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Mutant Epidermal Growth Factor Receptor Signaling Down-Regulates p27 through Activation of the Phosphatidylinositol 3-Kinase/Akt Pathway in Glioblastomas[1]

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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 G_1 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*[3] 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 Ki-67 staining (3, 7, 8), suggesting accelerated progression from G_1 into 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 CDKI, 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 G_1-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 tumorigenesis 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 G_1 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 LNZ308 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% CO_2 atmosphere at 37°C. U87MG cells, LNZ308 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 regenerated 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 (5×10^5) 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, 1×10^6 cells of the various cell lines in 0.1 ml of PBS were injected into the right flanks of nude mice. Tumor volumes were defined as (longest diameter) × (shortest diameter)^2 × 0.5. All of the procedures were approved by the animal care and use committee of the University of California, San Diego.

Flow Cytometric Analysis. U87MG.ΔEGFR cells were transiently transfected with control pcDNA, pcDNA KD-Akt, or pcDNA p27, cultured for 48 h under 2% serum, and harvested by trypsinization. Nuclei of transfected or nontransfected cells under various conditions were stained with propidium

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[3] The abbreviations used are: EGFR, EGF receptor; EGF, epidermal growth factor; PI3-K, phosphatidylinositol 3-kinase; CDK, cyclin-dependent kinase; PIP2, phosphatidylinositol biphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; CDKI, CDK inhibitor; KD-Akt, kinase dead Akt; GST, glutathione *S*-transferase.

iodide as described previously (26). A total of 20,000 nuclei were analyzed in a FACS Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) and DNA histograms were obtained and analyzed using Modfit-software (Verity, Topsham, ME).

Immunohistochemistry. An assessment of tumor cell proliferation was performed by Ki-67 immunohistochemistry on formalin-fixed paraffin-embedded tumor tissues as described previously (7). The Ki-67(MIB-1) staining index was determined as the percentage of Ki-67-labeled nuclei in the total number of nuclei in a high-power field (×400). Approximately 2000 nuclei were counted in each case by a process of systematic random sampling.

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); phosphotyrosine 4G10 (UBI, Waltham, MA); PI3-K p85 (UBI); 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 autoradiogram.

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-^{32}P]ATP with 200 μg immunocomplexes of CDK2, cyclin A and cyclin E, respectively. CDK4-cyclin D, CDK6-cyclin D, and cyclin D1-CDK4/6 kinase activities were also examined by incubating GST-RB protein and [γ-^{32}P]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).

PI3-K Assay. PI3-K assays were performed as described previously (6). Cells or frozen tissue specimens were lysed in PI3-K lysis buffer [137 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% (v/v) NP40, 0.1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride]. Lysate (500 μg) was incubated with anti-p85 antibody and protein G/protein A-Sepharose 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 Na_3VO_4], and twice with reaction buffer [25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6.25 mM $MgCl_2$, and 0.625 mM EDTA]. The beads were resuspended in 40 μl of reaction buffer containing 2 μg PIP2 and 30 μCi [γ-^{32}P]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 $CHCl_3$:CH_3OH (1:1), and the $CHCl_3$ layer was saved. The lipids were chromatographed on thin-layer chromatography plates (precoated with potassium oxalate and baked at 100°C for 1 h) in $CHCl_3$:CH_3OH:2.5 M NH_4OH (9:7:2, v/v/v). PI3-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, A1207, and A1207.ΔEGFR cells or LNZ308 and LNZ308.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 cells (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).

U87MG.ΔEGFR Cells Have Lower Expression Levels of p27 Than Parental U87MG Cells *in Vitro* and *in Vivo*. To understand 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 p27were 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 cells under serum starvation conditions (Fig. 3*A*). 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. 3*A*). 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 with *in vivo* tumors because

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

	Tumor volume (mm^3)	Ki-67 index
U87MG	2.5 ± 0.4	22.3 ± 2.5
U87MG.ΔEGFR	193.9 ± 15.9	46.6 ± 4.5
A1207	45.1 ± 4.2	26.3 ± 4.6
A1207.ΔEGFR	246.6 ± 23.1	46.5 ± 3.9
LNZ308	4.4 ± 0.8	26.8 ± 5.5
LNZ308.ΔEGFR	181.7 ± 15.0	56.3 ± 7.0

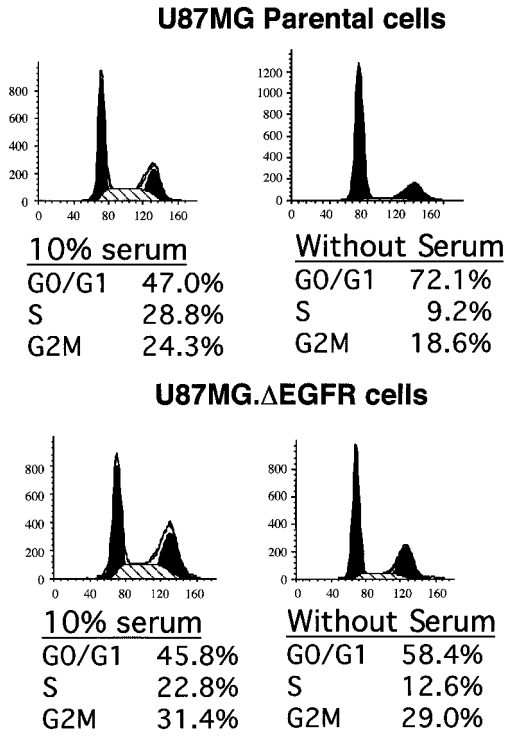

Fig. 1. ΔEGFR enhances cell proliferation *in vitro*. U87MG and U87MG.ΔEGFR cells were cultured in 10% serum or serum starvation conditions for 48 h. Their nuclei were stained with propidium iodide, and cell cycle analysis was performed by flow cytometry.

U87MG.ΔEGFR tumors showed a 3-fold higher activity of CDK2-cyclin A and increased hyperphosphorylated RB protein (Fig. 3*B*) when compared with parental U87MG and U87MG.DK tumors. CDK4-cyclin D, CDK6-cyclin D kinase activities were not altered among these tumors.

U87MG.ΔEGFR Cells Have More PI3-K Activity and Maintain Phosphorylation of Akt in Contrast with Parental U87MG Cells *in Vitro* and *in Vivo*. Because wild-type EGFR signaling leads to PI3-K activation and subsequent Akt phosphorylation, we examined these parameters in U87MG and U87MG.ΔEGFR cells. PI3-K activity was decreased along with phosphorylated Akt levels in parental U87MG cells, grown under serum starvation conditions (Fig. 4). In contrast, these levels were unaltered under identical growth conditions for U87MG.ΔEGFR cells *in vitro*. U87MG.ΔEGFR tumors expressed similarly greater levels of activated PI3-K and phosphorylated Akt *in vivo* (Fig. 4).

Inhibition of PI3-K or Akt in U87MG.ΔEGFR Cells Induces G_1 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. 5*A*). 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. 5*B*) and a G_1 arrest (Fig. 5*C*), 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. 5*C*), which indicated that p27 levels increased by least 2-fold are sufficient for the G_1 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 activity 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

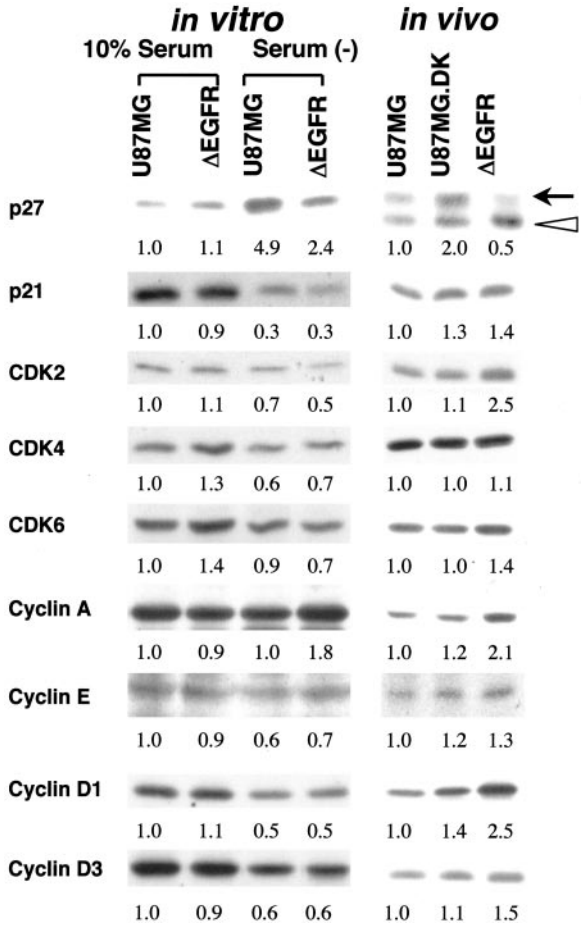

Fig. 2. Expression of cell cycle-related proteins in U87MG and U87MG.ΔEGFR cells *in vitro* and *in vivo*. U87MG and U87MG.ΔEGFR cells were cultured in 10% serum or serum starvation conditions for 48 h, and then cell cycle-related proteins were analyzed by immunoblotting. In a similar fashion, tumors 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. Below each panel, relative ratios to U87MG cells under 10% serum or parental U87MG-generated brain tumors measured by densitometry.

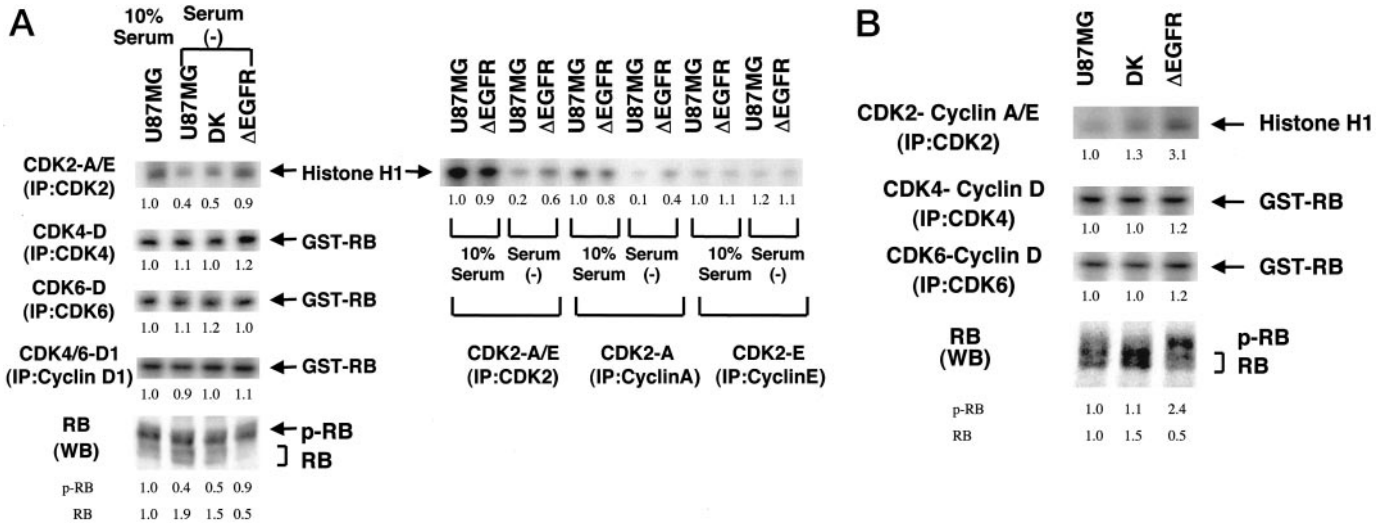

Fig. 3. CDK-cyclin Kinase activities and phosphorylation of the RB protein. In *A*, CDK2 and CDK4/CDK6 kinase activities were measured using Histone H1 or GST-RB protein as substrates, respectively. CDK2-cyclin A/E kinase activities were higher in U87MG.ΔEGFR cells than in parental U87MG cells under 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.ΔEGFR cells. Consistent with high CDK2-cyclin A kinase activity, the RB protein was hyperphosphorylated even under serum starvation conditions in U87MG.ΔEGFR 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.ΔEGFR 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.

proliferation index. There was no obvious difference in cell growth *in vitro* between parental U87MG cells and U87MG.ΔEGFR cells under normal serum conditions. However, parental U87MG cells underwent a G_1 arrest in serum starvation conditions, whereas U87MG.ΔEGFR cells were not completely arrested in identical conditions. To elucidate the mechanism by which ΔEGFR 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 ΔEGFR-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.ΔEGFR cells *in vitro* without serum than the activity in parental U87MG cells. Tumors derived from U87MG.ΔEGFR 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.ΔEGFR 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 ΔEGFR-signaling phenotype mediated *in vivo* can be mimicked by serum starvation conditions *in vitro*.

p27 is a member of the CDKIs, which also includes $p21^{WAF1}$ and $p57^{KIP1}$. These molecules interact with cyclin A, E, and D, which bind to CDK 2, 4 and 6. CDK-cyclin complexes mediate the G_1-S phase transition by phosphorylating the RB protein, which in turn leads to the activation of the E2F family of transcription factors. We demon-

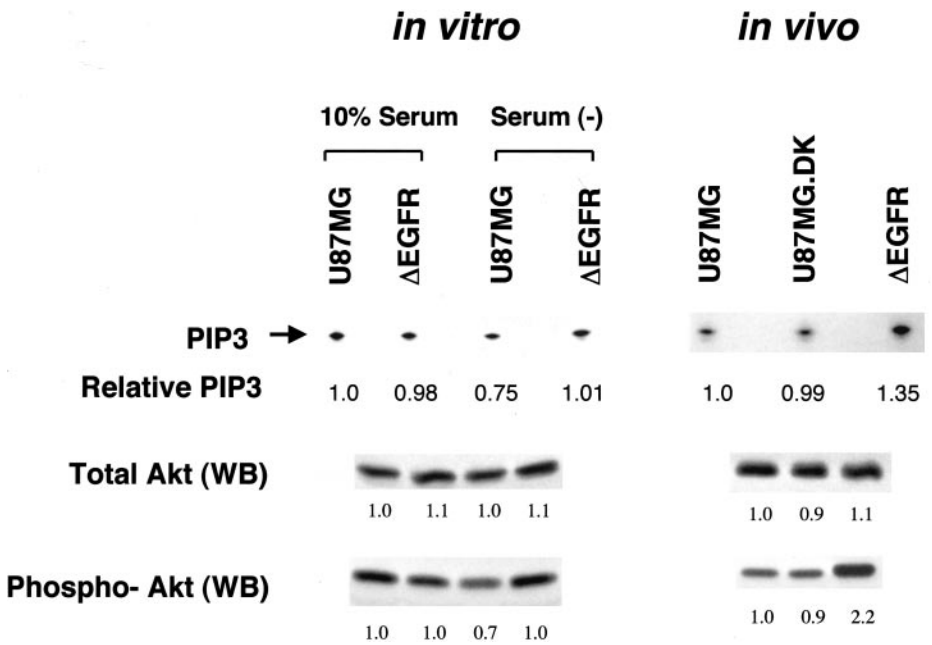

Fig. 4. PI3-K activity and phosphorylation of Akt. PI3-K activity was obtained from anti-p85 immunoprecipitates. ^{32}P-phosphorylated PIP3 product was analyzed using thin-layer chromatography method as described in "Materials and Methods." Relative PI3-K activity, compared with that in U87MG cells cultured 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.ΔEGFR cells. Levels of total Akt and phosphorylated Akt were compared in each cell line by immunoblotting. Phosphorylation of Akt was not altered in U87MG.ΔEGFR cells even under serum starvation conditions *in vitro*. Activated PI3-K and phosphorylated Akt levels were also determined for U87MG-, U87MG DK-, and U87MG.ΔEGFR-generated tumors.

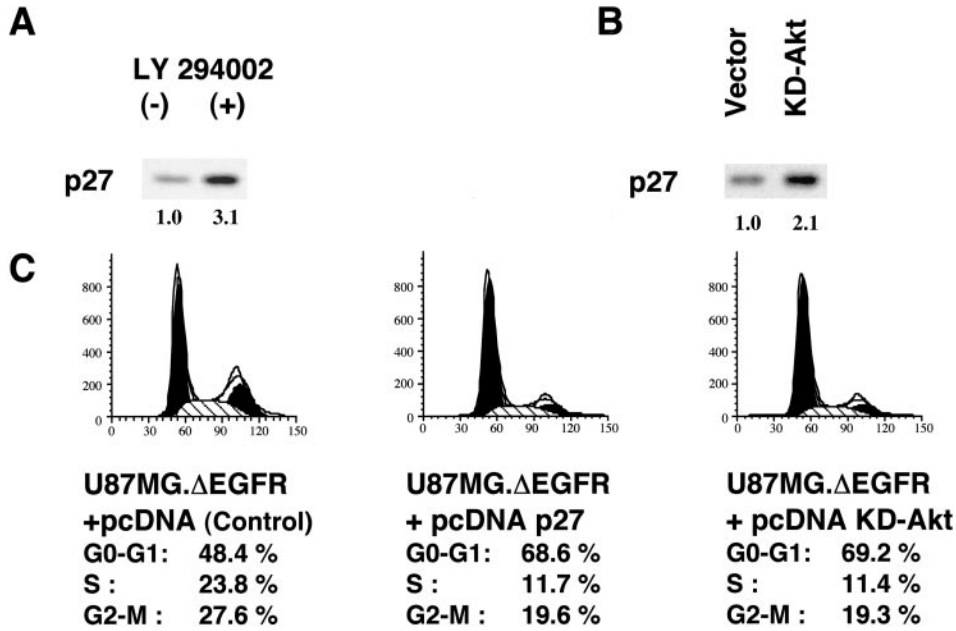

Fig. 5. Effects of inhibiting PI3-K/Akt activity on *in vitro* growth of the U87MG.ΔEGFR cells. In *A*, U87MG.ΔEGFR cells were cultured for 24 h without serum and then treated with 20 μM LY294002. Treatment with LY294002 up-regulated levels of p27. In *B*, U87MG.ΔEGFR cells were transiently transfected with control pcDNA, pcDNA KD-Akt, or pcDNA p27 and cultured for 48 h under 2% serum. Overexpression of p27 in U87MG.ΔEGFR cells induced G_1 arrest and, similarly, overexpression of KD-Akt resulted in up-regulation of p27 and induced G_1 arrest (*C*).

strated that parental U87MG cells underwent a G_1 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 G_1 into S phase in these glioblastoma cells. *p27* has been shown to be a bona fide tumor suppressor gene because p27−/− 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).

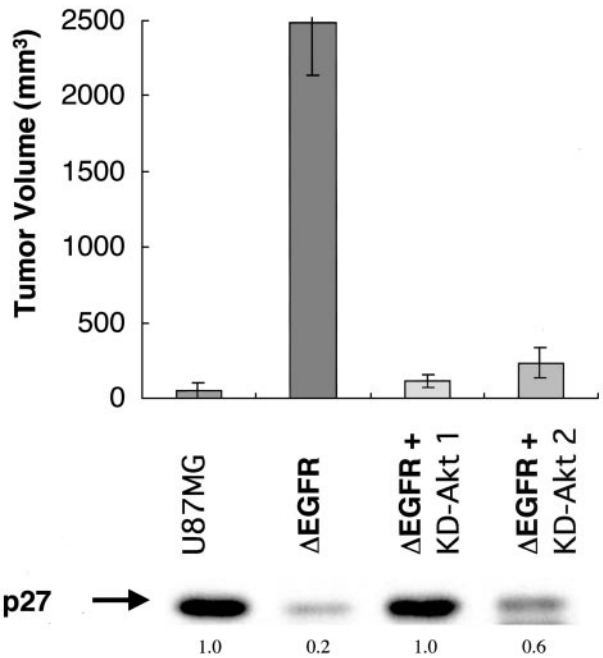

Fig. 6. The effect of inhibiting Akt activity on U87MG.ΔEGFR tumorigenicity. U87MG.ΔEGFR cells were transfected with control pcDNA or pcDNA KD-Akt, and the stable transfectants, KD-Akt 1 and KD-Akt 2, were obtained. Cells (1×10^6) of parental U87MG, U87MG.ΔEGFR with pcDNA, KD-Akt 1, and KD-Akt 2 cells in 0.1 ml of PBS were injected s.c. into the right flanks of nude mice. Tumor volumes were compared 3 weeks after the injection. Expression of KD-Akt in the U87MG.ΔEGFR cells resulted in up-regulation of p27 and substantial down-regulation of tumorigenicity *in vivo*.

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 G_1 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 G_1 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

mutation of the *PTEN* gene, are likely and may provide additional insight into tumor growth regulation by this signal transduction cascade. In any case, its central and substantial role in affecting tumorigenic behavior suggests that this pathway may be a suitable target for therapeutic intervention in tumors expressing this mutated receptor.

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