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Epidermal Growth Factor Receptor Transcriptionally Up-Regulates Vascular Endothelial Growth Factor Expression in Human Glioblastoma Cells via a Pathway Involving Phosphatidylinositol 3′-Kinase and Distinct from That Induced by Hypoxia[1]

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ABSTRACT

Glioblastomas are highly vascular malignant brain tumors that often overexpress vascular endothelial growth factor (VEGF). They also frequently overexpress epidermal growth factor receptor (EGFR) and contain regions of hypoxia, both conditions that can induce VEGF. We examined VEGF regulation in U87 MG human glioblastoma cells and in U87/T691 cells, a clonal derivative that contains a truncated erbB2/Neu receptor that interferes with EGFR signaling through the formation of nonfunctional heterodimeric receptor complexes. U87/T691 cells contained approximately one-half as much VEGF mRNA as did U87 MG cells under normoxic conditions (21% oxygen). Pharmacological inhibition of EGFR, Ras, or PI(3) kinase, but not MAP kinase, led to a significant decrease in VEGF mRNA levels in U87 MG cells. VEGF promoter activity in transient transfections was decreased by either pharmacological or genetic inhibition of EGFR, Ras, or phosphatidylinositol 3′-kinase [PI(3) kinase]. However, inhibition of PI(3) kinase or EGFR did not completely abolish induction of VEGF mRNA by hypoxia (0.2% oxygen). Likewise, VEGF mRNA expression was induced 3-fold by hypoxia in EGFR-inhibited U87/T691 cells, comparable with the fold induction seen in parental U87 MG cells, although the absolute level of message under hypoxia was higher in U87 MG cells. In transient transfections, a luciferase reporter construct containing a 1.2-kb fragment of the VEGF promoter, lacking the known hypoxic-responsive element (HRE), showed up-regulation after EGF stimulation to the same degree as the full-length, 1.5-kb VEGF promoter construct retaining the HRE. Furthermore, activity of the HRE-deleted, 1.2-kb promoter luciferase reporter was down-regulated by PI(3) kinase inhibition. Therefore, in glioblastoma cells, transcriptional regulation of the VEGF promoter by EGFR appears to involve Ras/PI(3) kinase and to be distinct from signals induced by hypoxia.

INTRODUCTION

Glioblastoma multiforme is the most common primary adult brain tumor. Although these tumors rarely metastasize, they almost always recur locally because of their inherent tendency for diffuse infiltration. Despite aggressive therapy, patients with these tumors have a poor prognosis with a median survival of <1 year (1). Pathologically, glioblastomas display vascular and endothelial cell proliferation (2). Therefore, blocking angiogenesis has been suggested as a potential means of inhibiting glioblastoma growth, as has been suggested for other tumors (reviewed in Ref. 3).

VEGF[3] is a potent angiogenic factor that has been implicated in the pathogenesis of glioblastomas (reviewed in Ref. 4). VEGF is commonly expressed in glioblastomas, as determined by *in situ* hybridization, but rarely expressed in low-grade gliomas (5, 6). Other studies have confirmed this correlation between tumor grade and VEGF expression in gliomas (7, 8). Therefore, VEGF is an attractive target for antiangiogenic therapy (reviewed in Ref. 9). Numerous studies in animal models have shown that inhibiting VEGF function using neutralizing antibodies (10), dominant-negative VEGF receptor mutants (11, 12), and antisense constructs (13–15) inhibits growth of glioma cells *in vivo* and causes regression of blood vessels (16).

Given the importance of VEGF in the growth of glioblastomas, we were interested in understanding its regulation in these tumors. Hypoxia, which is commonly observed in the microenvironment of solid tumors (17), is a well-known stimulus for inducing VEGF (6). Palisading cells in areas next to necrosis within glioblastomas are thought to up-regulate VEGF secondary to hypoxia (18). Hypoxia increases VEGF mRNA levels by transactivating the promoter via the transcription factor HIF1. HIF1 consists of an α subunit that is induced by hypoxia and a β subunit that is constitutively present (reviewed in Ref. 19). HIF1 binds to a specific consensus sequence, 5′-RCGTG-3′, which is found within the HRE in the VEGF promoter (20).

However, many glioblastoma cells express high levels of VEGF, even under normoxic conditions. In one study, U87 MG cells were found to express seven times the amount of VEGF mRNA as NIH3T3 fibroblasts or human fetal astrocytes under tissue culture conditions (21). This suggests that there are factors intrinsic to glioblastomas that lead to VEGF up-regulation independent of the environment. One of these factors could be EGFR, which is overexpressed as a result of gene amplification in 40–50% of malignant human glioma specimens (22–24). Many gliomas contain a mutant form of the EGFR known as deltaEGFR or EGFRvIII, which is missing exons 2–7, resulting in an in-frame deletion of 801 bp of the coding sequence of the extracellular domain, rendering the receptor constitutively active (25–27).

EGF has been shown to induce VEGF protein secretion in glioblastoma cells. Blockade of EGFR using a neutralizing antibody in A549 squamous carcinoma cells led to a decrease in the level of VEGF (28). Although the association between EGF and VEGF has been made, relatively little is known about the signaling pathway connecting the two. An extensive body of literature indicates that in nonglioma cell lines, Ras mutations can lead to increased VEGF levels (reviewed in Ref. 29). Although glioblastomas do not generally contain Ras mutations (30), they do have activation of the Ras pathway (31), presumably because of overexpression of EGFR or other tyrosine kinase growth factor receptors.

In this study, we used a matched pair of human glioblastoma cell lines, U87 MG, the parental line, and U87/T691, a derivative clonal line into which a truncated erbB2/Neu receptor protein containing a premature stop codon at T691 has been introduced. We have shown previously that introduction of the mutant erbB2/Neu protein inhibits kinase activation of EGFR through the formation of nonfunctional heterodimeric receptor complexes (32, 33). EGFR-inhibited U87/

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; HIF1, hypoxia inducible factor 1; EGFR, epidermal growth factor receptor; PI(3) kinase, phosphatidylinositol 3′-kinase; HRE, hypoxia-responsive element; MAP, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; FTI, farnesyltransferase inhibitor.

T691 subclones exhibit a less transformed phenotype by a variety of tumorigenicity assays and also display elevated apoptosis in response to DNA damage (34). In our studies, we found that the U87/T691 cells contain lower basal levels of VEGF mRNA and protein than the parental U87 MG cells. Using chemical inhibitors and transient transfections with a reporter construct containing the VEGF promoter, we provide evidence that EGFR regulates *VEGF* at the level of transcription in glioblastoma cells via a pathway involving Ras and PI(3) kinase, which is distinct from the pathway induced by hypoxia. The two pathways can, however, act to increase VEGF mRNA levels in an additive manner.

MATERIALS AND METHODS

Tissue Culture and Reagents. U87 MG cells were cultured in DMEM (4500 mg/l glucose; Life Technologies, Inc.) containing 10% fetal bovine serum (Atlanta Biologicals) and incubated under normoxic conditions in an incubator with 5% carbon dioxide and 21% oxygen or under hypoxic conditions as described below. U87/T691 cells were maintained in DMEM containing 10% fetal bovine serum and 0.4 mg/ml of G418 (Life Technologies, Inc.). U87 MG.deltaEGFR cells (27) were obtained from Dr. H. J. Huang (Ludwig Institute for Cancer Research, La Jolla, CA) and maintained in DMEM containing 10% fetal bovine serum and 0.4 mg/ml of G418 (Life Technologies, Inc.). For serum starvation, regular media were removed, dishes were washed twice with 5 ml of PBS, and then media without serum were added back.

Human recombinant EGF was obtained from Sigma and reconstituted in 10 mM acetic acid containing 0.1% BSA at a stock concentration of 20 μg/ml LY294002 and tyrphostin AG1478 (Alexis Biochemical) were dissolved in DMSO at stock concentrations of 19 and 31.6 mM, respectively. The stock concentrations of wortmannin (Sigma) and FTI-277 were 2 and 10 mM, respectively, both dissolved in DMSO. PD98059 (Alexis Biochemical) was prepared in DMSO at a stock concentration of 93.6 mM. The doses of inhibitors used in this study were determined from the literature and from preliminary experiments in our laboratory. Tyrphostin AG1478 at a concentration of 10 μM has been shown to block EGFR signaling in U87 MG cells (35). We have found that LY294002 and wortmannin inhibit PI(3) kinase activity, as measured by phosphorylated AKT at the doses used in this study (data not shown). We found that PD98059 at a concentration of 25 μM blocked MAP kinase phosphorylation in U87 MG cells using an antibody against phosphorylated ERK1/2 (Fig. 3*F*). FTI-277 at a concentration of 10 μM inhibited Ras farnesylation in these cells (data not shown).

Hypoxic Conditions. For hypoxia experiments, 5–7 $\times$ 10^5 cells were seeded on day 1 into 60-mm Permanox dishes (Nunc) and maintained in a 5% CO_2 incubator. Permanox plastic was used because of its high permeability to oxygen, permitting the efficient evacuation of oxygen from the dishes during hypoxia induction. On day 2, immediately before the induction of hypoxia, we replaced the standard media with media containing 50 mM HEPES, 0.15% (w/v) glucose, and 10 mM NaOH to maintain pH. Dishes were then placed into airtight aluminum chambers. The oxygen concentration was decreased by sequentially replacing a given percentage of the total gas within a chamber with 95% nitrogen/5% CO_2 using a precision vacuum gauge (36). In this way, the concentration was reduced from 21 to 5% with one gas exchange, from 5 to 1% with two exchanges, and then with an additional gas exchange to 0.2%. The oxygen content within the chambers was verified using a polarographic oxygen electrode (37). The aluminum chambers were then placed on an orbital shaker in a warm room maintained at 37°C. Because hypoxia can cause shifts in pH, we measured the pH of the media at the end of the hypoxia incubations. The HEPES buffered media used during these incubations prevented the pH from falling more than 0.3 pH unit during the hypoxic incubation.

mRNA Stability Assay. To measure the half-life of the VEGF message, we added actinomycin D to dishes of U87 MG cells maintained under normoxic conditions. Samples were harvested for RNA at intervals thereafter.

Northern Blot Analysis. Total RNA was isolated with Trizol (Life Technologies), using the manufacturer's directions. Ten to 15 μg of RNA were denatured with formaldehyde and formamide and run on a 0.9% agarose gel containing formaldehyde. RNA was transferred by capillary action in 20$\times$ SSC (1$\times$ SSC is 0.15 M NaCl, 0.15 M sodium citrate, pH 7) to a Duralon-UV membrane (Stratagene) and UV cross-linked prior to hybridization. Labeling of radioactive probes was performed using [^{32}P]dCTP and a Prime-It kit (Stratagene) using the manufacturer's directions. Hybridization was carried out at 65°C, after which the membranes were washed to a stringency of 0.1$\times$ SSC, 0.1% SDS at 65°C. Autoradiography was carried out at $-$80°C with intensifying screens. A 200-bp VEGF cDNA fragment excised with *Eco*RI from the pGEMh204 plasmid (Ref. 38; gift from Dr. B. Berse, Boston University School of Medicine, Boston, MA) was used to make radioactive probes for hybridization. To verify equal loading between lanes, all gels were stained with ethidium bromide. Furthermore, the membranes were probed with rpL32 (39), which is a ribosomal protein the mRNA level of which we have found to remain relatively constant under differing oxygen concentrations (40).

Protein Extraction, Western Blot Analysis, and ELISA. For protein isolation, cells were trypsinized and then pelleted by centrifugation. The pellets were suspended in PBS and then centrifuged again. The pellets were then solubilized in 0.3–0.5 ml of 1$\times$ sample lysis buffer (2% SDS, 60 mM TRIS, pH 6.8), Samples were boiled for 5 min and then passed repeatedly through a 26-gauge needle. They were centrifuged at 10,000 $\times$ *g*, and the supernatants were retained. Protein concentrations were determined using a BCA Protein Assay kit (Pierce).

For Western blotting, equal amounts of total protein were run in each lane of an SDS-PAGE gel (12% acrylamide). Each protein sample was mixed with an equal volume of 2$\times$ Laemmli buffer and boiled at 95°C for 5 min before loading onto the gel. After completion of gel electrophoresis, protein was transferred to a Hybond nitrocellulose membrane (Amersham) over 1 h using a blotting apparatus. For detection of the VEGF protein, we used a monoclonal antihuman VEGF antibody (PharMingen) at a dilution of 1:1000, followed by a goat antimouse antibody (Bio-Rad) at a dilution of 1:500. For detection of phosphorylated ERK-1 and ERK-2 kinases, we used the M8159 antibody (Sigma) at a dilution of 1:1000, followed by a goat antimouse antibody (Bio-Rad) at a dilution of 1:500. As a loading control, the blot was reprobed with an anti-β-actin antibody (Sigma) at a 1:1000 dilution, followed by a goat antimouse antibody (Bio-Rad) at a dilution of 1:500.

VEGF ELISA Assays. Conditioned media were removed for storage at $-$80°C. VEGF protein concentration in the media was determined by ELISA using a commercial kit (R&D Systems) .

Quantitation of Blots and Data Analysis. Gels were scanned on an Agfa Arcus II photoscanner using Adobe Photoshop 4.0. Bands on the gels were quantitated using NIH Image 1.54 software. Curve fitting was performed using Cricketgraph III, version 1.5.1 (Computer Associates). All results shown are representative of at least two independent, reproducible experiments.

Plasmid Constructs and Transient Transfections. We obtained a portion of the human VEGF promoter (41) from Dr. J. Abraham (Scios, Inc., Sunnyvale, CA). A 1.5-kb fragment of the promoter was excised using *Sac*I and subcloned into the *Sac*I site in the promoterless pGL3-Basic vector (Promega) in the proper orientation to make the plasmid pGL3–1.5kbVEGFprom (Fig. 9*B*). The pGL3–1.5kbVEGFprom plasmid contains the 47-bp HRE spanning from $-$985 to $-$939 (20). To delete the HRE, we constructed the plasmid pGL3–1.2kbVEGFprom by excising a 1.2-bp fragment from the VEGF promoter with the enzymes *Pst*I and *Sac*I and subcloning it into the *Pst*I/*Sac*I sites of pBluescript (Stratagene). The 1.2-kb fragment was then removed using *Kpn*I and *Sac*I and subcloned into these sites in pGL3-Basic (Fig. 9*B*).

We obtained the pcDNA3/RasN17 plasmid (42) from Dr. K. Hedin (Mayo Clinic) and the pGEX/deltap85 (43) from Dr. W. Ogawa (Kobe University School of Medicine, Kobe, Japan). The deltap85 cDNA was excised from this plasmid using *Bam*HI and *Eco*RI and inserted into the polylinker cloning site of pcDNA3 using the same restriction sites to make the plasmid pcDNA3/deltap85.

Transfections were performed using Fugene (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cells were split into 60-mm dishes so that 24 h later, they were ~50% confluent. At this time, each dish was transfected using 6 μl of Fugene and 2 μg of the reporter plasmid and, to control for transfection efficiency, 1 μg of pSV-β-galactosidase (Promega). Cells were harvested by removing the media, washing twice with PBS, and directly adding 200 μl of lysis buffer/dish. Of this lysate, 100 μl was used for luciferase determination, and 5 μl were used for β-galactosidase determinations. These determinations were performed using the LucLite kit (Packard Instrument Company) and the β-galactosidase Enzyme Assay System (Promega). Luciferase readings were performed on a TopCount Microplate Scintillation and Luminescence Counter (Packard Instrument Co.).

RESULTS

VEGF mRNA Levels in U87/T691 Cells Are Decreased Compared with U87 MG Cells. To determine the contribution of the EGFR to VEGF expression, we compared levels of VEGF mRNA in U87 MG cells to levels in U87/T691 cells. Two bands hybridized to the VEGF probe as described previously in U87 MG cells (13), one migrating at ~3.8 kb and the other at 1.4 kb. Under normoxic conditions, U87/T691 cells with functionally inactivated EGFR signaling expressed both mRNA species; however, the amount of VEGF mRNA was half that seen in U87 MG cells (Fig. 1*A*, compare *Lanes 1* and *3*). This difference was reflected at the protein level as well with U87/T691 cells containing 49% as much VEGF protein as U87 MG cells (Fig. 1*B*). These findings support the notion that EGFR kinase activation plays a role in maintaining high VEGF levels in U87 MG cells.

Despite the reduced level of VEGF mRNA in U87/T691 cells, these cells displayed a robust up-regulation of VEGF mRNA in response to hypoxia (Fig. 1*A*, compare *Lanes 3* and *4*). In U87/T691 cells, the amount of induction under hypoxia relative to normoxia was similar to that seen in U87 MG cells, 5.8-fold (2.9/0.5) *versus* 4.0-fold.

EGF and Hypoxia Additively Increase VEGF mRNA Levels in U87 MG Cells. As an alternate means of showing that VEGF can be regulated by EGF in these cells, we serum starved U87 MG cells and then restimulated them with EGF. Fig. 2 shows that stimulation with EGF increased VEGF mRNA levels 2-fold under normoxic conditions (*Lane 2 versus Lane 1*). We also determined the effect of hypoxia in conjunction with EGF stimulation on VEGF mRNA induction. EGF stimulation and 1% oxygen were both able to up-regulate VEGF mRNA levels; however, the two stimuli together had an additive effect in increasing VEGF mRNA levels compared with either alone (Fig. 2, *Lanes 2–5*). The same additive effect was observed with 5% oxygen and EGF stimulation (Fig. 2, *Lanes 2*, *3*, *6*, and *7*).

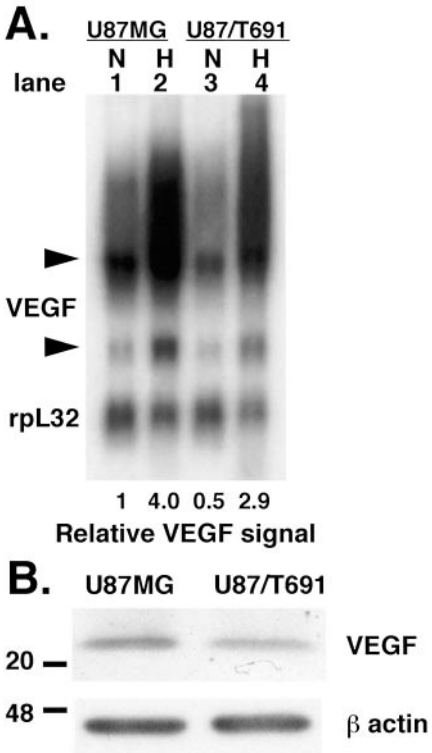

Fig. 1. VEGF mRNA levels in U87 MG and U87/T691 cells. *A*, cells were subjected to hypoxia (0.2% oxygen; *H*) or normoxia (21% oxygen; *N*). After 16 h, RNA was harvested, and then Northern blotting was performed. Ethidium bromide staining of the gel confirmed equal loading of the lanes by visual inspection (data not shown). Northern blot was probed for both VEGF and rpL32, a loading control (see "Materials and Methods"). The numbers shown at the *bottom* of the figure (relative VEGF signal) represent the ratio of intensity of the lower VEGF band to the rpL32 band. The level in the *first lane* was arbitrarily designated 1, and the values in the remaining lanes are relative to this. In this particular figure, the upper VEGF bands could not be quantitated because they were overexposed. *B*, protein lysates from both cell lines were run on a SDS-PAGE gel, and Western blotting was performed as described in "Materials and Methods."

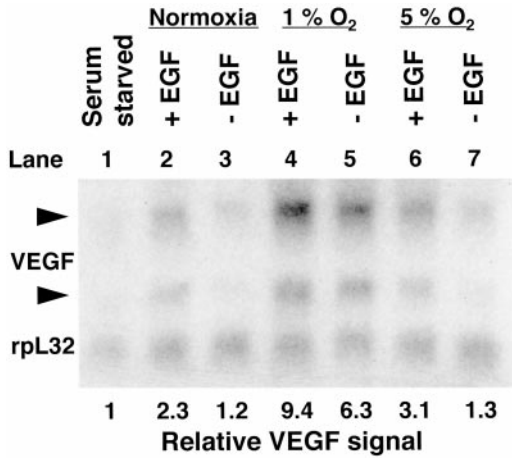

Fig. 2. VEGF mRNA induction in response to EGF and hypoxia in U87 MG cells. U87 MG cells were seeded on day 1. On day 2, the media were removed and replaced with serum-free media. Forty-eight h later, EGF (20 ng/ml) was added to half the dishes but not to the other half. Of the EGF-treated dishes, one-third were subjected to 5% oxygen, one-third were subjected to 1% oxygen, and one-third were kept in normoxia (21% oxygen). The control dishes not treated with EGF were similarly subjected to 5, 1, or 21% oxygen. Nine h later, RNA was harvested, and Northern blotting was performed. Ethidium bromide staining of the gel confirmed equal loading of the lanes by visual inspection (data not shown). The Northern blot was probed for both VEGF and rpL32, a loading control (see "Materials and Methods"). The numbers shown at the *bottom* of the figure (relative VEGF signal) represent the ratio of intensity of the upper VEGF band to the rpL32 band. The level in the *first lane* was arbitrarily designated 1, and the values in the remaining lanes are relative to this.

Pharmacological Blockade of EGFR, Ras, and PI(3) Kinase but not MAP Kinase Leads to Down-Regulation of VEGF mRNA Levels. To define the signaling pathway downstream of EGFR, we treated U87 MG cells with various pharmacological inhibitors. Treatment of U87 MG cells with tyrphostin AG1478, a drug that selectively inhibits EGFR tyrosine phosphorylation (35, 44, 45), led to a reduction of VEGF mRNA to ~50% of the control level (Fig. 3*A*), which confirms that EGFR kinase activity influences VEGF mRNA regulation. Because the Ras pathway has been shown to be activated in many glioblastoma cell lines, including U87 MG (31), we blocked Ras activity by using FTI-277, a farnesyltransferase inhibitor that has been used in other cell lines (46–48). FTI treatment decreased VEGF mRNA expression to ~30% of baseline (Fig. 3*B*).

Because Ras can activate the PI(3) kinase pathway (reviewed in Ref. 49), we investigated the consequence of PI(3) kinase inhibition. Treatment of U87 MG cells with LY294002, a PI(3) kinase inhibitor (50, 51), decreased VEGF mRNA to ~20% of the control level (Fig. 3*C*), as did treatment with wortmannin (Fig. 3*D*), which inhibits PI(3) kinase by a different mechanism (52–54). Another well-characterized signaling pathway downstream of Ras is the MAP kinase pathway (55), which regulates cell growth in many cell types. However, treatment of U87 MG cells with the MEK inhibitor PD98059 (56) had no effect on VEGF mRNA levels (Fig. 3*E*). We confirmed that the drug was blocking the MAP kinase pathway at this dose by showing inhibition of ERK phosphorylation (Fig. 3*F*).

VEGF Protein Levels in U87 MG Cells. To show that the effects we saw at the VEGF mRNA level corresponded to changes in protein levels, we performed ELISA assays on cell culture supernatants. As shown in Fig. 4*A*, EGF stimulation of serum-starved U87 MG cells led to an increase in secreted VEGF protein levels (Fig. 4*A*). Treat-

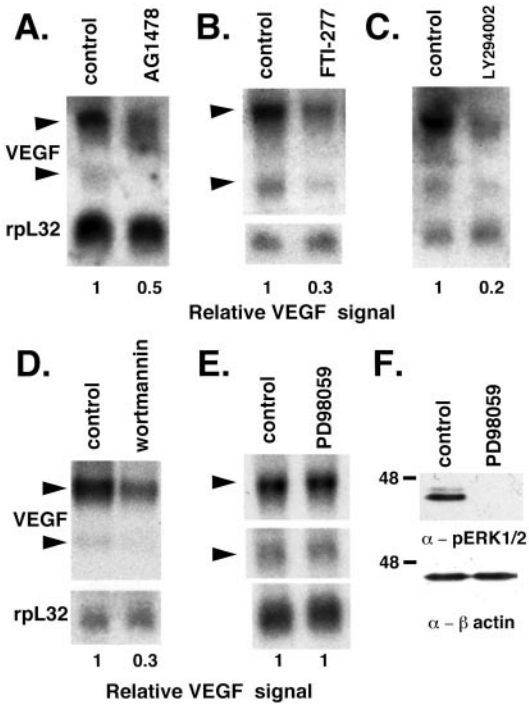

Fig. 3. Effect of pharmacological inhibitors on VEGF mRNA levels in U87 MG cells. *A–E*, Northern blots probed for VEGF and rpL32. Cells were treated with indicated drug for 24 h and then harvested for RNA. Ethidium bromide staining of the gel confirmed equal loading of the lanes by visual inspection (data not shown). The Northern blot was probed for both VEGF and rpL32, a loading control (see "Materials and Methods"). Numbers shown at the *bottom* of the figure (relative VEGF signal) represent the ratio of intensity of the upper VEGF band to the rpL32 band. For *A–E*, the VEGF:rpL32 ratio in the *first lane* was arbitrarily designated as 1, and the value in the *second lane* is relative to this. *A: Lane 1*, control (DMSO treated); *Lane 2*, treated with tyrphostin AG1478 (10 μM). *B: Lane 1*, control (DMSO treated); *Lane 2*, treated with FTI-277 (10 μM). *C: Lane 1*, control (DMSO treated); *Lane 2*, treated with LY294002 (20 μM). *D: Lane 1*, control (DMSO treated); *Lane 2*, wortmannin (100 nM). *E: Lane 1*, control (DMSO treated); *Lane 2*, treated with PD98059 (25 μM). *F*, Western blot using protein lysates harvested from the same experiment shown in *E*. Protein was run on a Western blot that was probed using an anti-phospho ERK1/2 antibody (see "Materials and Methods").

ment of U87 MG cells with LY294002 resulted in an ~50% decrease in VEGF protein secretion into the cell media (Fig. 4*B*). These data support the association between EGF stimulation, PI(3) kinase catalytic activation, and induction of VEGF mRNA and secreted protein.

Expression of Mutant EGFRvIII Results in Increased VEGF mRNA Levels in U87 MG Cells. We examined VEGF mRNA levels in U87 MG cells engineered to express the constitutively active mutant form of EGFR, known as the vIII mutant or deltaEGFR (27). These U87 MG.deltaEGFR cells display increased tumorigenicity compared with the parental U87 MG cells. By Northern blotting (Fig. 5*A*), we found that U87 MG.deltaEGFR cells expressed more VEGF mRNA parental U87 MG cells under normal tissue cell culture conditions, and that this difference was even more marked under serum starvation conditions, consistent with the constitutive signaling induced by deltaEGFR (33, 57). Treatment of U87 MG.deltaEGFR cells with LY294002 significantly down-regulated VEGF mRNA, whereas treatment with PD98059 did not (Fig. 5*B*). Given that deltaEGFR expression has been found to lead to constitutive activation of the PI(3) kinase pathway (58), these results lend further support to the connection between PI(3) kinase and VEGF up-regulation in these cells. These observations also suggest that EGFR/PI(3) kinase signals regulate the VEGF promoter independently of ERK activation.

Decreased VEGF mRNA Levels in EGFR Inhibited U87/T691 Glioblastoma Cells Are Attributable to Differences in Transcriptional Activity, Not mRNA Stability. Regulation of VEGF by hypoxia has been shown to occur both at the level of transcription and RNA stability (59, 60). To determine whether the regulation of VEGF by EGFR might involve RNA stability, we inhibited the transcription of newly synthesized mRNA using actinomycin D (Fig. 6*A*). We plotted the relative VEGF mRNA levels (Fig. 6*B*), from which we calculated that the half-lives of the 3.8- and 1.4-kb species were 4 and <h, respectively, in both cell lines. The half-life of the upper band is similar to the half-life of 3.8–4.8 h obtained in 293T cells (61). Therefore, altered VEGF mRNA stability does not account for the difference in VEGF levels in the two cell lines.

This observation suggested, by exclusion, that there might be differences in transcriptional regulation of the *VEGF* gene between the two cell lines. A luciferase reporter containing the VEGF promoter, pGL3–1.5kbVEGFprom (see "Materials and Methods"), was transfected into U87 MG and U87/T691 cells. The activity of this promoter in U87/T691 cells was approximately half that in U87 MG

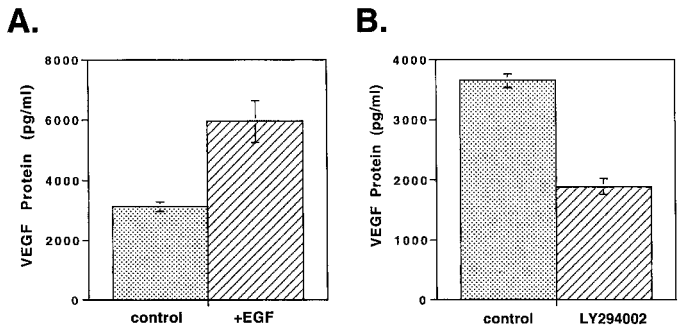

Fig. 4. VEGF protein secretion by U87 MG cells in response to EGF and LY294002. *A*, U87 MG cells were seeded into 60-mm dishes at a density of 2×10^5 cells/dish and incubated in regular media for 24 h and then in serum-free media for 48 h. At this time, EGF was added to treated but not the control dishes. Twenty-four h later, aliquots were collected from the cell culture supernatants for VEGF protein determination by ELISA (see "Materials and Methods"). *B*, U87 MG cells were seeded into 60-mm dishes at a density of 2×10^5 cells/dish. After 24 h, LY294002 or DMSO (control) was added, and then 24 h later, samples were collected. Bars, SD.

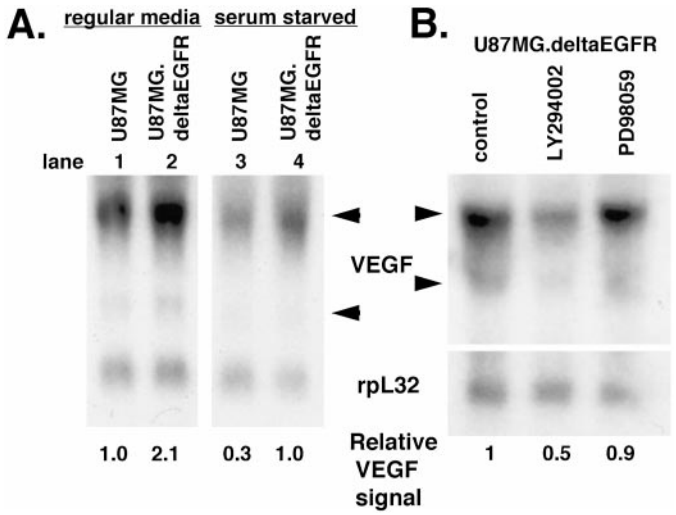

Fig. 5. VEGF mRNA levels in U87 MG.deltaEGFR cells. *A* and *B*, Northern blots probed for both VEGF and rpL32, a loading control (see "Materials and Methods"). Ethidium bromide staining of both gels confirmed equal loading of the lanes by visual inspection (data not shown). The numbers shown at the *bottom* of the figure (relative VEGF signal) represent the ratio of intensity of the upper VEGF band to the rpL32 band. The level in the *first lane* for both panels was arbitrarily designated 1, and the values in the remaining lanes are relative to this. *A*, U87 MG and U87 MG.deltaEGFR cells were grown either in regular media containing 10% FCS or no serum. After 48 h, RNA was harvested. Note that all four lanes were run on the same gel and probed simultaneously; therefore, the intensities of the signals can be directly compared among all four lanes. *B*, DMSO (control), LY294002 (20 μM), or PD98059 (25 μM) was added to dishes containing U87 MG.deltaEGFR cells. Twenty-four h later, RNA was harvested.

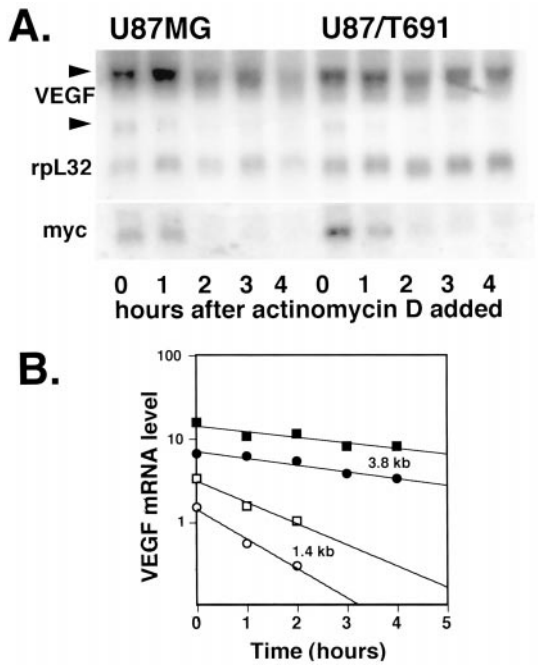

Fig. 6. VEGF mRNA decay in U87 MG and U87/T691 cells with actinomycin D. *A*, cells were seeded on day 1. The next day, the media were removed and replaced with fresh media containing actinomycin D (10 μg/ml). Samples were obtained at regular intervals after addition of actinomycin D. RNA was extracted, and Northern blotting was performed. Staining of the gel with ethidium bromide confirmed equal loading of the lanes (data not shown). The Northern blot was probed for both VEGF and rpL32, which is a loading control (see "Materials and Methods"). To verify that actinomycin D was inhibiting transcription of new mRNA, the blot was reprobed for myc, which showed the expected rapid decay (75). *B*, the bands on the gel from *A* were quantitated and then plotted using Cricket Graph 3.0. Curves were fitted using the assumption of a first-order exponential decay. *X axis*, hours after addition of actinomycin D. *Y axis*, VEGF mRNA level. ■ and □, U87 MG cells; ● and ○, U87/T691 cells. ■ and ●, 3.8-kb band; □ and ○, 1.4-kb band. The half-lives of the 3.8-kb VEGF message in U87 MG cells and U87/T691 cells were calculated to be 4.5 h ($r^2 = 0.84$) and 3.7 h ($r^2 = 0.93$), respectively. The half-lives of the 1.4-kb VEGF message in U87 MG cells and U87/T691 cells were calculated to be 1.2 h ($r^2 = 0.97$) and 0.9 h ($r^2 = 0.98$), respectively.

cells (Fig. 7*A*). Thus, differences in VEGF mRNA levels between these two isogenic glioblastoma cell lines differing with respect to functional activation of the EGFR kinase can be attributed solely to differences in activation of the VEGF promoter.

Inhibition of the PI(3) Kinase Pathway Leads to Decreased VEGF Promoter Activity. We investigated the effect of pharmacological inhibitors on VEGF promoter activity. Treatment with tyrphostin AG1478, an EGFR kinase inhibitor, and FTI-277, a FTI, both led to a decrease in VEGF promoter activity to less than half the control level, consistent with the effects of these drugs on VEGF mRNA levels; however, blockade of the MAP kinase pathway with PD98059 failed to inhibit VEGF transcriptional activity (Fig. 7*B*). In contrast, inhibition of PI(3) kinase with either LY94002 or wortmannin significantly decreased VEGF promoter activity (Fig. 7*C*). As an alternate means of showing the involvement of the Ras/PI(3) kinase pathway in VEGF promoter regulation, we used a dominant-negative Ras construct, pcDNA3/RasN17, and a dominant-negative p85 construct, pcDNA3/delta p85. Transient transfection with either construct into U87 MG cells along with the VEGF promoter construct led to a progressive decrease in promoter activity with increasing amounts of input plasmid (Fig. 7*D*).

Inhibition of PI(3) Kinase Does Not Abolish Induction of VEGF mRNA or the VEGF Promoter by Hypoxia. Having shown that inhibition of the EGFR/PI(3) kinase pathway could lead to decreased VEGF levels under normoxic conditions, we determined whether this pathway played any role in the induction of VEGF mRNA under hypoxic conditions. We pretreated U87 MG cells with the EGFR kinase inhibitor tyrphostin AG1478 (Fig. 8*A*) or the PI(3) kinase inhibitor LY294002 (Fig. 8*B*) for 2 h and then subjected them to hypoxia with 0.2% oxygen. The level of VEGF mRNA under normoxic conditions dropped after incubation with tyrphostin AG1478 (Fig. 8*A*, compare *Lanes 3* and *1*) and LY294002 (Fig. 8*B*, compare *Lanes 3* and *1*). However, even in the presence of tyrphostin AG1478, VEGF mRNA was strongly induced by hypoxia (Fig. 8*A*, compare *Lanes 3* and *4*). Thus, the hypoxic induction of VEGF mRNA in U87 MG cells can occur despite pharmacological inhibition of EGFR. These results are consistent with those shown in Fig. 1 in which U87/T691 cells that have functional blockade of the EGFR pathway are still inducible for VEGF mRNA under hypoxic conditions.

Likewise, blockade of the PI(3) kinase pathway using the drug LY294002 also did not abolish the hypoxic induction of the VEGF mRNA, although it did lower the levels under both normoxic and hypoxic conditions compared with controls (Fig. 8*B*). Because we were interested specifically in the effect of PI(3) kinase on VEGF promoter activity, we transiently transfected the 1.5-kb VEGF promoter reporter construct into cells, pretreated the cells with LY294002, and then subjected them to hypoxia. The luciferase levels under normoxia and hypoxia were less with LY294002 than the corresponding levels in the control set (Fig. 9*A*). However, the induc-

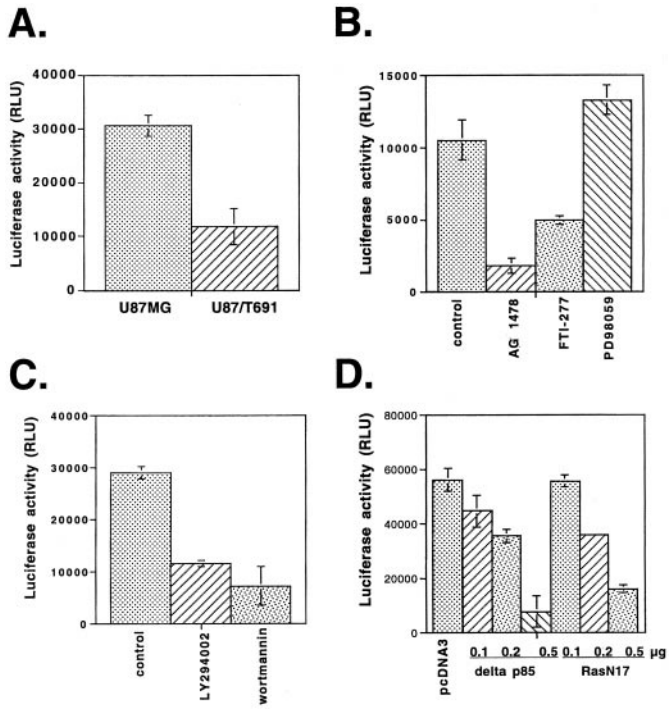

Fig. 7. VEGF promoter activity in U87 MG and U87/T691 cells. For *A–D*, normalized luciferase (luciferase:β-galactosidase ratios) are plotted on the *Y axis* in relative luciferase units (*RLU*). For all four panels, values represent the mean of three independent transfections; bars, 1 SD of the mean. *A*, both U87 MG and U87/T691 cells were cotransfected with pSV-β-galactosidase (Promega) and pGL3–1.5kbVEGFprom (see "Materials and Methods"). Forty-eight h later, samples were collected and analyzed for luciferase and β-galactosidase activity. *B* and *C*, U87 MG cells were transfected with pSV-β-galactosidase (Promega) and pGL3–1.5kbVEGFprom (see "Materials and Methods"). Forty-eight h later, cells were treated with drugs as listed below. After 12 h, cells were harvested for luciferase and β-galactosidase activity. *B: column 1*, control (DMSO treated); *column 2*, tyrphostin AG1478 (10 μM); *column 3*, FTI-277 (10 μM); *column 4*, PD98059 (25 μM). *C: column 1*, control (DMSO treated); *column 2*, LY294002 (20 μM); *column 3*, wortmannin (100 nM). *D*, U87 MG cells were cotransfected with pSV-β-galactosidase (Promega), pGL3–1.5kbVEGFprom (see "Materials and Methods"), and a third plasmid as listed below. Forty-eight h later, samples were harvested. *Column 1*, pcDNA3 (control plasmid); *columns 2–4*, pcDNA3/delta p85 in increasing amounts (0.1–0.5 μg); *columns 5–7*, pcDNA3/RasN17 in increasing amounts (0.1–0.5 μg). Total amount of DNA was 0.5 μg; therefore, pcDNA3 was added as a filler DNA as needed. *A–D, bars*, SD.

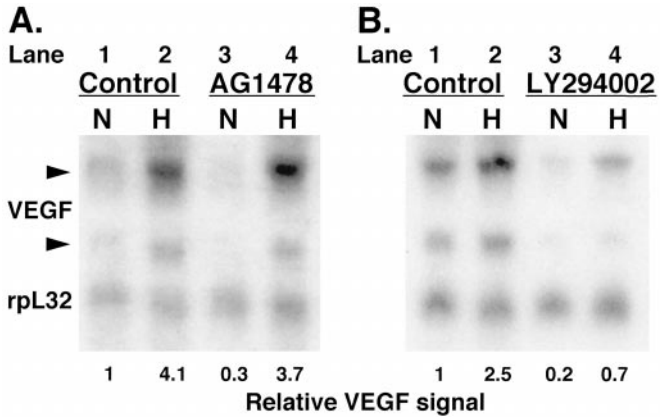

Fig. 8. VEGF mRNA levels after hypoxia in U87 MG cells pretreated with AG1478 or LY294002. U87 MG cells were treated with DMSO (control) or LY294002 (20 μM) for 2 h prior to exposure to normoxia (*N*) or hypoxia (0.2% oxygen; *H*) for 6 h. Samples were harvested for RNA at the end of the experiment, which was run on an agarose/formaldehyde gel. Ethidium bromide staining of the gel was performed, which confirmed equal loading of the lanes by visual inspection (data not shown). The gel was transferred to a Northern blot that was probed for VEGF and rpL32. The numbers shown at the *bottom* of the figure (relative VEGF signal) represent the ratio of intensity of the upper VEGF band to the rpL32 band. The level in the *first lane* was arbitrarily designated 1, and the values in the remaining lanes are relative to this.

tion from normoxia to hypoxia was similar in the two cases, 2–2.5-fold. These results mirror the Northern blot results shown in Fig. 8*B*. Although LY294002 can decrease VEGF promoter activity by interfering with EGFR/Ras/PI(3) kinase signaling, it does not completely abolish the hypoxia-induced rise in VEGF message and promoter activity.

VEGF Promoter Lacking the HRE Is Still Responsive to EGF and PI(3) Kinase Inhibition. In all of the previously discussed experiments using the VEGF promoter, we used the plasmid pGL3–1.5kbVEGFprom, which contains 1.5 kb of the VEGF promoter including the HRE, which contains a HIF-1 binding site (Fig. 9*B*). To separate effects mediated by the HRE, we created another luciferase reporter vector, pGL3–1.2kbVEGFprom, in which the HRE sequence was deleted (see "Materials and Methods" and Fig. 9*B*). When this reporter was transfected into U87 MG cells, its activity was still induced in response to EGF (Fig. 9*C*) and down-regulated in response to PI(3) kinase inhibition (Fig. 9*D*). These experiments show that EGF stimulation and PI(3) kinase responsiveness of the VEGF promoter can occur independently of the HRE.

DISCUSSION

Because glioblastomas commonly overexpress EGFR and also express high levels of VEGF, they provide an ideal system for examining the relationship between the two. We established a link between EGFR kinase activation and VEGF up-regulation in U87 MG cells by showing that inactivation of the EGFR kinase by introduction of a truncated $p185^{erbB2/Neu}$ receptor led to a decrease in the level of VEGF mRNA and protein by approximately one-half. We also used a pharmacological approach to confirm the dependence of VEGF expression on EGFR function. Treating U87 MG cells with the EGFR kinase inhibitor tyrphostin AG1478 led to a 50% decrease in VEGF levels. Although the difference in VEGF levels between the two cell lines may not appear very dramatic, in fact it may be enough to affect growth *in vivo*. For example, a 50% decrease in VEGF mRNA and secreted protein using an anti-VEGF monoclonal antibody was sufficient to block the *in vivo* growth of A431 epidermoid cancer cells (28).

We showed that blockade of EGFR in U87 MG cells led to a down-regulation of VEGF mRNA levels, not through alteration in message stability but through decreased transcription at the level of the VEGF promoter. This is an important distinction because VEGF has been shown to be regulated via changes in RNA stability in some situations such as hypoxia (59, 60). To better define the signaling pathway leading to transcription of the VEGF promoter, we used several different pharmacological agents. Treatment with the EGFR inhibitor tyrphostin AG1728 led to a significant decrease in promoter activity, even greater than that seen at the mRNA level. The reason for this is unclear but may relate to factors that independently regulate VEGF mRNA stability.

Treatment of U87 MG cells with a FTI led to a decrease in VEGF mRNA levels as well as in VEGF promoter activity. FTIs have been used to inhibit Ras function in various systems (reviewed in Ref. 62) and have been shown to decrease the level of VEGF in H-*ras*-transformed intestinal cells to levels seen in non-*ras*-transformed cells (63). The action of FTIs is not specific for Ras because these drugs will inhibit the function of any protein that requires farnesylation to be active. However, we found there was a progressive decrease in VEGF promoter activity with increasing amounts of transfected RasN17 plasmid, providing specific evidence for the involvement of the Ras pathway in VEGF signaling in glioblastoma cells.

Both the MAP kinase pathway and the PI(3) kinase pathway are known to be downstream of Ras. We have demonstrated that in U87 MG cells, EGF stimulation can activate both the MAP kinase pathway (64) and the PI(3) kinase pathway (65). In the current study, we have shown that the MAP kinase pathway is not involved in the regulation of VEGF mRNA in these glioblastoma cells by using the specific MEK inhibitor PD98059 (56). This is in direct contrast to results obtained by other investigators using rodent fibroblasts in which transfection with elements from the Ras/MAP kinase pathway resulted in up-regulation of VEGF expression (66, 67), and treatment with the MEK inhibitor PD98059 led to a decrease in VEGF levels (68).

However, in U87 MG human glioblastoma cells, we found that the PI(3) kinase inhibitors LY294002 and wortmannin both decreased VEGF mRNA levels. Conversely, in U87 MG cells transfected with the mutant vIII EGFR, which is known to constitutively activate PI(3) kinase, VEGF mRNA was increased relative to parental U87 MG cells. Therefore, in human glioblastoma cells, our data argue that VEGF mRNA levels are regulated by the PI(3) kinase pathway, not the MAP kinase pathway. We believe that the difference between our results and those cited above (66–68) using rodent fibroblasts are attributable to cell-type differences. In support of the importance of cell context, VEGF regulation was similarly found to be dependent on PI(3) kinase but independent of MAP kinase in *ras*-transformed intestinal epithelial cells (68). PI(3) kinase has also been implicated in VEGF regulation in Ras transformed endothelial cells (69), as well as in its regulation by platelet-derived growth factor in human umbilical vein endothelial cells (70). Zundel *et al.* (71) reported in human glioblastoma cells that the tumor suppressor gene *PTEN* regulates PI(3) kinase induced VEGF expression in a HIF1α-dependent fashion. Zhong *et al.* (72) found that EGF could up-regulate the level of HIF1α protein in some prostate carcinoma cells via a PI(3) kinase-dependent pathway. However, we found that a reporter construct in which the HRE containing the HIF1 binding site was deleted was still up-regulated by EGF and down-regulated by inhibition of PI(3) kinase. Therefore, our results indicate that there also exists a pathway involving PI(3) kinase, but independent of HIF1α, by which EGF can transactivate the VEGF promoter.

We found that hypoxia could interact with EGF stimulation to increase VEGF levels in an additive manner. Hypoxic induction of VEGF still occurred when the EGFR/PI(3) kinase pathway was inhibited. U87/T691 cells with defective EGFR kinase function still up-regulated VEGF upon exposure to hypoxia. Likewise, pretreat-

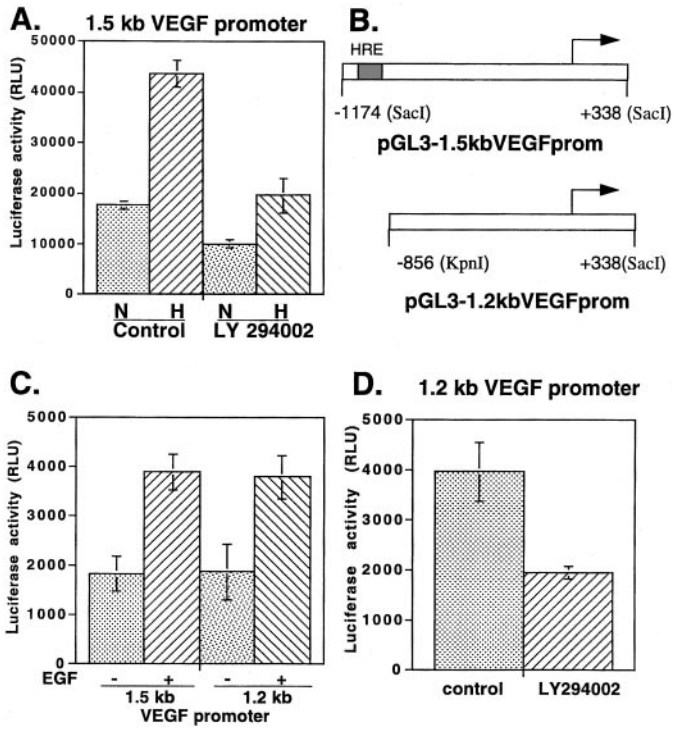

Fig. 9. VEGF promoter activity after hypoxia and/or EGF stimulation in U87 MG cells. For *A*, *C*, and *D*, normalized luciferase levels (luciferase:β-galactosidase ratios) are plotted on the *Y axis* in relative luciferase units (*RLU*). Values represent the mean of three independent transfections; *bars*, 1 SD of the mean. *A*, U87 MG cells were transfected with pSV-β-galactosidase (Promega) and pGL3–1.5kbVEGFprom (see "Materials and Methods"). Forty-eight h later, samples were treated with LY294002 (20 μM) or DMSO (control). After 2 h, half the LY294002-treated dishes were subjected to hypoxia (0.2% oxygen; *H*) and half to normoxia (*N*). Likewise, half the DMSO treated dishes were subjected to hypoxia and half to normoxia. After 6 h, dishes were harvested for both luciferase and β-galactosidase activity. *B*, schematic of pGL3–1.5kbVEGFprom and pGL3–1.2kbVEGFprom plasmids. Only pGL3–1.5kbVEGFprom contains the 47-bp HRE located from −985 to −939, which is *shaded* (20). *C*, U87 MG cells were transfected with either of the two plasmids shown in *B*, along with pSV-β-galactosidase (Promega). In each of the two groups, half the dishes were stimulated with EGF (20 ng/ml) and half were not. After 8 h, dishes were harvested for both luciferase and β-galactosidase activity. *D*, U87 MG cells were cotransfected with pGL3–1.2kbVEGFprom along with pSV-β-galactosidase (Promega). Forty-eight h later, cells were treated with LY294002 or DMSO (control). After 12 h, cells were harvested for luciferase and β-galactosidase activity. *Bars*, SD.

ment
of U87 MG cells with the EGFR inhibitor tyrphostin AG1478 or the PI(3) kinase inhibitor LY294002 failed to completely abolish the induction of VEGF by hypoxia. We found that hypoxia in the presence of an intact EGFR/PI(3) kinase pathway resulted in higher absolute levels of VEGF mRNA than hypoxia with EGFR or PI(3) kinase inactivation. However, the fold-induction of the VEGF mRNA from normoxia to hypoxia was similar, regardless of whether the EGFR/PI(3) kinase pathway was intact. This indicates that the induction of VEGF by hypoxia can occur despite PI(3) kinase inhibition. At first glance, these results appear to be at odds with recent results showing that PI(3) kinase can regulate HIF1 activity (71, 72). However, Zhong *et al.* (72) found that HIF1α levels under hypoxic conditions were only partly inhibited by PI(3) kinase inhibitors at doses that completely inhibited expression under normoxic conditions. Therefore, it is likely that even if PI(3) kinase plays a role in the induction of HIF1α by hypoxia, other signaling mechanisms are involved as well.

These findings open the way for defining the *cis*-acting element(s) in the VEGF promoter, other than the HRE and *trans*-acting factors downstream of the PI(3) kinase pathway, that are involved in transactivation in response to EGF. To date, there have only been a few transcription factors other than HIF1α that have been shown to be regulated by PI(3) kinase signaling, including the forkhead family members (73) and nuclear factor-κB (74). Further work on defining the signal transduction pathways downstream of EGF might have potential clinical applications in suggesting ways of decreasing VEGF levels in glioblastomas. For example, our data suggest that therapeutic approaches that target EGFR in glioblastomas may also have the added benefit of reducing tumor angiogenesis.

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