Mutant Epidermal Growth Factor Receptor Signaling Down-Regulates p27 through Activation of the Phosphatidylinositol 3-Kinase/Akt Pathway in Glioblastomas

Yoshihata Narita, Motoo Nagane, Kazuhiko Mishima, H-J. Su Huang, Frank B. Furnari, and Webster K. Cavenee

ABSTRACT

Alterations of the epidermal growth factor receptor (EGFR) gene are common in some forms of cancer and the most frequent is a deletion of exons 2-7. We have previously shown that this mutant receptor, called ΔEGFR, confers enhanced tumorigenicity to glioblastoma cells through elevated proliferation and reduced apoptotic rates of the tumor cells in vivo. To understand the molecular mechanisms that underlie ΔEGFR-enhanced proliferation, we examined the gene products that control cell cycle progression. We found that levels of the cyclin-dependent kinase (CDK) inhibitor, p27, were lower in U87MG/ΔEGFR tumors than in parental U87MG or control U87MG.DK tumors. Consequently, CDK2-cyclin A activity was also elevated, concomitant with the RB protein hyperphosphorylation. In addition, activated phosphatidylinositol 3-kinase (PI3-K) and phosphorylated Akt levels were also elevated in the U87MG/ΔEGFR tumors. U87MG/ΔEGFR cells failed to arrest in G1 in response to serum starvation in vitro and while maintaining high levels of PI3-K activity and hyperphosphorylated RB. Treatment of U87MG/ΔEGFR cells with LY294002, a PI3-K inhibitor, caused reduced levels of phosphorylated Akt and concomitantly up-regulated levels of p27. Expression of a kinase dead dominant-negative Akt mutant in the U87MG/ΔEGFR cells similarly resulted in up-regulation of p27 and down-regulation of tumorigenicity in vivo. These results suggest that the constitutively active ΔEGFR can enhance cell proliferation in part by down-regulation of p27 through activation of the PI3-K/Akt pathway. This pathway may represent another therapeutic target for treatment of those aggressive glioblastomas expressing ΔEGFR.

INTRODUCTION

Patients harboring glioblastoma multiforme have 5-year survival rates of less than 10% (1), despite treatment with combinations of surgery, radiotherapy, and chemotherapy. About 30-40% of glioblastomas have alterations of the EGFR gene (2, 3), the most frequent of which is an in-frame deletion of exons 2-7 that is named ΔEGFR or EGFRvIII (4). This truncated receptor lacks part of the extracellular ligand binding domain and is constitutively activated despite being unable to bind to ligands such as EGF (5, 6). We have previously shown that ΔEGFR enhances the growth of glioblastoma cells as xenografts in immunodeficient mice and that these tumors showed an elevated proliferation rate as indicated by Ki67 staining (3, 7, 8), suggesting accelerated progression from G1 to S phase. This transition requires phosphorylation and activation of CDKs by cyclins. As a counterbalance to this, CDKIs can associate with CDK-cyclin complexes and inhibit their activities (9, 10). Glioblastomas have been shown to express increased levels of cyclin D (11, 12), contain amplification of CDK4/6 genes (13, 14), or have reduced levels of the CDK1, p27 (15-17), suggesting that CDK-cyclin kinase activities are elevated in glioblastomas. However, whether ΔEGFR signaling influences the levels or activities of these molecules involved in G1-S phase transition is not known.

ΔEGFR has been shown to activate PI3-K, one of the signal transducers that plays an important role in tumorogenesis and cell cycle progression (6, 18, 19). PI3-K transduces cellular signals by phosphorylating PIP2 to generate the second messenger PIP3, which leads to the activation of Akt kinase activity (20). Recent studies show that activated and phosphorylated Akt, in turn, phosphorylates and promotes mobilization of the Forkhead transcription factor from the nucleus to the cytoplasm, which reduces the transcription levels of the cell cycle inhibitor p27 (21-24). p27 induces G1 arrest in tumor cells by binding to CDK-cyclin complexes and inhibiting their activities. Therefore PI3-K/Akt signaling can lead to cell cycle progression in tumor cells through the down-regulation of p27.

Here, we present evidence that active ΔEGFR can enhance cell proliferation in vitro and in vivo in various glioblastoma cell lines, by down-regulation of p27, through the activation of the PI3-K/Akt pathway. A direct demonstration of the importance of this cascade arises from data showing that chemical PI3-K inhibitors or dominant-negative KD-Akt mutants reverse ΔEGFR-mediated cell or tumor growth enhancement. These results provide additional insight into the basic mechanisms of tumor cell growth regulation by ΔEGFR and indicate that the PI3-K/Akt pathway may be a target for intervention.

MATERIALS AND METHODS

Cell Culture and Biological Reagents. The human glioblastoma cell lines U87MG, A1207, and LN229 were cultured in DMEM with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in a 95% air/5% CO2 atmosphere at 37°C. U87MG cells, LN229 cells, and A1207 cells, which express ΔEGFR, and U87MG.DK cells that express kinase-defective ΔEGFR, used in this study, were described previously (3). PIP2 and LY294002 (PI3-K inhibitor), were purchased from Sigma Chemical Co. (St. Louis, MO) and Histone H1 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The pcDNA KD-Akt vector expressing KD-Akt was generated from pCMV5-HA-Akt(K179M), which was a gift of Dr. Philip Tsichlis (Thomas Jefferson Medical College, Philadelphia, PA; Ref. 25). To generate KD-AKT-U87MG/ΔEGFR cells, U87MG/ΔEGFR cells were transfected with pcDNA KD-Akt by the calcium phosphate method (26) and were selected under 100 µg/ml Zeocin and 10% serum. Several stable transfectants were obtained.

Tumors in Nude Mice. Cells (5 x 10^6) of each cell line in 5 µl PBS were implanted into the right corpus striatum of 4-6-week-old female nude BALB/c mice (Simonsen Laboratories, Gilroy, CA) as described previously (3). Mice were killed on the development of neurological symptoms. For s.c. tumors, 5 x 10^6 cells of the various cell lines in 0.1 ml of PBS were selected under 100 µg/ml Zeocin and 10% serum. Several stable transfectants were obtained.

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immunocomplexes were washed three times with PI3-K lysis buffer, twice with PI3-K lysis buffer, and once with PI3-K lysis buffer. PhosphorImager quantitation of the PIP3 spot was compared with that in (Cell Signaling, Beverly, MA); phosphorylated Akt (Cell Signaling); phospho-unphosphorylated RB (PharMingen); phosphorylated RB (PharMingen); Akt (PharMingen); cyclin D3 (Neo Markers); cyclin E (Santa Cruz Biotechnology); CDK6 (Santa Cruz Biotechnology); CDK2 (Santa Cruz Biotechnology); cyclin D1 (Neo Markers, Fremont, CA); cyclin D2 (Neo Markers, Fremont, CA); CDK6 (Santa Cruz Biotechnology); p21 (PharMingen, San Diego, CA); p27 (BD Biosciences, San Jose, CA); cyclin A (Santa Cruz Biotechnology); cyclin D1 (Neo Markers, Fremont, CA); cyclin D2 (Neo Markers); cyclin D3 (Neo Markers); cyclin E (Santa Cruz Biotechnology); unphosphorylated RB (PharMingen); phosphorylated RB (PharMingen); Akt (Cell Signaling, Beverly, MA); phosphorylated Akt (Cell Signaling); phospho-tyrosine 4G10 (UBL, Waltham, MA); P13-K p85 (UBL); β-actin (Sigma).

Western blotting. Tissue specimens of intracranial xenografts were snap-frozen in liquid nitrogen immediately after surgical resection and stored at −80 °C until use. Western blotting was performed as described previously (7). Each protein sample (20 µg) was separated with appropriate polyacrylamide/SDS gels and electroblotted onto nitrocellulose membranes (ECL membrane; Amersham Pharmacia Biotech). Primary antibodies used in this study were CDK2 (Santa Cruz Biotechnology, Santa Cruz, CA); CDK4 (Santa Cruz Biotechnology); CDK6 (Santa Cruz Biotechnology); p21 (PharMingen, San Diego, CA); p27 (BD Biosciences, San Jose, CA); cyclin A (Santa Cruz Biotechnology); cyclin D1 (Neo Markers, Fremont, CA); cyclin D2 (Neo Markers); cyclin D3 (Neo Markers); cyclin E (Santa Cruz Biotechnology); unphosphorylated RB (PharMingen); phosphorylated RB (PharMingen); Akt (Cell Signaling, Beverly, MA); phosphorylated Akt (Cell Signaling); phospho-tyrosine 4G10 (UBL, Waltham, MA); P13-K p85 (UBL); β-actin (Sigma). Western blotting for each antibody was performed more than three times, and representative blots are shown. β-actin was used as loading control for each result (data not shown). Quantitation was performed using laser scanning densitometry of the autoradiograms.

CDK2-, CDK4- or CDK6-associated Kinase Assays. CDK2-, CDK4- or CDK6-associated kinase activity assays were performed as described previously (27).

Briefly, CDK2-cyclin A/E, CDK2-cyclin A and CDK2-cyclin E kinase activities were assayed by incubating Histone H1 and [γ-32P]ATP with 200 µm immunocomplexes of CDK2, cyclin A and cyclin E, respectively. CDK4-cyclin D, CDK6-cyclin D, and cyclin D1-CDK6/¡6 kinase activities were also examined by incubating GST-RB protein and [γ-32P]ATP with immunocomplexes of CDK4, CDK6, and cyclin D1, respectively. This RB fusion protein was generated in Escherichia coli (27). Relative kinase activity signals were determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

P13-K Assay. P13-K assays were performed as described previously (6). Cells or frozen tissue specimens were lysed in P3-K lysis buffer [137 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM CaCl2, 1 mM MgCl2, 1% (v/v) NP40, 0.1 mM Na3VO4, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 100 µg/ml phenylmethylsulfonyl fluoride]. Lysates (500 µg) were incubated with anti-p85 antibody and protein G/protein A-agarose for 4 h at 4 °C with rotation. Immunocomplexes were washed three times with PI3-K lysis buffer, twice with PI3-K washing buffer [0.5 M LiCl in 100 mM, Tris-HCl (pH 7.5), and 100 µM Na3VO4], and twice with reaction buffer [25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6.25 mM MgCl2, and 0.625 mM EDTA]. The beads were resuspended in 40 µl of reaction buffer containing 2 µg PIP2 and 30 µCi [γ-32P]ATP and then were incubated at room temperature for 10 min. The reaction was terminated by the addition of 80 µl of 1 M HCl. Phospholipids were extracted with 160 µl of CHCl3/CH3OH (1:1), and the CHCl3 layer was saved. The lipids were chromatographed on thin-layer chromatography plates (proceded with potassium oxalate and baked at 100°C for 1 h) in CHCl3/CH3OH:2.5:5 NIH-3T3 (9:2, v/v/v). P13-K activity was determined by PhosphorImager quantitation of the PIP3 spot and was compared with that in U87MG cells cultured under 10% serum.

RESULTS

ΔEGFR Increases the Proliferation of Glioblastoma Cells. We initially examined the effect of the introduction of ΔEGFR in various human glioma cell lines in vitro and in vivo. Mice bearing U87MG/ΔEGFR, A1207/ΔEGFR, or LNZ308/ΔEGFR cells showed symptoms such as hemiparesis or body weight loss 3–5 weeks after the inoculation. To compare tumor sizes between parental cells and their ΔEGFR transfectants, mice bearing U87MG and U87MG/ΔEGFR cells, were killed 3, 4, or 5 weeks after the injection of tumor cells, respectively. ΔEGFR enhanced tumor formation by all of the cell lines (Table 1). The tumor volumes of ΔEGFR-expressing U87MG, A1207, and LNZ308 were about 80, 5, and 40 times larger, respectively, than those of the parental cell lines (Table 1).

To assess tumor cell proliferation, the Ki-67(MIB) staining index was determined for each tumor sample. The Ki-67 indices of U87MG/ΔEGFR cells, A1207/ΔEGFR cells, and LNZ308/ΔEGFR were 46.6, 46.5, and 56.3%, respectively, values that were approximately 2-fold greater than those of parental cell lines (Table 1). Because growth enhancement and proliferation were similarly affected in each of the three cell types by ΔEGFR, we concentrated further on the U87MG series.

To determine whether cell cycle profiles differed between parental cells and ΔEGFR transfectants, the DNA content in U87MG and U87MG/ΔEGFR cells cultured with and without serum was compared. No obvious differences between cells cultured with 10% serum were detected, whereas, compared with parental U87MG cells, U87MG/ΔEGFR cells arrested to a much lesser extent after serum starvation (Fig. 1).

To elucidate the molecular mechanisms that underlie ΔEGFR-enhanced proliferation, we examined the gene products that control cell cycle progression in vitro and in vivo (Fig. 2). There was no obvious difference in the expression levels of CDK2, CDK4, CDK6, cyclin D1, cyclin D3, cyclin E, and p21 when examined in cell lines cultured with or without serum (cyclin D2 was not detected in U87MG cells). Lower levels of p27 (0.5-fold) and slightly elevated cyclin A levels were detected in U87MG/ΔEGFR cells compared with parental U87MG cells only under serum starvation conditions. Similarly, the expression levels of p27 were 0.5-fold lower and the levels of cyclin A, D1, and D3 and of CDK2 were more than 2-fold higher in U87MG/ΔEGFR- than in parental U87MG- or U87MG-DK- generated tumors. There was no obvious difference in the expression levels of the other cell cycle-related proteins among these tumors.

U87MG/ΔEGFR Cells Have Higher CDK2-Cyclin A Activity and Maintenance of Hyperphosphorylated RB Proteins Than Parental U87MG Cells under Serum Starvation Conditions. Because p27 binds to CDK2-cyclin A/E complexes and suppresses their activities, we examined these kinase activities by incubating Histone H1 with immunocomplexes of CDK2. CDK2-cyclin A/E kinase activities in U87MG/ΔEGFR were selectively 2-fold higher than in parental cell lines under serum starvation conditions (Fig. 3A). Consistent with this higher kinase activity, the RB protein in U87MG/ΔEGFR cells was hyperphosphorylated even under serum starvation conditions. When CDK2-cyclin A and CDK2-cyclin E kinase activities were measured separately, only CDK2-cyclin A kinase activity was up-regulated in vitro (Fig. 3A). There was no obvious difference in CDK4-cyclin D, CDK6-cyclin D, and cyclin D1-CDK4/¡6 kinase activities between cell lines.

Similar results were observed in vivo tumors because of the lower expression of the other cell cycle-related proteins among these tumors.

Table 1. Tumor volume and Ki-67 index in intracranial brain tumors of nude mice from parental and ΔEGFR engineered cell lines

<table>
<thead>
<tr>
<th>Tumor volume (mm³)</th>
<th>Ki-67 index</th>
</tr>
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<tbody>
<tr>
<td>U87MG</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>U87MG/ΔEGFR</td>
<td>193.9 ± 15.9</td>
</tr>
<tr>
<td>A1207</td>
<td>45.1 ± 4.2</td>
</tr>
<tr>
<td>A1207/ΔEGFR</td>
<td>246.6 ± 23.1</td>
</tr>
<tr>
<td>LNZ308</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>LNZ308/ΔEGFR</td>
<td>181.7 ± 15.0</td>
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Note: Data are the mean ± SEM of three independent experiments.
Inhibition of PI3-K or Akt in U87MG.ΔEGFR Cells Induces G1 Arrest and Up-Regulation of p27. To test whether activation of PI3-K and phosphorylation of Akt influences the expression of p27, U87MG.ΔEGFR cells were cultured for 24 h without serum and then treated with 20 μM LY294002, a PI3-K inhibitor. This treatment caused reduced levels of phosphorylated Akt and also 3-fold up-regulated levels of p27 (Fig. 5A). In a similar fashion, overexpression of the dominant-negative KD-Akt mutant in U87MG.ΔEGFR cells also produced 2-fold increased levels of p27 (Fig. 5B) and a G1 arrest (Fig. 5C), when compared with vector control (pcDNA) transfected cells. Exogenous expression of p27 in U87MG.ΔEGFR cells led to a cell cycle block similar to that of KD-Akt (Fig. 5C), which indicated that p27 levels increased by least 2-fold are sufficient for the G1 arrest.

Inhibition of Akt Causes Decreased Tumorigenicity of U87MG.ΔEGFR Cells in Vivo. Because U87MG.ΔEGFR tumors enhance tumorigenicity in vivo and maintain high PI3-K and phosphorylation of Akt, thus leading to low expression levels of p27, we examined whether Akt inhibition, by exogenous expression of KD-Akt, inhibits their tumorigenicity. U87MG.ΔEGFR cells were transfected with pcDNA KD-Akt, and several stable transfectants were obtained. There was no obvious difference in cell growth among U87MG.ΔEGFR, U87MG.ΔEGFR-KD-Akt 1, and U87MG.ΔEGFR-KD-Akt 2 cells under 10% serum conditions in vitro. When these transfectants were injected s.c. into nude mice, the KD-Akt-transfected clones produced substantially smaller tumors when compared with U87MG.ΔEGFR cells and, like our in vitro results, expressed up-regulated p27 (Fig. 6). These results indicate that ΔEGFR-mediated down-regulation of p27 expression, through the PI3-K/Akt pathway, plays a direct role in the enhanced tumor growth of U87MG.ΔEGFR cells.

**Discussion**

We have demonstrated that expression of ΔEGFR can increase the proliferation of various glioma cell lines in vivo. The volume of brain tumors obtained from each ΔEGFR transfectant was more than five times greater than that of parental cells, mirrored by a higher Ki-67 labeled Ki-67 immunostaining (Fig. 2A). This suggests that ΔEGFR enhances cell proliferation in vivo, even when expressed in tumors derived from wild-type EGFR-expressing cells. This effect may be due to increased activation of PI3-K and downstream signaling pathways that enhance cell proliferation, such as the Akt/mTOR pathway. Further studies are needed to identify the specific mechanisms by which ΔEGFR enhances cell proliferation in vivo.

**Figure 1.** ΔEGFR enhances cell proliferation in vivo. U87MG and U87MG.ΔEGFR cells were cultured in 10% serum or serum starvation conditions for 48 h. Their nuclei were stained with propidium iodide, and cell cycle analysis was performed by flow cytometry.

**Figure 2.** Expression of cell cycle-related proteins in U87MG and U87MG.ΔEGFR cells in vitro and in vivo. U87MG and U87MG.ΔEGFR cells were cultured in 10% serum or serum starvation conditions for 48 h, and then cell cycle-related proteins were analyzed by immunoblotting. In a similar fashion, tumors derived from U87MG, U87MG.DK, or U87MG.ΔEGFR cells were examined. Down-regulation of p27 and up-regulation of cyclin A levels in cultured U87MG.ΔEGFR cells under serum starvation conditions. Levels of p27 (arrow) were lower, and the levels of cyclin A and D1 were higher, in U87MG.ΔEGFR than in parental U87MG- or U87MG.DK-generated brain tumors. Triangle indicates immunoglobulin light chain, which originates from mouse tissue. There was no obvious difference in the expression levels of other proteins among these tumors.

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proliferation index. There was no obvious difference in cell growth in vitro between parental U87MG cells and U87MG.H9004 EGFR cells under normal serum conditions. However, parental U87MG cells underwent a G1 arrest in serum starvation conditions, whereas U87MG.H9004 EGFR cells were not completely arrested in identical conditions. To elucidate the mechanism by which H9004 EGFR signaling increased proliferation in vivo and in vitro under serum starvation conditions, we initially compared the expression levels of cell cycle-related proteins and found that H9004 EGFR-expressing cells maintained low expression levels of p27 and high levels of cyclin A. Consistent with these results, CDK2-cyclin A kinase activity was higher in U87MG.H9004 cells in vitro without serum than the activity in parental U87MG cells. Tumors derived from U87MG.H9004 cells also showed higher CDK2-cyclin A kinase activity than did parental tumors, which led to hyperphosphorylation of the RB protein and to cell cycle progression. Our study showed that high levels of PI3-K activity and phosphorylated Akt are found in U87MG.H9004 cells in vivo and in vitro even under serum starvation conditions. All of the results illustrating the expression levels and kinase activities of cell cycle-related proteins in cultured cells in serum starvation conditions are very similar to the levels and activities in intracranial brain tumors. This suggests that some of the H9004 EGFR-signaling phenotype mediated in vivo can be mimicked by serum starvation conditions in vitro.

p27 is a member of the CDKIs, which also includes p21WAF1 and p57KIP1. These molecules interact with cyclin A, E, and D, which bind to CDK2, 4 and 6. CDK-cyclin complexes mediate the G1-S phase transition by phosphorylating the RB protein, which in turn leads to the activation of the E2F family of transcription factors. We demon-

Fig. 3. CDK-cyclin Kinase activities and phosphorylation of the RB protein. In A, CDK2 and CDK4/CDK6 kinase activities were measured using Histone H1 or GST-RB protein as substrates, respectively. CDK2-cyclin A/E kinase activities were higher in U87MG.H9004 cells than in parental U87MG cells under serum starvation conditions. CDK2-cyclin A and CDK2-cyclin E kinase activities, obtained from immunocomplexes of cyclin A or cyclin E, were also measured, and only CDK2-cyclin A kinase activity was maintained in U87MG.H9004 cells. Consistent with high CDK2-cyclin A kinase activity, the RB protein was hyperphosphorylated even under serum starvation conditions in U87MG.H9004 cells. There was no obvious difference in CDK4-cyclin D, CDK6-cyclin D, and cyclin D1-CDK4/6 kinase activities among these cells with or without serum. In B, similarly, U87MG.H9004 tumors showed higher activity of CDK2-cyclin A and hyperphosphorylated RB proteins, compared with parental U87MG and U87MG.DK tumors. CDK4-cyclin D and CDK6-cyclin D kinase activities were not altered among these tumors.

Fig. 4. PI3-K activity and phosphorylation of Akt. PI3-K activity was obtained from anti-p85 immunoprecipitates. 32P-phosphorylated PIP3 product was analyzed using thin-layer chromatography method as described in “Materials and Methods.” Relative PI3-K activity, compared with that in U87MG cells cultured under 10% serum, was determined by PhosphorImager quantitation of PIP3 spots. Whereas PI3-K activity in parental U87MG was decreased to 75% when cells were cultured without serum, it was highly maintained in U87MG.H9004 cells. Levels of total Akt and phosphorylated Akt were compared in each cell line by immunoblotting. Phosphorylation of Akt was not altered in U87MG.H9004 cells even under serum starvation conditions in vitro. Activated PI3-K and phosphorylated Akt levels were also determined for U87MG, U87MG.DK, and U87MG.H9004-generated tumors.

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strated that parental U87MG cells underwent a G₁ cell cycle arrest under serum starvation conditions mediated by the up-regulation of p27, which resulted in a decrease in CDK2-cyclin A kinase activity, and hypophosphorylation of the RB protein. CDK4-cyclin D and CDK6-cyclin D kinase activities were not altered under serum starvation conditions, presumably because of U87MG being mutated for p16 (28), which mainly binds CDK4-cyclin D and CDK6-cyclin D complexes and inhibits their activities. Because the higher activities of CDK4-cyclin D and CDK6-cyclin D were maintained even under serum starvation conditions, the expression levels of p27, followed by the alteration of CDK2-cyclin A kinase activity, appear to play an important role in regulating cell cycle progression from G₁ into S phase in these glioblastoma cells. p27 has been shown to be a bona fide tumor suppressor gene because p27/H11002 mice develop pituitary tumors spontaneously (29), similar to RB mutant mice (30). Mutations of p27 are rare in glioblastomas (15); however, the expression of p27 is reduced in high-grade gliomas and is inversely related to the prognosis of the patients (15–17).

We also demonstrated that treatment with LY294002 caused reduced levels of phosphorylated Akt and concomitantly up-regulated levels of p27, and that overexpression of KD-Akt in the U87MG.ΔEGFR cells restored p27 levels similar to parental U87MG cells, which resulted in a G₁ arrest. In contrast to U87MG.ΔEGFR cells, p27 is up-regulated in U87MG.wt EGFR cells under serum starvation conditions similar to U87MG parental cells, but it is also decreased after stimulation with EGF (data not shown). However, pretreatment with LY294002 can completely block phosphorylation of Akt and decrease p27 even after stimulation with EGF in U87MG.wt EGFR cells (data not shown). LY294002 also completely inhibits the phosphorylation of Akt and increases p27 levels in U87MG, DK, and ΔEGFR cell lines (data not shown). These results suggest that the activation of PI3-K, followed by phosphorylation of Akt, regulates the expression levels of p27 in glioblastoma cells. It has been reported that Akt phosphorylates and inactivates the Forkhead transcription factors, which mediate the transcription of p27 (21–24). Therefore the activation of the ΔEGFR/PI3-K/Akt pathway inhibits the expression levels of p27, which contributes to enhanced proliferation of the tumor cells.

Thirty to 40% of glioblastomas have a mutated PTEN gene (31, 32), which can inhibit PI3-K/Akt signaling by dephosphorylating PIP3. Introduction of PTEN in glioblastoma cells mediates a G₁ cell cycle arrest by up-regulating p27 through the inhibition of the PI3-K/Akt pathway (33, 34). About 60% of glioblastomas have amplification of EGFR or mutated PTEN genes (32), and, thus, the PI3-K/Akt pathway is activated in many, if not most, glioblastomas. Because Akt is phosphorylated in most glioblastomas (35) and the PI3-K/Akt pathway plays a pivotal role in tumor proliferation in glioblastomas (35, 36), other mechanisms for activating the PI3-K/Akt pathway, in addition to amplification or rearrangement of the EGFR gene or...
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