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Acidic Extracellular pH Induces Vascular Endothelial Growth Factor (VEGF) in Human Glioblastoma Cells via ERK1/2 MAPK Signaling Pathway

MECHANISM OF LOW pH-INDUCED VEGF*

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Overexpression of vascular endothelial growth factor (VEGF) is associated with disease progression in human glioblastomas. We recently showed that VEGF promoter activity is inversely correlated with tumor extracellular pH (pH_o) *in vivo* in the human glioma (U87 MG) xenografts. Here we show that substitution of the neutral culture medium (pH 7.3) with acidic pH medium (pH 6.6) up-regulates VEGF mRNA and protein production in human glioblastoma cells as reflected by Northern blot analysis and enzyme-linked immunosorbent assay. Functional analysis of the VEGF promoter reveals that the sequence between −961 bp and −683 bp upstream of the transcription start site is responsible for the transcriptional activation of the *VEGF* gene by acidic pH. This region contains the binding site for AP-1. Consequently, AP-1 *luciferase* reporter gene was activated by acidic pH. Gel-shift analysis confirmed that AP-1 DNA binding activity is induced under acidic pH. While investigating the upstream signaling pathways, we found that ERK1/2 MAPK is activated and translocates to the nucleus to activate Elk-1, and inhibition of the activation of ERK by specific inhibitors of MEK1 blocks the up-regulation of *VEGF* by low pH. Dominant negative forms of Ras and Raf abolished the activation of VEGF promoter by acidic pH. These results show that acidic pH activates Ras and the ERK1/2 MAPK pathway to enhance *VEGF* transcription via AP-1, leading to increased VEGF production.

VEGF[1] is a multifunctional cytokine, originally discovered as a tumor-secreted protein that promotes vascular permeability (1). VEGF promotes angiogenesis by inducing migration and proliferation of endothelial cells (2, 3). It is expressed in virtually all tumor types, but its expression is highly up-regulated in glioblastoma multiforme and positively correlates with tumor grade in gliomas (4, 5). The critical role of VEGF in tumor growth has been suggested by the targeted deletion of the *VEGF* gene (6, 7), by blocking antibodies (8) and by introduction of antisense *VEGF* constructs into neoplastic cells (9).

Hypoxia and glucose deprivation, common features of solid tumors, up-regulate the expression of VEGF in a wide range of tumor cell lines (10, 11). Acidic extracellular pH (pH_o) is another common feature of solid tumors (12, 13). Tumor pH_o is on an average 0.5 pH unit lower than the corresponding normal tissue pH_o (14). Although a great deal is known about the effect of hypoxia on gene expression and function in tumors, our knowledge of the effect of low pH on gene expression is still in its infancy. Recently, we have shown that VEGF promoter activity inversely correlates with tumor pH_o in a human glioma xenograft *in vivo* (15). Yet to be determined are whether there is a causal relationship between tumor acidic pH_o and VEGF expression in human glioma cells and the underlying mechanisms thereof.

MAPKs play a key role in the transcriptional and post-transcriptional regulation of VEGF (16). MAPKs are a family of serine/threonine kinases that are activated through a signaling pathway triggered by growth factors, stress, hormones, lymphokines, extracellular matrix components, and tumor promoters (17). Protein kinase phosphorylation events are involved in the regulation of gene expression by activating transcription factors and through post-transcriptional mechanisms (18). Mammalian cells express several groups of MAPKs, including extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK) and p38 proteins. The best-characterized function is their regulation of transcription factor AP-1 (19). The MAPK activity is regulated through three-tiered cascades: MEK1/2 activates ERK1/2, MKK3/6 activates p38, and MKK4/7 (JNK1/2) activates JNK. Each MAPKK can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signaling (17). Ras triggers activation of MAPK; Raf-1 (MEK) serine/threonine kinase is one of the downstream effectors of Ras (20, 21). Ras has been shown to play a significant role in the expression of *VEGF* (22).

Here, we hypothesize that acidic pH up-regulates VEGF expression in human glioblastoma cells. We show that VEGF mRNA and protein production are increased with incubation in acidic pH medium. Then, we show that the induction of VEGF involves activation of the *VEGF* gene transcription. The transcriptional activation of *VEGF* is mediated by AP-1, and ERK1/2 MAPK is required. By blocking Ras and Raf with the use of their dominant negative mutants, we demonstrate that

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor A; pH_o, extracellular pH; HIF, hypoxia-inducible factor; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MEK, MAPK/ERK kinase; AP-1, activating protein-1; JNK, Jun amino-terminal kinase; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; FBS, fetal bovine serum; SAPK, stress-activated protein kinase.

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Ras and Raf act as important intermediary molecules in acidic pH-mediated overexpression of VEGF.

EXPERIMENTAL PROCEDURES

Reagents—U0126 and PD098059 (Calbiochem, San Diego, CA) were used at final concentrations of 10 and 50 mM, respectively. Rabbit polyclonal antibodies to phospho-ERK1/2, ERK1/2, and phospho-Elk-1 were purchased from Cell Signaling, Inc. (Beverly, MA).

Cell Culture—The U87 MG human glioma cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The tumor cell line was maintained in monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Gaithersburg, MD). To vary the medium's pH, serum-free medium (BioWhitaker, Rockland, ME) was used; pH was adjusted with 20 mM MES and 20 mM Tris (23).

Northern Blot Analysis—Northern blot was performed as described previously (24). *VEGF* and *β-actin* cDNA probes were synthesized by PCR using primers for *VEGF*: 5′-TCC GGA TCC ATG AAC TTT C-3′ and 5′-TGG CTC ACC GCC TTG GCT-3′; and for *β-actin*: 5′-TGT ATG CCT CTG GTC GTA CC-3′ and 5′-CAA CGT CAC ACT TCA TGA TGG-3′.

Measurement of VEGF Protein Production by ELISA—VEGF protein production was analyzed by ELISA using the Quantikine VEGF ELISA kit (R&D Systems) according to the manufacturer's instructions.

Plasmid Constructs—All deletion constructs were derived from the 2.8-kb *hVEGF* fragment described previously (GenBank™ M63971) (25). 5′ deletions to −1451, −961, −683, −495, −165, −41 bp were obtained by PCR using primers: −1451: 5′-GGA ATT CAC TAG TCC AGG AAT TTT TCC AAG CTG-3′, −961: 5′-GGA ATT CAC TAG TAC AGG TCC TCT TCC CTC C-3′, −683: 5′-GGA ATT CAC TAG TAG CTG TTT GGGAGG TCA GAA-3′, −495: 5′-GGA ATT CAC TAG TGT GGG TGA GTG AGT GTG TGC-3′, −165: 5′-GGA ATT CAC TAG TCT TCA CTG GGC GTC CGC A-3′, −41: 5′-GGA ATT CAC TAG TCC CCC CCT TTT TTT TTT A-3′. The reverse primer was: 5′-GTG GTG CAG ATG AAC TTC A-3′. All PCR products were sequenced and confirmed to be identical to the published sequence of VEGF promoter. The PCR products were digested with *Spe*I and *Hin*dIII and subcloned into the peak12 *luciferase* reporter gene vector (A gift from Dr. Brian Seed, Massachusetts General Hospital). 4xTRE-luc is a gift from Dr. Shu Chien, University of California, San Diego) (26). RasV12, RasN17, RafCAAX, and RafS621A were obtained from CLONTECH (Palo Alto, CA).

Transient Transfections and Reporter Gene Assays—Transient transfection and reporter gene assays were performed as described previously (24). The cells were co-transfected with 20 mg of the serial deletion constructs of VEGF promoter and 2 mg of pRL-TK/plate using the calcium phosphate method. pRL-TK, obtained from Promega (Madison, WI) contains the herpes simplex virus thymidine kinase promoter region upstream of *Renilla luciferase* and was used as an internal control for transfection efficiency. The transfected cells were then exposed for 6 h to neutral or acidic pH conditions. Cell lysates were prepared using the Dual Luciferase Assay System (Promega). The light intensity was measured on 20 ml of cell lysates using a luminometer.

Electrophoretic Mobility Shift Assay—EMSA was performed using nuclear extracts prepared from U87 MG cells cultured for various time periods under neutral or acidic pH conditions. For EMSA experiments, the following double-stranded oligonucleotides were used: HIF-1a, 5′-TGC ATA CGT GGG CTC CAA CAG-3′; AP-2, 5′-GAT CGA ACT GAC CGC CCG CGG CCC GT-3′; Sp-1, 5′-ATT CGA TCG GGG CGG GGC GAG C-3′; AP-1, 5′-GTA GGT TTG AAT CAT CAC GCA GGC-3′; AP-1 mutant, 5′-GTA GGT T**A**G AAT CAT CAC GCA-3′. Bold indicates mutated base pair. For competition assays, 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. Where indicated, anti-c-Jun, anti-c-Fos, anti-JunB, and anti-JunD antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were added to the binding reaction in supershift analysis (24).

Western Blot Analysis—U87 MG cells were incubated in serum-free medium overnight and then incubated in either neutral pH (pH 7.3) or acidic pH (pH 6.6) for different time points. Then the cells were scraped from plates, pelleted, and resuspended in lysis buffer. 30 mg of protein per sample was separated on 10% SDS-polyacrylamide gels. The expression of ERK1/2 was detected by polyclonal antibodies against ERK1/2 (1:1000) and phosphorylated ERK1/2 (1:1000).

Immunofluorescence Analysis—U87 MG cells grown on a glass slide were fixed with cold (−20 °C) methanol for 20 min at room temperature without previous PBS wash. After blocking with 2% normal goat serum with 0.3% Triton-X in PBS for 1 h at room temperature, fixed cells were incubated with 1:250 diluted anti-phospho-ERK1/2 antibody at 4 °C overnight. Then cells were washed three times with PBS and incubated with fluorescence isothiocyanate-conjugated anti-rabbit IgG antibody (1:300) for 1 h at room temperature. Finally, cells were washed three times with PBS, mounted under glass coverslides with Vectashield mounting media (Vector, Fremont, CA), and examined under epifluorescent illumination with excitation-emission filters for fluorescence isothiocyanate.

Protein Kinase Assay—Subconfluent cultures of U87 MG cells were grown in Dulbecco's modified Eagle's medium containing 0.5% FBS for 24 h at 37 °C. U87 MG cells were then incubated in neutral pH or acidic pH medium for different time periods. Cells were then washed rapidly with ice-cold PBS and immediately lysed in lysis buffer. Two-hundred microliters of total cellular extract was immunoprecipitated with an immobilized phospho-ERK1/2 antibody, washed, and incubated with an Elk-1 fusion protein as a substrate. This allows immunoprecipitated active ERK1/2 to phosphorylate Elk-1. Phosphorylation of Elk-1 was measured by Western blotting using a phospho-Elk-1 antibody.

Statistical Analysis—The significance of the data was analyzed by the Student's *t* test (two tailed).

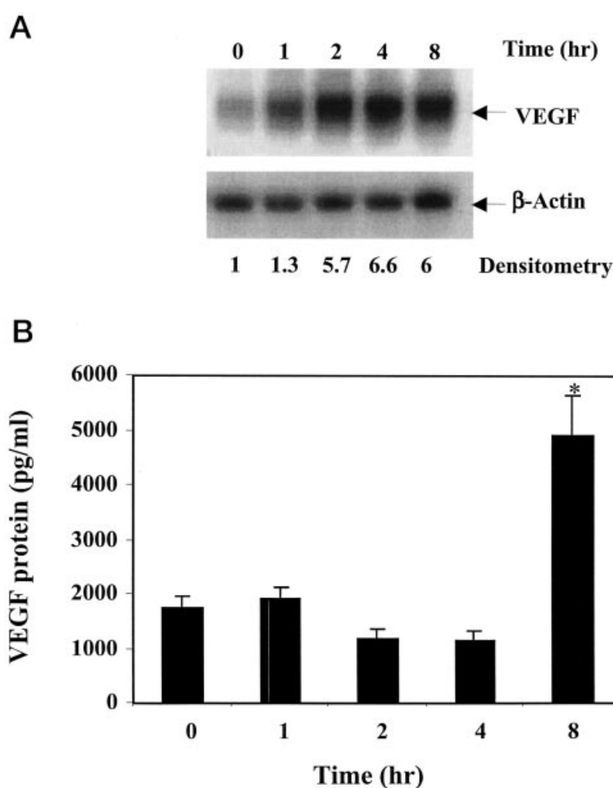

FIG. 1. *A*, VEGF expression under acidic pH. Northern blot analysis of *VEGF* mRNA in acidic human glioblastoma cells. U87 MG cells were incubated in neutral or acidic pH for different time periods as indicated. Northern blot analysis was performed. Densitometry of *VEGF* mRNA level normalized by the level of *β-actin* is presented. *B*, production of VEGF protein by human glioblastoma cells exposed to acidic pH. U87 MG cells were incubated in neutral or acidic pH medium for different time periods. VEGF contents in supernatants were determined by ELISA. Data are expressed as the mean of triplicate ± S.D. *, statistically significant difference as compared with the baseline ($t = 0$) value ($p < 0.001$). These are representative of at least three independent experiments.

RESULTS

Induction of VEGF Production in Human Glioma Cells by Acidic pH—U87 MG cells were incubated in acidic pH (pH 6.6) medium for different time period. Then, supernatant were collected for ELISA, and the cells were lysed for Northern blot analysis. Incubation in acidic pH resulted in an increase in *VEGF* mRNA in a time-dependent manner. A rapid increase of *VEGF* transcripts was observed 1 h following incubation in acidic pH medium and reached a plateau after 2 h (Fig. 1*A*). As

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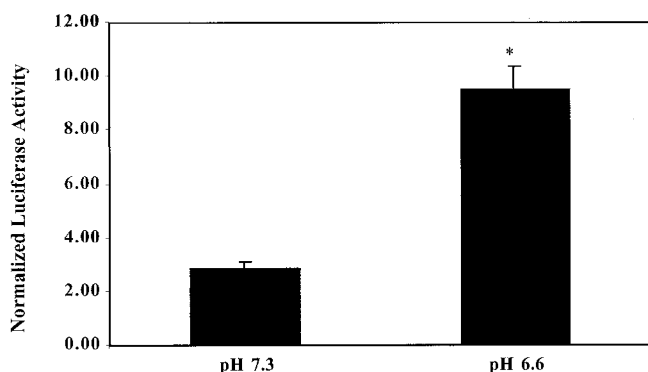

FIG. 2. **VEGF promoter activity analysis by luciferase reporter gene assay.** U87 MG cells were transiently transfected with VEGF-*luciferase* reporter gene followed by incubation in neutral or acidic pH conditions for 6 h. The normalized luciferase activities are the luminometer readings of firefly luciferase activity normalized for transfection efficiency on the basis of *Renilla* luciferase activity. *, statistically significant difference ($p < 0.001$). Data are expressed as the mean of three independent experiment ± S.D.

shown in Fig. 1*B*, acidic pH stimulation of U87 MG cells led to an increase in VEGF protein levels in the culture medium, from 1749 ± 87 pg/ml in neutral pH medium to 4910 ± 245 pg/ml in acidosis for 8 h. The induction of VEGF protein is not obvious before 8 h, presumably due to the time (4–6 h) required for the protein to accumulate. These data support the association between acidic pH stimulation and induction of VEGF mRNA and protein secretion.

Activation of VEGF Promoter by Acidic pH in Vitro—To test whether acidic pH activates the VEGF promoter *in vitro*, 2.8 kb of the VEGF promoter region were subcloned and tested using the luciferase reporter gene. Transiently transfected U87 MG cells were incubated in neutral or acidic pH medium for 6 h. Fig. 2 shows that VEGF promoter was activated up to 3-fold upon incubation in acidic pH, indicating that the induction of VEGF production involves transcriptional activation.

Deletional Analysis of the Acidic pH Enhancer in VEGF Promoter—Based on the data obtained from the reporter gene assay, we expected *cis*-acting elements to control the transcriptional activation of the *VEGF* gene in response to acidic pH. To determine the sequences in the VEGF promoter that contribute to the acidic pH response in U87 MG cells, we constructed and analyzed the activities of different regions of the VEGF promoter. Fig. 3*A* shows the different constructs used in the reporter gene assays. Acidic pH induction was significantly decreased ($p < 0.001$) by shortening the promoter to −683 bp (Fig. 3*B*). The loss of promoter activation suggests a truncation of transcription factor binding sites in the −961 bp to −683 bp region that are required to response to acidic pH. This region contains the AP-1 DNA binding sequence. Next we used 4xTRE-luc to study the response of the AP-1 response element to acidic pH. 4xTRE-luc was co-transfected with pRL-TK into U87 MG cells. Transiently transfected cells were then split and incubated in pH 7.3 or pH 6.6 medium. Fig. 3*C* shows that the luciferase reporter driven by TRE was induced about 4-fold by acidic pH.

Acidic pH Induced Activation of DNA-binding Proteins AP-1—To confirm the specific role of AP-1 transcription factor in the regulation of the VEGF promoter, we utilized an oligonucleotide probe corresponding to the AP-1 binding sequence in the VEGF promoter in the EMSA experiments. Radioactively labeled AP-1 probes were incubated with nuclear protein extracts from U87 MG cells that had been exposed to either neutral or acidic pH medium. Nuclear proteins extracted from unstimulated cells did not form any complexes. Exposure of the

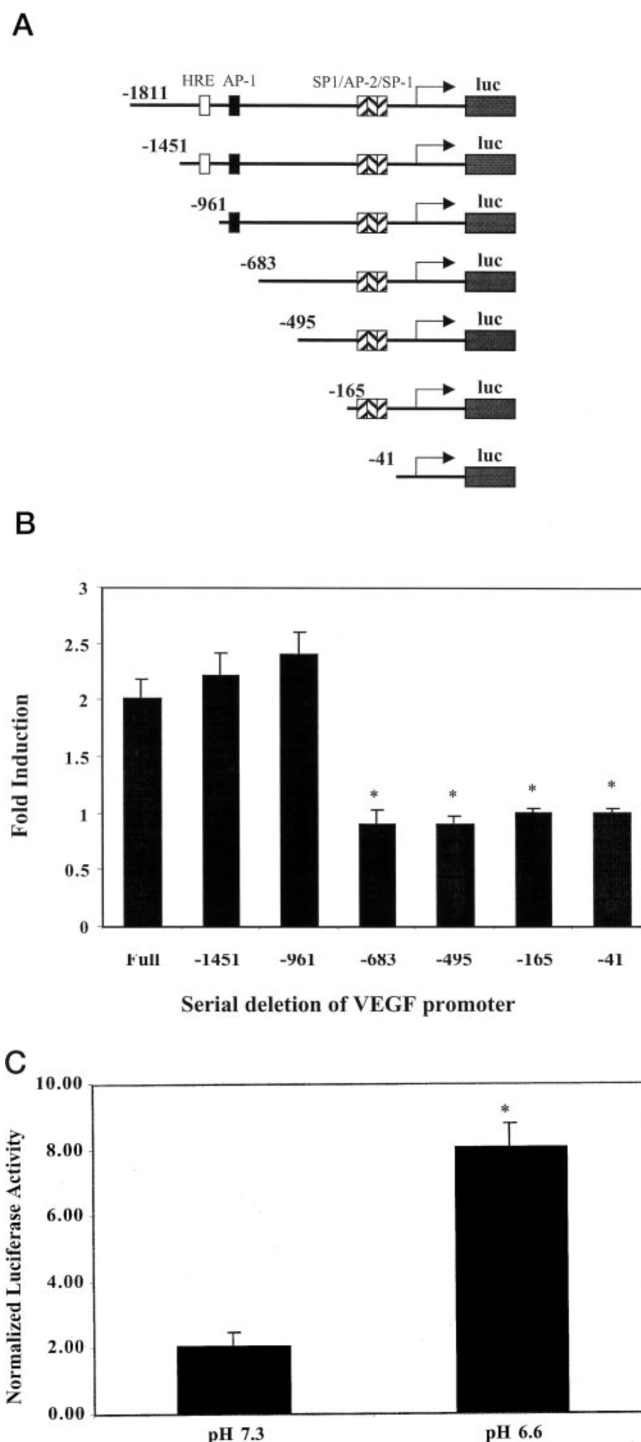

FIG. 3. **Deletional analysis of the acidic pH enhancer in the VEGF promoter.** *A*, serial deletion of the 5′-flanking region of the *VEGF* gene. *B*, the effects of deletions on inducibility of luciferase activity by acidic pH were determined by luciferase reporter gene assay. The -fold induction was calculated by comparing the normalized luciferase activity from cells incubated in pH 6.6 medium to that of cells incubated in pH 7.3 medium. *C*, the response of AP-1 response element to acidic pH. Data are expressed as the mean of three independent experiments ± S.D. *, statistically significant difference ($p < 0.001$).

U87 MG cells to acidosis resulted in a substantial increase in the binding activity of AP-1, which peaked at 1 h after incubation in acidic medium (Fig. 4*A*). The binding specificity of the complexes formed was determined by competition with a excess (50 M) of unlabeled oligonucleotide containing the AP-1 consensus binding site and unrelated NF-κB oligonucleotides. The

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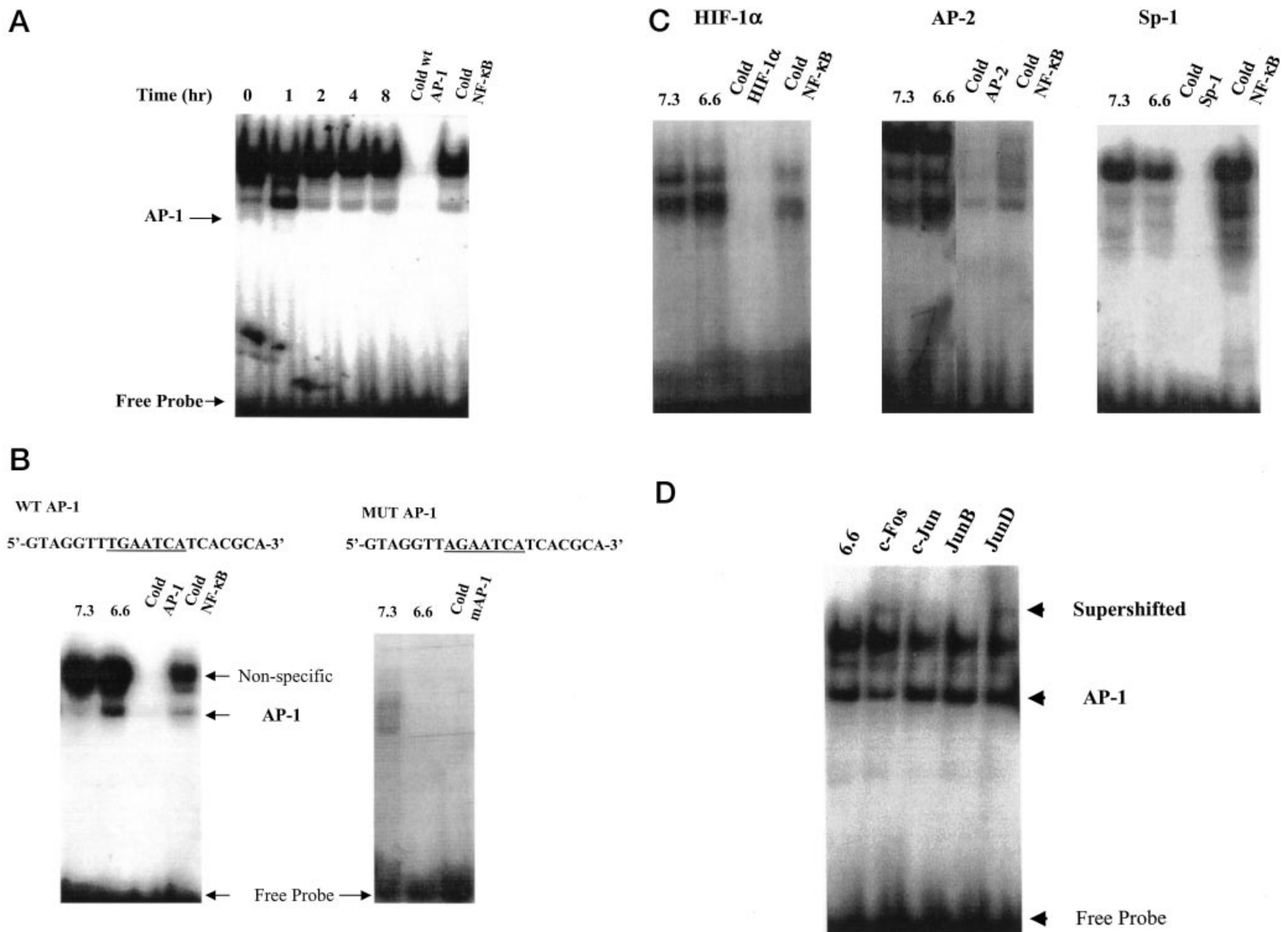

FIG. 4. **Gel mobility shift assay.** *A*, nuclear proteins were extracted from U87 MG cells incubated in normal or acidic pH medium for 1, 2, 4, and 8 h. Binding reactions were performed. All *lanes* contain the labeled AP-1 probe. *B*, acidic pH-induced AP-1 DNA binding activity is prevented by mutation in the AP-1 probe. Binding reactions were performed with the same nuclear extracts as above. All *lanes* contain the radiolabeled probe with wild-type AP-1 or mutant AP-1 binding site. *C*, binding reactions with HIF-1a, AP-2, and Sp-1 probes. *D*, supershift assay. Antibodies against c-Fos, c-Jun, JunB, and JunD were incubated with the gel shift binding reaction. Images shown are representative of at least three independent experiments.

formation of the complex was inhibited by the unlabeled AP-1 probe and unaffected by NF-κB probe. Point mutations introduced into the AP-1 oligonucleotide prevented the formation of the shifted complex (Fig. 4*B*). In combination with the reporter gene data, this finding suggests that AP-1 complex mediates the acidic pH activated *VEGF* transcription.

Addition of radiolabeled HIF-1a, Sp-1, and AP-2 oligonucleotide probes to the nuclear extract did not reveal any differences in the binding pattern and affinity of the DNA-protein complexes in neutral and acidic pH medium (Fig. 4*C*). This result agrees with the serial deletion promoter assay and suggests that HIF-1a, AP-2, and Sp-1 binding sites are not involved in VEGF induction by acidic pH in U87 MG cells.

The transcription factor AP-1 is composed of members of the Fos family (c-Fos, Fos-B, Fra-1, and Fra-2) and the Jun family (c-Jun, JunB, and JunD) that form restricted homo- or heterodimers (27). Therefore, we performed supershift assays to determine which AP-1 family member is activated and involved in the formation of the complexes by using antibodies against c-Jun, c-Fos, JunB, and JunD. Supershifted bands were detectable with antibody against c-Fos and JunD (Fig. 4*D*). Altogether, these data demonstrate that AP-1 transcription factors are activated and bind to the VEGF promoter in acidic pH medium.

Acidic pH Activates ERK 1/2 MAPK Pathway—MAPKs are involved in the induction of c-Fos and Jun, which heterodimerize to form AP-1 complexes and bind to the promoter region to activate gene transcription (18). Thus, we sought to determine whether MAPKs are activated during acidic pH and mediate VEGF induction. Using an anti-phospho-ERK1/2 antibody, which recognizes the active forms of ERK1/2 (p42/p44), a hyperphosphorylated form of ERK1/2 could be detected after 10-min incubation in acidic medium (Fig. 5*A*). Using antibodies raised against total ERK1/2, no modification in the level of ERK MAPK expression was detected (Fig. 5*A*). This result indicates that the increase in ERK1/2 phosphorylation was not due to an increase in total ERK1/2 proteins production. Furthermore, the phosphorylation of ERK1/2 was inhibited by pre-treatment of the cells with specific inhibitors of MEK1, PD98059 (50 μM), and U0126 (10 μM) (Fig. 5*B*). We observed a more potent inhibitory effect of U0126 on phosphorylation of ERK1/2, because U0126 inhibits constitutively active MEK1 with an IC_{50} of 0.07 μM, whereas PD98059 inhibits at an IC_{50} of 10 μM (28). A Western blot for phospho-JNK/SAPK and p38 MAPK did not reveal any detectable phosphorylated kinases (data not shown).

Because many of the nuclear substrates of MAPK are transcription factors, MAPK nuclear translocation is thought to represent a crucial step in the modulation of gene expression. Therefore, we tested whether ERK1/2 translocates to the nucleus under acidic pH. Fluorescent immunostaining shows basal levels of phospho-ERK1/2 predominantly cytoplasmic in

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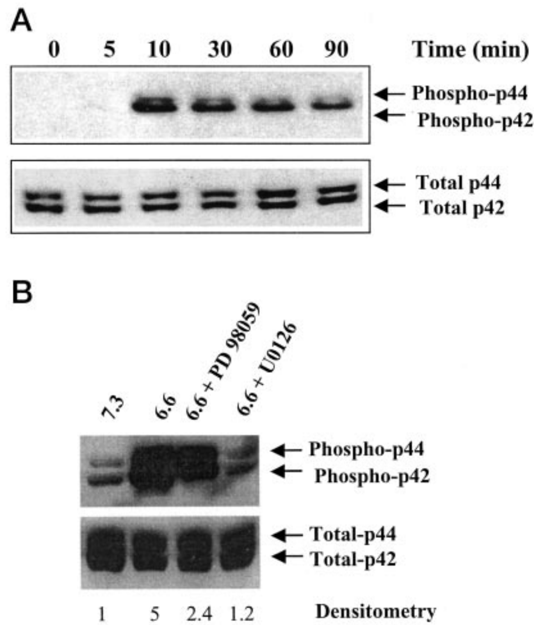

FIG. 5. **Western blot of ERK1/2 MAPK in acidic pH.** *A*, U87 MG cells were exposed to either neutral pH or acidic pH for different time points. Expression of phosphorylated ERK1/2 was determined by Western blot analysis. *B*, U87 MG cells were incubated in neutral or acidic pH for 1 h, or pretreated with PD98059 (50 μM) or U0126 (10 μM) for 1 h in neutral pH and then incubated in acidic pH medium with the corresponding inhibitors for 1 h. Western blots were performed to determine phospho-ERK1/2 expression. Images shown are representative of at least three independent experiments.

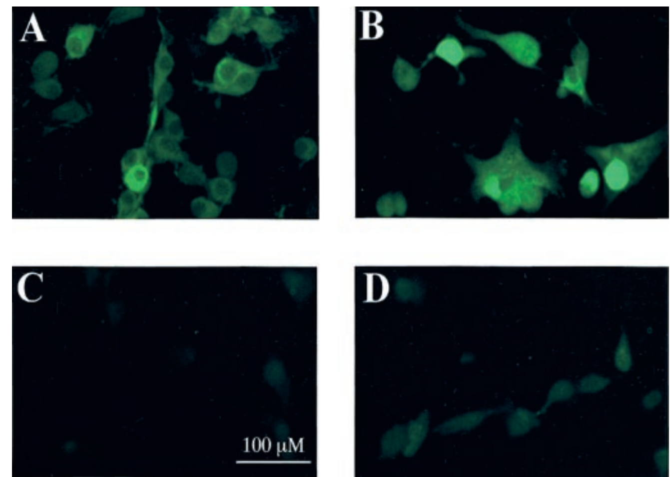

FIG. 6. **Immunofluorescence analysis of phosphorylated ERK1/2 MAPK.** U87 MG cells were grown on glass slides in 0.5% FBS for 24 h. Then: *A*, cells were incubated in pH 7.3 medium for 1 h; *B*, cells were incubated in pH 6.6 medium for 1 h; *C*, cells were pretreated with 50 μM PD98059 for 1 h and then incubated in pH 6.6 medium with PD98059 for 1 h; *D*, cells were pretreated with 10 μM U0126 for 1 h and then incubated in pH 6.6 medium with U0126 for 1 h. Staining condition and exposures for the fluorescence photomicrographs were identical for all the panels. *Scale bar*, 100 μM. Images shown are representative of three independent experiments.

cells incubated in pH 7.3 medium (Fig. 6*A*), whereas it is relocated to the nucleus in cells incubated under acidic pH (Fig. 6*B*). Addition of the specific MEK inhibitor PD98059 and U0126 was sufficient to block the phosphorylation and nuclear translocation of ERK1/2 MAPK in response to acidic pH.

To investigate whether this hyperphosphorylated ERK1/2 MAPK correlated with functional activation of MAPK, an *in vitro* MAPK assay was performed. U87 MG cells were incubated in neutral or acidic pH medium for different time periods. Phosphorylation of Elk-1 by immunoprecipitated phospho-ERK1/2 was detected by a phospho-specific anti-Elk-1 antibody. At time 0 (before incubation in acidic pH medium) a basal level of phosphorylated Elk-1 was detected; a significant induction in the level of phosphorylated Elk-1 was detected 15 min after incubation in acidic pH medium (Fig. 7), indicating phosphorylation of ERK1/2 is accompanied by its functional activation.

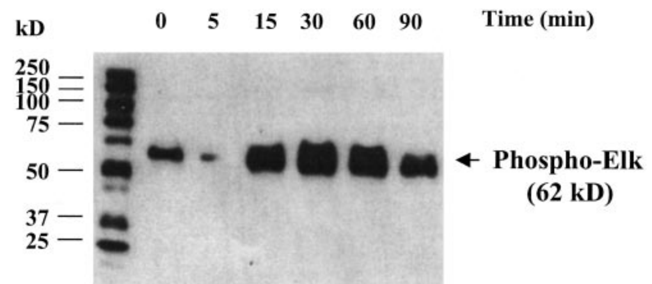

FIG. 7. ***In vitro* MAPK assay.** Subconfluent cultures of U87 MG cells were grown in 0.5% FBS medium for 24 h at 37 °C. U87-MG cells were incubated in neutral or acidic pH medium for different time periods. *In vitro* kinase assay was performed. Images shown are representative of at least three independent experiments.

To further confirm that the ERK pathway is involved in the acidic pH-induced VEGF expression, we tested whether the induction of *VEGF* transcripts was inhibited by the MEK1 inhibitors, PD98059 and U0126. Northern blot analysis showed that the induction of *VEGF* mRNA by acidic pH was decreased by treatment with the MEK1 inhibitors (Fig. 8). These results further confirmed that ERK MAPK is required in acidic pH up-regulation of VEGF.

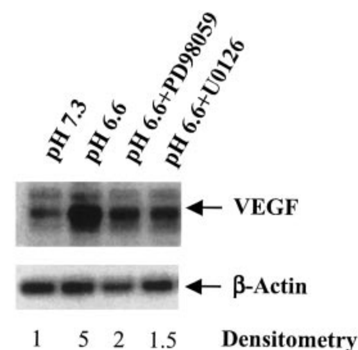

FIG. 8. **MEK1 inhibitors inhibit acidic pH-induced *VEGF* mRNA expression.** U87 MG cells were pretreated with PD98059 (50 μM) or U0126 (10 μM) for 1 h in neutral pH, and then incubated in acidic pH medium with the corresponding inhibitors for 3 h. For controls, U87 MG cells were preincubated with neutral pH medium without the inhibitors, and then incubated in either neutral pH or acidic pH medium without the inhibitors for 3 h. mRNA were extracted for Northern blot analysis. Images shown are representative of at least three independent experiments.

Finally, we demonstrated that Ras and Raf promoted the *VEGF* transcriptional activation in human glioblastoma cells. U87 MG cells were co-transfected with the full-length VEGF promoter-luciferase, pRL-TK, and plasmid containing RasV12 (constitutively active form of the Ras) or RasN17 (dominant negative form of the Ras) or RafCAAX (constitutively active Raf) or RafS621A (dominant negative form of the Raf). Cells transfected with only VEGF-luc and pRL-TK were used as control. VEGF promoter activity was increased up to 3-fold under acidic pH condition. With the transfection of constitutively activated Ras or Raf, the induction of VEGF promoter activity by acidic pH increased to 4- to 5-fold, whereas transfection of a plasmid that expresses the dominant negative form of Ras and Raf completely abolished the induction of VEGF

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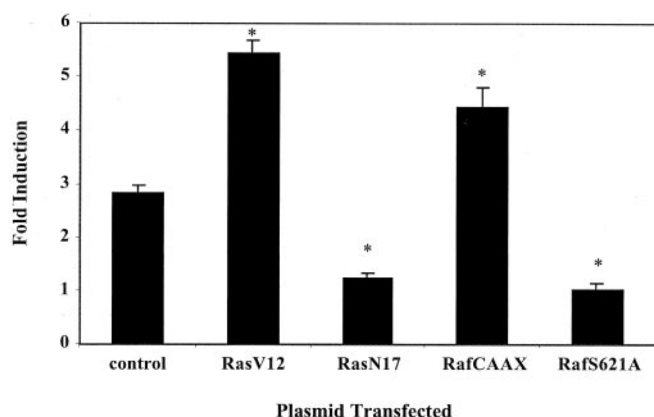

FIG. 9. **Effect of Ras and Raf on VEGF promoter activity.** 3×10^5 U87 MG cells were plated in a 10-cm dish and co-transfected with full-length VEGF promoter-*luciferase* (15 μg), pRL-TK (3 μg), and 15 μg of each of the RasV12, RasN17, RafCAAX, or RafS621A. Cells were split into six-well plates and incubated in low serum medium until confluent. Then cells were incubated in pH 7.3 or pH 6.6 for 6 h before harvesting for luciferase assays. The -fold induction is calculated by comparing the normalized luciferase activity from cells incubated in pH 6.6 medium to that of cells incubated in pH 7.3 medium. Data are expressed as the mean ± S.D. of three independent experiments. *, statistically significant difference ($p < 0.001$).

promoter activity by acidic pH (Fig. 9), suggesting that Ras and Raf are critical for mediating *VEGF* transcriptional activation by acidic pH.

DISCUSSION

Many recent studies showed that the factors most influential in the control of the expression of VEGF are those associated with tumor microenvironmental conditions. Because glioblastomas commonly overexpress VEGF and have a low extracellular pH of 6.78–6.93 (14), they provide an ideal system for examining the mechanisms that up-regulate VEGF by the host microenvironment. We established a link between tumor acidic pH and VEGF up-regulation in U87 MG human glioma cells. Acidic pH has been shown to up-regulate VEGF and basic fibroblast growth factor expression in human endothelial cells (29) and various tumor cells (15, 30), as well as interleukin-8 expression in human ovarian carcinoma cells (31) and in human pancreatic cancer cells (32). On the other hand, some studies also show that acidic pH decreases the expression of VEGF and extracellular matrix proteins gene expression in mouse osteoblasts (33, 34). It has been suggested that the lower pH and decreased VEGF production serve to regulate osteogenesis negatively in areas that first require resorption by resident osteoclasts (33). These observations suggest that variable responses of different cells to the same stimuli are relevant to different biological functions. In solid tumors, which often have insufficient blood flow, an effective means of overcoming the limitations of poor perfusion is to up-regulate VEGF and stimulate the angiogenic response.

To better define the signaling pathway leading to the transcriptional activation of the VEGF promoter by acidic pH, we used reporter gene analysis with serial deletion constructs of the VEGF promoter. The human VEGF promoter contains sequences corresponding to the HIF-1 binding site, 5′-TACGTGGG (−975 to −968 bp), which has been shown to be essential for VEGF induction by hypoxia (35). It contains the AP-1 binding site, 5′-TGACTAA (−937 to −931 bp), which has been shown to also be involved in hypoxic induction of VEGF in glioma cells (36). It also contains AP-2 and Sp-1 binding sites located in the promoter region −85 to −50 bp that confers responsiveness to transforming growth factor-α, interleukin-1β, and platelet-derived growth factor (37–40). Our deletional analysis of the VEGF promoter showed that deletion of the region containing the AP-1 binding site abolished the activation of VEGF promoter by acidosis. However, whether this region also contains a specific pH response element different from the AP-1 binding site is not known, and further deletion within this region is required.

We observed that the DNA binding activity of AP-1 is induced in acidosis. With the use of specific antibodies, we showed that c-Fos and JunD members of the AP-1 family are components of the complex that was induced by acidic pH. AP-1 is known to potentiate the HIF-1-mediated hypoxia-induced transcriptional activation of VEGF in C6 glioma cells (36). It also mediates the induction of VEGF expression by hypoglycemia in human hepatoblastoma cells (41) and by ionizing radiation in human glioblastoma cells (42). The increase in AP-1 activity in response to stimuli, such as UV and tumor necrosis factor-α, could be due to increased Jun and c-Fos synthesis and phosphorylation. We are presently investigating whether this increased binding affinity is regulated by phosphorylation or mediated by an increase in the amounts of both factors.

ERK1/2 MAPK phosphorylates proteins from the plasma membrane to the nucleus (43). In unstimulated cells, ERK1/2 are largely cytoplasmic and relocalize to the nucleus upon stimulation (44–46). The ERK group of MAPKs phosphorylates Elk-1. Elk-1 activates c-Fos, which, upon translocation to the nucleus, combines with pre-existing Jun proteins to form AP-1 dimers that leads to higher levels of AP-1 DNA binding activity (47, 48). Most cell types contain some c-Jun protein prior to their stimulation, which is activated by SAPK/JNK phosphorylation (48). Our study shows that only ERK MAPK activity is activated in acidic pH conditions, and it translocates to the nucleus to phosphorylate its downstream pathway member, Elk-1. However, ERK MAPK may also activate other transcription factors that contribute to the activation of AP-1 and mediate VEGF induction by acidic pH.

The role of ERK MAPK in regulating cell proliferation and promoting cell survival is well defined (49–51). Recent studies show that ERK MAPK is also activated during hypoxia in human endothelial cells (52), normal and transformed fibroblasts (16), and human cervical carcinoma cells (53). Its activation is involved in phosphorylation of HIF-1α (16, 52, 54) and induces transcription of *c-fos* (53). Our results are in agreement with these studies suggesting that the functions of ERK MAPK are not limited to cell proliferation and survival but are also involved in cellular response to extracellular stress. Phosphorylated p38 MAPK and JNK/SAPK were undetectable by Western blot in our study. However, our results suggest that, even with the inhibition of ERK1/2 MAPK, VEGF mRNA up-regulation by acidic pH was not completely inhibited. These results indicate that other signaling pathways may also contribute to the VEGF induction.

Ras mediates Raf activation and, thus, initiates MAPK activation. This leads to an increase in the expression of c-Jun and c-Fos and therefore to an increased AP-1 activity (55). The expression and secretion of VEGF are induced by activated oncogenes such as *ras* and *raf* (56, 57). Ras and Raf activation has also been shown to be involved with hypoxia in up-regulating VEGF (55). We analyzed the effect of the dominant negative form of Ras and Raf on the induction of VEGF by acidic pH. Our results showed that dominant negative forms of Ras and Raf abolished the acidic pH-induced *VEGF* transcriptional activation. Several studies have shown that the phosphatidylinositol 3-kinase/Akt signaling pathway mediates hypoxia-induced VEGF expression in *ras*-transformed cells (58). Whether phosphatidylinositol 3-kinase/Akt is involved in acidic

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pH signal transduction and what are the roles of other signaling pathways are currently under investigation.

Our results have strong implications in our understanding of the pathophysiological mechanisms involved in the acquisition of the angiogenic phenotype. The data presented here provide evidence that acidic pH activates *VEGF* gene transcription. The transcriptional activation of *VEGF* gene is mediated by AP-1 and requires the ERK MAPK. There is now urgent need to decipher how the acidic pH signal is sensed by the cells and which transduction pathways are implicated in VEGF induction.

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Additions and Corrections

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Acidic extracellular pH induces vascular endothelial growth factor (VEGF) in human glioblastoma cells via ERK1/2 MAPK signaling pathway. Mechanism of low pH-induced VEGF.

Lei Xu, Dai Fukumura, and Rakesh K. Jain

Page 11369, paragraphs 6 and 8 under "Experimental Procedures": "mg" should be "μg."

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