER fusion protein, as reported previously (23). EGFPΔRaf-1:ER induction was noted within 24 h of E2 addition and was sustained for 4 days (Fig. 1a). E2 incubation also led to an increase in phosphorylated ERK1/2 in NHA/RafER cells, but not NHA or NHA containing the kinase-inactive version of Raf-1 (NHA/Raf301:ER), as determined by Western blot analysis using a phosphospecific p42/p44 antibody (Fig. 1a and data not shown). This increase in phosphorylated ERK1/2 is consistent with p42/p44 ERK1/2 pathway stimulation following Raf activation. The level of phosphorylated ERK1/2 remained high throughout the experiment. As shown in Fig. 1a, the addition of 4HT resulted in induction of EGFPΔRaf-1:ER and phosphorylated ERK1/2 similar to that seen with E2.

Activation of EGFPΔRaf-1:ER also led to changes in cell proliferation and morphology. Beginning ~48 h following EGFPΔRaf-1:ER activation, cells became smaller and refractile but remained adherent and viable (determined by trypan blue exclusion, data not shown). [3H]thymidine incorporation was reduced by 90% after 4 days of EGFPΔRaf-1:ER activation (Fig. 1b). Incorporation of [3H]thymidine by uninfected NHA and by NHA/Raf301:ER was not affected by the addition of E2 to the medium (Fig. 1b). Additionally, 4 days after EGFPΔRaf-1:ER activation by the addition of either 4HT or E2, 45–50% of cells stained positively for SA-β-gal, a common marker of senescent cells, while control NHA/RafER cells and parental NHA had fewer than 10% SA-β-gal positive cells (Fig. 1c).

Senescent cells have been shown to have increased expression of p16Ink4a, p21Cip1, and p53 (20, 25, 26), and human fibroblast studies have demonstrated that elevation of p16Ink4a expression is associated with the maintenance of senescence (18, 26). Therefore, we assessed the effect of Raf activation on the expression of these cell cycle regulatory proteins in NHA. As shown in Fig. 1d, incubation of uninfected NHA with E2 had no effect on expression of p16Ink4a, p21Cip1, or p53. Similarly, incubation of NHA/Raf301:ER with 4HT did not affect p16Ink4a expression (data not shown). EGFPΔRaf-1:ER activation in NHA/RafER cells similarly did not significantly increase levels of p21Cip1 expression or p53 expression 4 days following Raf

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**Fig. 1. Analysis of Raf-induced senescence in NHA.** NHA and NHA/RafER were incubated for 24–96 h with E2 or 4HT to activate Raf and then were harvested and analyzed as described under “Experimental Procedures.” a, Western blot analysis of changes in expression of chimeric RafER and phosphorylated ERK1/2 following E2-mediated Raf activation. 4HT incubation was for 96 h. 96* values were derived from cells washed extensively to remove E2 and then grown 4 days in control medium before analysis. b, inhibition of proliferation following Raf activation was assessed by [methyl-3H]thymidine incorporation. All values are the mean ± S.D. of at least three experiments. E2* values were derived from cells washed extensively to remove E2 and then grown 4 days in control medium before analysis. c, expression of the senescence marker SA-β-gal following Raf activation was determined by incubation with a 1 mg/ml X-gal solution. All values are the mean ± S.D. of at least three experiments. E2* values were derived from cells washed extensively to remove E2 and then grown 4 days in control medium before analysis. d, Western blot analysis of changes in protein expression following E2-mediated Raf activation. 96* values were derived as described for a.
Activation of p16 Ink4a expression suggest that activation of Raf and then were harvested and analyzed as described under “Experimental Procedures.” 

Western blot analysis of changes in expression of chimeric EGFP\(\Delta\)Raf-1:ER and phosphorylated ERK1/2 following E2-mediated Raf activation. 

Inhibition of proliferation following Raf activation was assessed by [\(\text{methyl-}\)\(^{3}\text{H}\)]thymidine incorporation. All values are the mean ± S.D. of at least three experiments.

Fig. 2. Analysis of Raf-induced growth arrest in NHA/E7/RafER. NHA/E7 and NHA/E7/RafER were incubated for 4 days with 4HT to activate Raf and then were harvested and analyzed as described under “Experimental Procedures.” 

Western blot analysis of changes in expression of chimeric EGFP\(\Delta\)Raf-1:ER protein and phosphorylated ERK1/2 following Raf activation (Fig. 1d). 

To address whether the Raf-induced growth arrest in NHA/RafER cells was permanent and irreversible, NHA/RafER cells were incubated with E2 for 4 days, washed extensively with PBS to remove E2 from the culture, and grown in control medium for an additional 4 days. E2 was used to activate EGFP\(\Delta\)Raf-1:ER in these experiments, since it is more easily removed from the cells than 4HT. As shown in Fig. 1a, hormone removal resulted in decreases of both EGFP\(\Delta\)Raf-1:ER protein and phosphorylated ERK1/2 levels to those seen in control cells. 

Following E2 removal, however, cells maintained an altered morphology, and thymidine incorporation remained reduced by 90% (Fig. 2a). Additionally, there was no change in the percentage of cells that stained positively for SA-\(\beta\)-gal (Fig. 1b). 

Expression of p16\(^{ink4a}\) was elevated in NHA/E7/RafER cells as compared with NHA/RafER cells (Fig. 2c). Expression of p16\(^{ink4a}\) was induced with E2 in NHA/E7/RafER cells (Fig. 2a). E2-mediated activation of EGFP\(\Delta\)Raf-1:ER led to increased expression of both chimeric EGFP\(\Delta\)Raf-1:ER protein and phosphorylated ERK1/2 (Fig. 2a). 

EGFP\(\Delta\)Raf-1:ER activation also resulted in a 60% inhibition of [\(\text{methyl-}\)\(^{3}\text{H}\)]thymidine incorporation, as compared with control cells (Fig. 2b). EGFP\(\Delta\)Raf-1:ER activation did not, however, alter cell morphology, and after 4 days of 4HT exposure fewer than 5% of cells stained positively for SA-\(\beta\)-gal.

To better understand the difference in Raf-induced arrest in NHA/E7/RafER cells compared with NHA/RafER cells, Western blot analysis was done to assess changes in protein expression following EGFP\(\Delta\)Raf-1:ER activation. 

Expression of p16\(^{ink4a}\) was elevated in NHA/E7/RafER cells compared with NHA/RafER cells (Fig. 2a, lower panel), which is consistent with the destabilization of pRb by the E7 protein (27, 28). Unlike what was noted in NHA/RafER cells with a functional p16\(^{ink4a}\)/pRb pathway, however, there was no further increase in p16\(^{ink4a}\) levels, relative to control cells (average p16\(^{ink4a}\) levels were 65 ± 9% of control values) in the pRb-deficient NHA/E7/RafER cells following Raf activation (Fig. 2c).

There was, however, an increase in p21\(^{cip1}\) expression in NHA/E7/RafER cells following EGFP\(\Delta\)Raf-1:ER activation. This p21\(^{cip1}\) induction was apparent within 48 h (250 ± 43% of control values), persisted at least 4 days (250 ± 16% of control values), and appeared to be independent of p53, the levels of which remained unchanged throughout the experiment (Fig. 2c). These results indicate that activated Raf is able to induce growth arrest in NHA in the absence of a fully functional p16\(^{ink4a}\)/pRb pathway. This arrest, however, is associated with increased p21\(^{cip1}\) expression, is not associated with increased SA-\(\beta\)-gal, and appears to be functionally
different from senescence-like arrest seen in NHA.

Activation of Raf Results in Growth Arrest in High Grade Gliomas—Although NHA have two pathways leading to growth arrest following sustained Raf expression, glial tumors often have genetic defects that might influence their responses to Raf activation. For example, the vast majority of high grade (grade IV) gliomas have defects in the p16Ink4a/pRb pathway (13). To assess whether glioma cells retained cell cycle arrest pathways in response to Raf activation, high grade glioma cells were infected with EGFP\textunderscore D\textunderscore Raf-1:ER, incubated with 4HT or E2 to activate EGFP\textunderscore D\textunderscore Raf-1:ER, and monitored for cellular alterations and activation of cell signaling pathways. While \textsuperscript{3}H\textsuperscript{methyl} thymidine incorporation by U251 cells containing the kinase-inactive form of Raf-1 (U251/Raf301:ER) was not affected by incubation with 4HT (Fig. 3a), \textsuperscript{3}H\textsuperscript{methyl} thymidine incorporation was reduced by 80% in U251/RafER cells, by 90% in SF126/RafER cells, and by 85% in U87/RafER cells following 4 days of incubation with E\textsubscript{2} (Fig. 3a). In contrast to the NHA/RafER cells, however, which became smaller and refractile and SA-\beta-gal-positive following EGFP\textunderscore D\textunderscore Raf-1:ER activation, the tumor cells became elongated with long processes, and SA-\beta-gal activity did not increase following EGFP\textunderscore D\textunderscore Raf-1:ER activation.

The response of these cells was therefore more similar to that of pRb-deficient NHA/E7/RafER cells than that of NHA. Consistent with this idea, all cell lines showed elevated p21Cip1 expression, ranging from 2-fold increase in U87/RafER cells to 8-fold in U251/RafER and SF126/RafER cells, which began 24–48 h after 4HT exposure and persisted throughout the experiment (Fig. 3, b–e). As in the NHA/E7/RafER cells, p21\textsuperscript{cip1} induction appeared to be independent of p53, since 1) U251 and SF126 cells lack functional p53 (29, 30); 2) U87 cells containing functional p53 (30) showed no increase in p53 protein levels following 4 days of 4HT exposure and persisted throughout the experiment (Fig. 3d); and 3) U87 + E\textsubscript{6} cells, which lack functional p53, displayed p21\textsuperscript{cip1} induction and growth arrest similar to that noted in U87 cells (Fig. 3e). p16\textsuperscript{ink4a} did not have
also contained high levels of p16Ink4a. The possibility exists, however, levels of pRb were low but not absent, and these cells associated arrest, and vice versa). In E7-expressing astrocytes, 

suggest that high grade gliomas, which have lost the p16 Ink4a-absence of induced levels of p21Cip1 (18), a finding reinforced by both of these proteins (17, 18). In NHF, however, Raf-induced activation in the glioma cell lines resulted in growth arrest that lasted only as long as Raf was activated. This arrest also was associated with increased expression of p21Cip1, which returned to normal levels with the inactivation of EGFPΔRaf-1:ER. These data suggest that the Raf-induced, p21Cip1-associated arresting growth arrest in these glioma cell lines is reversible and is dissimilar to the senescence seen in NHF in response to Raf activation.

**DISCUSSION**

The data presented in this paper demonstrate the presence of two distinct inducible growth arrest pathways in astrocytic cells. These two pathways are qualitatively different in nature, with the choice of which is used dependent on the presence of a functional p16Ink4a/pRb pathway. In the case of NHA, which have an intact p16Ink4a/pRb pathway, Raf activation results in an irreversible, senescence-like growth arrest associated with increased p16Ink4a expression. However, when this pathway is disrupted, as is the case in the NHA/E7/RafER cells and in the high grade glioma cell lines, Raf activation leads to reversible growth arrest associated with increased p21Cip1 expression.

The finding that normal human cells contain two distinct growth arrest pathways in response to Raf activation is both novel and consistent with previous published work. The ability of cells to undergo arrest in response to exogenous expression of either p16Ink4a or p21Cip1 is widely appreciated (18, 31–33), and Ras or Raf activation in NHF leads to increased expression of both of these proteins (17, 18). In NHF, however, Raf-induced senescence-like growth arrest has been shown to occur in the absence of induced levels of p21Cip1 (18), a finding reinforced by our observation that in NHA, p16Ink4a levels, but not levels of p21Cip1, increase in response to EGFPΔRaf-1:ER activation. The novel finding of the present study is that p21Cip1, whose potential function in Ras- and Raf-induced arrest in NHF was not closely examined, is associated not with senescence-like growth arrest in NHF but rather with a second distinct arrest pathway that is not associated with markers of senescence and whose presence can be noted only in the absence of the primary p16Ink4a-associated arrest pathway. The two growth arrest pathways defined in NHF appear to be mutually exclusive (i.e., functional p16Ink4a/pRb appears to be unnecessary for p21Cip1-associated arrest, and vice versa). In E7-expressing astrocytes, however, levels of pRb were low but not absent, and these cells also contained high levels of p16Ink4a. The possibility exists, therefore, that the presumed p21Cip1-associated response might in fact be simply a muted version of the already described p16Ink4a-associated arrest. Additionally, since high levels of p16Ink4a have been suggested to displace p21Cip1 from cyclin D-Cdk4 complexes and to stimulate formation of growth-inhibitory p21Cip1-cyclin E-Cdk2 complexes (34), the possibility also exists that the presumed p21Cip1-associated response might in fact be directly dependent on high levels of p16Ink4a. The qualitative difference in response to EGFPΔRaf-1:ER activation in E7-expressing cells (p21Cip1-associated arrest in the absence of SA-β-gal expression or morphologic alterations) versus that in NHA (p16Ink4a-associated senescence-like arrest), however, argues against a single p16Ink4a-associated pathway. The presence of a p21Cip1-associated arrest pathway in p16Ink4a null tumor cell lines and the transient as opposed to permanent nature of this arrest further support the contention that two distinct arrest pathways exist in cells of glial origin.

A second important finding of the present work is that even tumors which have dismantled the p16Ink4a-dependent senescence-like growth arrest pathway retain the secondary, p21Cip1-associated growth arrest mechanism. Numerous tumor cell types have been shown to respond to Raf induction by undergoing growth arrest, although in these studies either the tumor cells used were p16Ink4a/pRb-proficient (35, 36) or the arrest did not involve p21Cip1 (21). We also have noted that activation of EGFPΔRaf-1:ER induces a SA-β-gal-associated arrest in p16Ink4a-proficient tumor cells derived from low grade human gliomas (data not shown). It therefore seems likely that p16Ink4a/pRb-proficient tumors retain senescence-like arrest pathways similar to those noted in normal human cells following activation of Raf. The arrest noted in the glioma cell lines used in the present study, however, was clearly different from the p16Ink4a-dependent arrest noted in NHF, since it was reversible, not associated with SA-β-gal expression, and activated even in tumor cells (U87, U251, and SF126) devoid of p16Ink4a. Significantly, the p21Cip1-associated arrest was also noted in tumor cells lacking functional p53 and p14ARF (U251, SF126). Recent studies have shown that Ras or Raf activation can induce expression of both p14ARF and the p53 inhibitor Mdm2 and that the balance of these actions may affect p53 levels and downstream p53-dependent effects (37). The presence of a Raf-induced, p21Cip1-associated growth arrest pathway in p14ARF and p53-deficient cells therefore suggests that the linkage between Raf induction and increased p21Cip1 expression is independent of not only p16Ink4a but also of effects of the Ras/Raf/MEK/ERK pathway on the p14ARF/Mdm2/p53 pathway.

Having established the presence of a secondary p21Cip1-associated, Raf-stimulated arrest pathway in NHF, the question arises as to how Raf induces p21Cip1 expression and how p21Cip1 induces growth arrest in the absence of pRb/p16Ink4a. With regard to the latter question, p21Cip1 has been shown to inhibit the action of E2F in human cervical and bladder carcinoma cell lines that lack functional pRb (38), suggesting that the p21Cip1-associated arrest noted in glialoma cell lines and, in particular, in the pRb/p16Ink4a-deficient SF126 line may similarly involve E2F. Alternatively, p21Cip1 may regulate the activities of cyclin E-Cdk2 and/or cyclin A-Cdk2 complexes (19). With regard to the former question, p53-independent induction of p21Cip1 has been associated with a variety of transcription factors including Sp1, Sp3, STAT proteins, and E2F2s (39–41). Raf-induced increases in any of these transcription factors could therefore lead to p53-independent increases in p21Cip1 levels. These possibilities and, in particular, the interplay between Raf, E2F, and p21Cip1, remain to be examined.

Finally, the identification of two distinct growth arrest pathways in astrocytes and astrocytic tumors may be important both to understanding the genesis of tumors and to effectively stopping their growth. Approximately half of grade III anaplas-
tic astrocytomas have inactivation of at least one component of the p16Ink4a/pRb pathway, and homozygous deletion of p16Ink4a is seen in up to 70% of grade IV glioblastoma multiforme (13). Thus, most glial tumors, by dismantling the p16Ink4a/pRb pathway, have eliminated the possibility of Ras/Raf-mediated p16Ink4a-associated permanent growth arrest. Inactivation of p21Cip1, however, has rarely been reported in gliomas or in other human tumors (13, 19, 42). These observations suggest that tumor development may select for cells with Ras/Raf/MEK/ERK pathway activation high enough to promote growth yet low enough to avoid triggering p21Cip1-dependent growth arrest. Indeed, this appears to be the case in experimental human gliomas created by overexpression of mutant Ras, which, despite having very high levels of activated Ras, have only modest levels of ERK1/2 activation.2 More importantly, these results support the idea that intact arrest pathways lie dormant in tumors (43), including gliomas. Selective activation of these pathways in tumor cells would represent an ideal, noncytotoxic way to prevent the growth and the ultimate fatal consequences of tumors. The definition of at least two distinct growth arrest pathways in cells is a first step toward this goal.

REFERENCES

1. Marshall, C. J. (1996) Curr. Opin. Cell Biol. 8, 197–204
2. Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Oncogene 17, 1395–1413
3. Hanahan, D., and Weinberg, R. A. (2000) Cell 100, 57–70
4. Katz, M. E., and McCormick, F. (1997) Curr. Opin. Genet. Dev. 7, 75–79
5. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Cell 77, 841–852
6. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woede, G. F., and Ahn, N. J. (1994) Science 265, 966–970
7. Stang, S., Bottorf, D., and Stone, J. C. (1997) Mol. Cell. Biol. 17, 3047–3055
8. Marais, R., Light, Y., Paterson, H., and Marshall, C. J. (1995) EMBO J. 14, 101–110
9. Marshall, C. J. (1994) Curr. Opin. Genet. Dev. 4, 82–89
10. Chen, R. H., Surnecki, C., and Blenis, J. (1992) Mol. Cell. Biol. 12, 915–927
11. Gille, H., Kortenjamn, M., Themae, O., Moonaw, C., Slaughter, C., Cobb, M. H., and Shaw, P. E. (1995) EMBO J. 14, 951–962
12. Shirasawa, S., Furuse, M., Yokoyama, N., and Sasaki, T. (1993) Science 260, 85–88
13. Louis, D. N. (1997) Brain Pathol. 7, 755–764
14. Olson, J. J., James, C. D., Krishit, A., Barnett, D., and Hunter, S. (1994) Neuropeuro 36, 740–748
15. Schlesinger, J. (2000) Cell 103, 211–225
16. Guha, A., Feldkamp, M. M., Lau, N., Boss, G., and Pawson, A. (1997) Oncogene 15, 2755–2765
17. Lin, A. W., Marradas, M., Stone, J. C., van Aelst, L., Serrano, M., and Lowe, S. W. (1998) Genes Dev. 12, 3008–3019
18. Zhu, J., Woods, D., McMahon, M., and Bishop, J. M. (1998) Genes Dev. 12, 2907–3007
19. Sherr, C. J. (1996) Science 274, 1672–1677
20. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Cell 88, 503–602
21. Ravi, R. K., Weber, E., McMahon, M., Williams, J. R., Baylin, S., Mal, A., Harter, M. L., Dillehay, L. E., Claudius, P. P., Giordano, A., Nelkin, B. D., and Mahry, M. (1998) J. Clin. Invest. 101, 153–159
22. Woods, D., Parry, D., Cherywinski, H., Bosch, E., Rees, E., and McMahon, M. (1997) Mol. Cell. Biol. 17, 5586–5611
23. Samuels, M. L., Weber, M. J., Bishop, J. M., and McMahon, M. (1993) Mol. Cell. Biol. 13, 6241–6252
24. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linsenm, M., Robelj, I., Pereira-Smith, O., Peacoche, M., and Campisi, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9363–9367
25. Dimri, G. P., Itahana, K., Acosta, M., and Campisi, J. (2000) Mol. Cell. Biol. 20, 273–285
26. Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 17472–17477
27. Khledi, S. N., DeGregori, J., Yee, C. L., Ottersen, G. A., Kaye, F. J., Nevins, J. R., and Howley, P. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4350–4354
28. Martin, L. G., Demers, G. W., and Galloway, D. A. (1998) J. Virol. 72, 975–985
29. Ishii, N., Maier, D., Merlo, A., Tada, M., Sawamura, Y., Diserens, A-C., and Van Meir, E. G. (1999) Brain Pathol. 9, 469–479
30. Costanzo-Strauss, E., Strauss, B. E., Naviaux, R. K., and Haas, M. (1998) Exp. Cell Res. 238, 51–62
31. Uhrbom, L., Nister, M., and Westermark, B. (1995) Oncogene 15, 505–514
32. Yang, Z. Y., Perkins, N. D., Ohno, T., Nabel, E. G., and Nabel, G. J. (1995) Nat. Med. 1, 1052–1056
33. Gorospe, M., Wang, X., Guyton, K. Z., and Holbrook, N. J. (1996) Mol. Cell. Biol. 16, 6654–6660
34. Mitra, J., Dai, C. Y., Somasundaram, K., El-Deiry, W. S., Satyamoorthy, K., Herlyn, M., and Enders, G. H. (1999) Mol. Cell. Biol. 19, 3916–3928
35. Ravi, R. K., McMahon, M., Yangang, Z., Williams, J. R., Dillehay, L. E., Nelkin, B. D., and Mahry, M. (1999) J. Cell. Biochem. 72, 458–469
36. Ravi, R. K., Thiagalingam, A., Weber, E., McMahon, M., Nelkin, B. D., and Mahry, M. (1999) Am. J. Respir. Cell Mol. Biol. 20, 543–549
37. Ries, S., Biedrer, C., Woods, D., Shifman, O., Shirasawa, S., Sasaki, T., McMahon, M., Oren, M., and McCormick, F. (2000) Cell 103, 321–330
38. Dimri, G. P., Nakanishi, M., Desprez, P-Y., Smith, J. R., and Campisi, J. (1996) Mol. Cell. Biol. 16, 2987–2997
39. Garrelt, A. L., and Tynier, A. L. (1999) Exp. Cell Res. 246, 280–289
40. Chin, Y. E., Kitagawa, M., Su, W-C. S., You, Z-H., Iwamoto, Y., and Fu, X-Y. (1996) Science 272, 719–722
41. Garrelt, A. L., Najmabadi, F., Goufman, E., and Tynier, A. L. (2000) Oncogene 19, 961–964
42. Hall, M., and Peters, G. (1996) Adv. Cancer Res. 68, 67–108
43. Weintraub, S. J. (1999) Am. J. Respir. Cell Mol. Biol. 20, 541–542

2 C. P. Fanton, M. McMahon, and R. O. Pieper, unpublished observation.
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