

# EGFR Activation Results in Enhanced Cyclooxygenase-2 Expression through p38 Mitogen-Activated Protein Kinase–Dependent Activation of the Sp1/Sp3 Transcription Factors in Human Gliomas

Kaiming Xu and Hui-Kuo G. Shu

Department of Radiation Oncology, Emory University, Atlanta, Georgia

## Abstract

Expression of cyclooxygenase-2 (COX-2) has been linked to many cancers and may contribute to malignant phenotypes, including enhanced proliferation, angiogenesis, and resistance to cytotoxic therapies. Malignant gliomas are highly aggressive brain tumors that display many of these characteristics. One prominent molecular abnormality discovered in these astrocytic brain tumors is alteration of epidermal growth factor (EGF) receptor (EGFR) through gene amplification and/or mutation resulting in excessive signaling from this receptor. We found that EGF-mediated stimulation of EGFR tyrosine kinase in human glioma cell lines induces expression of both COX-2 mRNA and protein. The p38 mitogen-activated protein kinase (p38-MAPK) pathway was a strong downstream factor in this activation with inhibition of this pathway leading to strong suppression of COX-2 induction. The p38-MAPK pathway can activate the Sp1/Sp3 transcription factors and this seems necessary for EGFR-dependent transactivation of the COX-2 promoter. Analysis of COX-2 promoter/luciferase constructs revealed that transcriptional activation of the COX-2 promoter by EGFR requires the Sp1 binding site located at –245/–240. Furthermore, Sp1/Sp3 binding to this site in the promoter is enhanced by EGFR activation both *in vitro* and *in vivo*. Enhanced DNA binding by Sp1/Sp3 requires p38-MAPK activity and correlates with increased phosphorylation of the Sp1 transcription factor. Thus, EGFR activation in malignant gliomas can transcriptionally activate COX-2 expression in a process that requires p38-MAPK and Sp1/Sp3. Finally, treatment of glioma cell lines with prostaglandin E<sub>2</sub>, the predominant product of COX-2 activity, results in increased vascular endothelial growth factor expression, thus potentially linking elevations in COX-2 expression with tumor angiogenesis in malignant gliomas. [Cancer Res 2007;67(13):6121–9]

## Introduction

Cyclooxygenases (COX) are crucial rate-limiting enzymes in the biosynthesis of prostaglandins (for review, see ref. 1). They exist in two isoforms, COX-1, which is constitutively expressed in most tissues and regulates physiologic level of prostaglandins, and COX-2, which is inducible and rapidly increases in response to various stimuli, including mitogens, cytokines, growth factors, and inflammatory signals in many types of cells. A growing body of

evidence indicates that COX-2 can play important roles in tumor onset and growth (2), metastasis (3), angiogenesis (4), and resistance to chemotherapy and radiotherapy (5, 6). COX-2 is aberrantly overexpressed in many human cancers, including those arising from the colon and lung (7–9).

Malignant gliomas are the most common primary brain tumor in adults. Previous studies revealed that COX-2 protein is often detectable in human glioma samples, especially those of higher grade, and that more mitotically active gliomas, in particular, display higher COX-2 expression than their lower grade and less proliferative counterparts (10, 11). Higher tumoral expression of COX-2 also strongly correlated with shorter survival in malignant glioma patients and this factor was found to be independently prognostic (11). Few investigators have focused on the biological significance of COX-2 expression in malignant gliomas; however, initial studies have shown that COX-2 inhibition can suppress the growth and migration of glioblastoma cell lines *in vitro* (10, 12). These results suggest that COX-2 could contribute to the malignant phenotype of gliomas *in vivo* and may offer a unique opportunity for therapeutic intervention. Therefore, understanding the regulation of COX-2 expression in these tumors is of great interest.

Epidermal growth factor (EGF) receptor (EGFR), a receptor tyrosine kinase, is commonly altered in malignant gliomas leading to abnormally regulated kinase activity and excessive downstream signaling, the result of which is believed to contribute to the aggressive phenotype seen in these tumors (13–17). Because abnormal signaling through EGFR and excessive COX-2 expression both seem to contribute to the malignancy of high-grade glioma, we hypothesized that these factors may be related. To date, it has not been determined whether EGFR signaling alters the expression of COX-2 in these tumors. In this study, we show that COX-2 expression is strongly induced by EGF stimulation of human glioma cell lines. We further investigated the mechanisms by which EGFR signaling regulates COX-2 expression in these cells and found that the p38 mitogen-activated protein kinase (p38-MAPK) is the major signaling pathway that contributes to EGFR-dependent COX-2 induction. The p38 pathway seems to increase COX-2 promoter activity through phosphorylation and activation of the Sp1/Sp3 transcription factors.

## Materials and Methods

**Plasmids.** The wild-type COX-2 promoter DNA from bases –1,122 to +27 relative to the transcriptional start site was obtained by PCR of human genomic DNA and sequence was confirmed. This fragment was inserted upstream of the luciferase open reading frame in an MuLV-based retroviral vector that also contains the neomycin resistance gene expressed from the 5' long terminal repeat (18). This constructed plasmid was labeled pCOX2/P1. The Sp1 mutant of COX-2 promoter construct, pCOX2/P1m, was created by mutating the Sp1 binding site at –245 to –240 in the pCOX2/P1 from GGGAGG to GTTAAC by PCR-based, site-directed mutagenesis. MKK3be

**Requests for reprints:** Hui-Kuo G. Shu, Department of Radiation Oncology, Emory University, 1365 Clifton Road, Northeast, Building C, Room C-3082, Atlanta, GA 30322. Phone: 404-778-2161; Fax: 404-778-5520; E-mail: hui-kuo@radonc.emory.org.

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and MKK6be, constitutively active forms of these kinases, inserted in the mammalian expression vector pcDNA3 (Invitrogen) were provided by Dr. Jiahui Han (The Scripps Research Institute, La Jolla, CA; ref. 19).

**Cell lines, culture conditions, and transfections.** Glioma cells lines (LN229 derivatives, U87, U343, U1242, SF188, and SF767) were cultured in high-glucose DMEM (Sigma-Aldrich) supplemented with 10% FCS (Sigma), sodium pyruvate (1 mmol/L), and penicillin (100 units/mL)/streptomycin (100 µg/mL) unless otherwise indicated. The LN229/puro and LN229/ER (high expression of EGFR) cell lines were previously described (20). The COX-2 promoter/luciferase constructs (pCOX2/P1, pCOX2/P1m) were stably introduced into the SF767 cell line for functional COX-2 promoter analysis. Each retroviral vector was transiently transfected into Phoenix cell (amphotropic retroviral packaging line) by a CaPO<sub>4</sub> precipitation method. SF767 cells were successively infected with conditioned supernatants containing virus thrice at 12 h intervals and subsequently selected in 1 mg/mL of G418 (Invitrogen) for a minimum of 10 days. To express constitutively active MKK3 and MKK6, pcDNA3/MKK3be and pcDNA3/MKK6be were transiently transfected into SF767 cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations. For small interfering RNA (siRNA) suppression, SF767 cells were transfected with commercially available siRNA against p38-MAPK and Sp1 (Santa Cruz Biotechnology) using LipofectAMINE 2000. The Stealth RNAi medium GC duplex negative control (Invitrogen) was used to control for sequence-independent effects of introducing short RNA duplexes into cells.

**Growth factor and inhibitor treatments.** EGF (100 ng/mL, Roche) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>; concentrations used are noted, Sigma) were used to treat glioma cell lines that were serum starved for 24 h before stimulation except as otherwise noted. DMSO stocks of inhibitors against the following kinases, EGFR (PD153035, Tocris Bioscience), p38-MAPK (SB202190, Calbiochem), MAP/ERK kinase (U0126, Calbiochem), phosphatidylinositol 3-kinase (PI3K; LY294002, Calbiochem), and c-Jun-NH<sub>2</sub>-kinase (JNK; SP600125, Calbiochem), were made and working concentrations for a particular inhibitor are noted in the figure legends with vehicle only (DMSO) used as a negative control. Inhibitors against the following transcription factors, Sp1/Sp3 (mithramycin, Sigma), nuclear factor-κB (NF-κB; SN50, Calbiochem), and NFAT (cyclosporine A, LKT), were also made as stock solutions in DMSO and used at concentrations noted for each experiment.

**Western blotting and antibodies used.** All Western blots were done according to standard procedures. SDS lysis buffer [62.5 mmol/L Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/L DTT, 0.01% bromophenol blue] was used. Genomic DNA was sheared in the lysates by sonication. Equal amount of lysate was resolved by SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membrane. Blots were probed with antibodies against human EGFR (Santa Cruz Biotechnology), COX-2 (Cayman Chemical), p38-MAPK/phosphorylated p38-MAPK (Cell Signaling Technology), and Sp1 and Sp3 (Santa Cruz Biotechnology). As loading controls, blots were also probed with antibodies against either actin (Santa Cruz Biotechnology) or EIF5α (Santa Cruz Biotechnology), where appropriate. Blots were detected using anti-rabbit or mouse IgG secondary antibody conjugated with horseradish peroxidase and chemiluminescent substrate.

**RNase protection assay.** RNase protection assay (RPA) was done by standard techniques as previously described (21). Briefly, 5 µg of total RNA were incubated with the <sup>32</sup>P-labeled riboprobe of interest in 10 µL hybridization buffer [40 mmol/L HEPES (pH 6.4), 300 mmol/L NaCl, 80% formamide, 1 mmol/L EDTA] overnight at 42°C and digested with RNase A/T<sub>1</sub>. Protected riboprobes were then precipitated by ethanol, resolved on a 5% urea/polyacrylamide gel, and detected by phosphor imaging on a Typhoon 9210 using ImageQuant software from Molecular Dynamics (GE Healthcare). Cyclophilin was used as the internal normalization control. The riboprobe template for detection of cyclophilin was obtained from Ambion. The riboprobe template for detection of COX-2 is made from a vector termed pKS/ribo/rec/COX-2 containing bases 61 to 378 of the human COX-2 cDNA linearized with *Xho*I. The COX-2 template produces a 380 base runoff transcript with a protected size of 317 bases. The riboprobe template for detection of vascular endothelial growth factor (VEGF) is made from a

vector termed pKS/ribo/VEGF containing bases 1,019 to 1,347 of the human VEGF cDNA linearized with *Nco*I (within coding region). A 317 base runoff transcript is produced with a protected size of 232 bases.

**Luciferase reporter assay.** The human COX-2 promoter/luciferase reporter constructs were introduced stably into the SF767 glioma cell line by retroviral transduction as described above. Two different pools of cells that stably express luciferase were made for each retroviral construct. For each construct, both pools were used and therefore the presented results were from each of the two pools done in triplicate. After overnight serum starvation, glioma cells were treated with EGF (100 ng/mL) for 5 h and harvested. Luciferase activity was measured as described previously (22).

**Electrophoretic mobility shift assay.** Electrophoretic mobility shift assays (EMSA) were done by standard techniques. Briefly, SF767 cells were treated with EGF for 30 min before harvesting. Nuclear protein extracts were prepared by standard techniques. Briefly, cells were lysed in hypotonic lysis buffer [10 mmol/L KCl, 20 mmol/L HEPES (pH 7.9), 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1× protease inhibitor cocktail (Sigma), and 10% glycerol] with Dounce homogenization. Nuclei was pelleted and resuspended in nuclear extract buffer [420 mmol/L NaCl, 10 mmol/L KCl, 20 mmol/L HEPES (pH 7.9), 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, 1× protease inhibitor cocktail (Sigma), and 20% glycerol] on ice for 30 min. Extract is spun and supernatant was used for binding reaction. A 22-mer containing the Sp1 consensus site (Santa Cruz Biotechnology) and two 22-mers from the human COX-2 promoter containing either the wild-type (COX/wtSp) or the mutated (COX/mtSp) Sp1 site were used. Sense sequences of oligonucleotides used are as follows: (Sp1 consensus site) 5'-attcgcggtggggcgagc-3', (COX/wtSp) 5'-gaggagagggaggatcaga ca-3', and (COX/mtSp) 5'-gaggagagT-TaACgatcagaca-3' (mutated bases are capitalized and italicized). Double-stranded oligonucleotides were end-labeled using T4 polynucleotide kinase and [<sup>γ</sup>-<sup>32</sup>P]ATP. Probes were purified using Sephadex G25 Quick Spin columns (Roche). Five micrograms of nuclear extracts were incubated in a final volume of 10 µL containing 20 mmol/L HEPES (pH 7.9), 4% Ficoll, 1 mmol/L MgCl<sub>2</sub>, 40 mmol/L KCl, 0.1 mol/L EGTA, 1 mmol/L DTT, 1 µg poly(deoxyinosinic-deoxycytidylic acid), and 1 ng labeled probe at room temperature for 20 min, and then resolved on a 5% nondenaturing polyacrylamide gel. The gels were dried and detected by phosphor imaging as described above. For detection of Sp1 or Sp3 by supershift on EMSA, antibodies against Sp1 or Sp3 (Santa Cruz Biotechnology) were premixed with nuclear extracts for 10 min before mixing with the labeled probe.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) assay was done according to standard protocol available from Upstate Biotechnology. Immunoprecipitation was done with antibodies against Sp1 or Sp3 (Santa Cruz Biotechnology). The amount of COX-2 promoter DNA associated with Sp1 or Sp3 was determined by hot semiquantitative PCR using the following primer pair: (forward) 5'-cgggcaaaagactcgaagaagaaa-3' and (reverse) 5'-aaccaagccatgtgacgaatgac-3'; this generates a 280 base product that encompasses the Sp1 site at -245 to -240. The forward primer was labeled using T4 polynucleotide kinase and [<sup>γ</sup>-<sup>32</sup>P]ATP and ~0.5 pmol of hot primer was added to a PCR reaction containing 10 pmol of each cold primers. PCR conditions are as follows: 94°C × 30 s, 58°C × 30 s, and 72°C × 45 s for 20 cycles. The PCR products were resolved on a 5% polyacrylamide gel. Gels were dried and detected by phosphor imaging as described above. A portion of the DNA recovered from an aliquot of sheared chromatin was used as the "input" sample. Normal rabbit serum was used as a negative control.

**In vivo <sup>32</sup>P labeling.** To detect phosphorylation of the Sp1 transcription factor, *in vivo* [<sup>32</sup>P]P<sub>i</sub> labeling of SF767 cells was done. After serum starvation for 24 h, SF767 cells were incubated in phosphate-free medium for 1 h, and then labeled in the medium containing 200 µCi [<sup>32</sup>P]P<sub>i</sub>/mL (Amersham) for 2 h. At the time of labeling, cells were pretreated with SB202190 (20 µmol/L), U0126 (20 µmol/L), LY294002 (25 µmol/L), or vehicle only (DMSO) for 2 h followed by stimulation with EGF (100 ng/mL) for 30 min. After stimulation, the cells were harvested and lysed in the lysis buffer containing 50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, protease inhibitor cocktail 111 (Sigma), and phosphatase inhibitor cocktail 1 and 11 (Sigma). For SF767 cells transiently

transfected with pcDNA3 (vector alone), pcDNA3/MKK3be, or pcDNA3/MKK6be, preparation for the labeling process was initiated 16 h after transfection (serum starvation for 24 h followed by incubation in phosphate-free medium  $\times$  1 h). Cells were subsequently *in vivo* labeled in the medium containing 200  $\mu$ Ci [ $^{32}$ P] $P_i$ /mL for 4 h and then lysed as described above. Immunoprecipitation of Sp1 was then done. Briefly, protein lysates were precleared with protein A agarose beads (Roche) and then incubated with protein A beads prebound to anti-Sp1 antibody (Santa Cruz Biotechnology) at 4°C overnight. Beads were subsequently washed five times with lysis buffer, resuspended in 50  $\mu$ L SDS sample buffer, and heated to 95°C for 5 min to release the immunoprecipitated protein. Samples were resolved by 7.5% SDS/PAGE and transferred onto PVDF membrane. Identification of  $^{32}$ P-labeled Sp1 was accomplished by overnight exposure to a phosphor imaging screen and detection as described in a previous section. Western blotting for Sp1 with chemiluminescent detection was then done on the filter to determine total Sp1 protein levels.

## Results

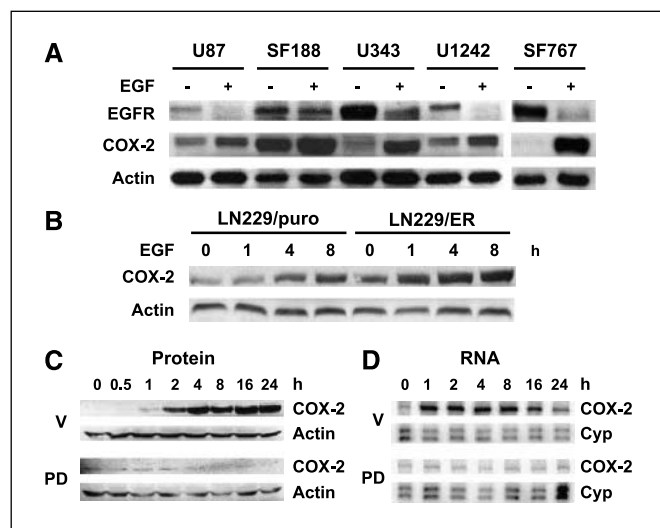
**EGF-dependent induction of COX-2 in multiple human glioma cell lines.** To determine whether COX-2 is expressed or induced by EGF in human glioma cells, we assessed five glioma cell lines by immunoblot assay. As shown in Fig. 1A, these glioma cells displayed a range of endogenous EGFR and basal COX-2 protein expression. Eight hours after stimulation with EGF, EGFR expression was consistently decreased due to internalization and degradation as has been previously shown following ligand engagement and receptor activation (Fig. 1A; ref. 23). Interestingly, COX-2 protein was significantly induced to varying degrees in all the cell lines assayed (Fig. 1A). To determine whether EGFR expression levels correlate with the degree of COX-2 induction by

EGF, we used the LN229 glioma cell line engineered to overexpress EGFR (LN229/ER) along with its corresponding isogenic vector only control cells (LN229/puro), which only expresses low endogenous levels of EGFR (20). Western blot results indicate that although both cell types display EGF-induced COX-2 expression, EGFR overexpression resulted in a greater degree of COX-2 induction (Fig. 1B).

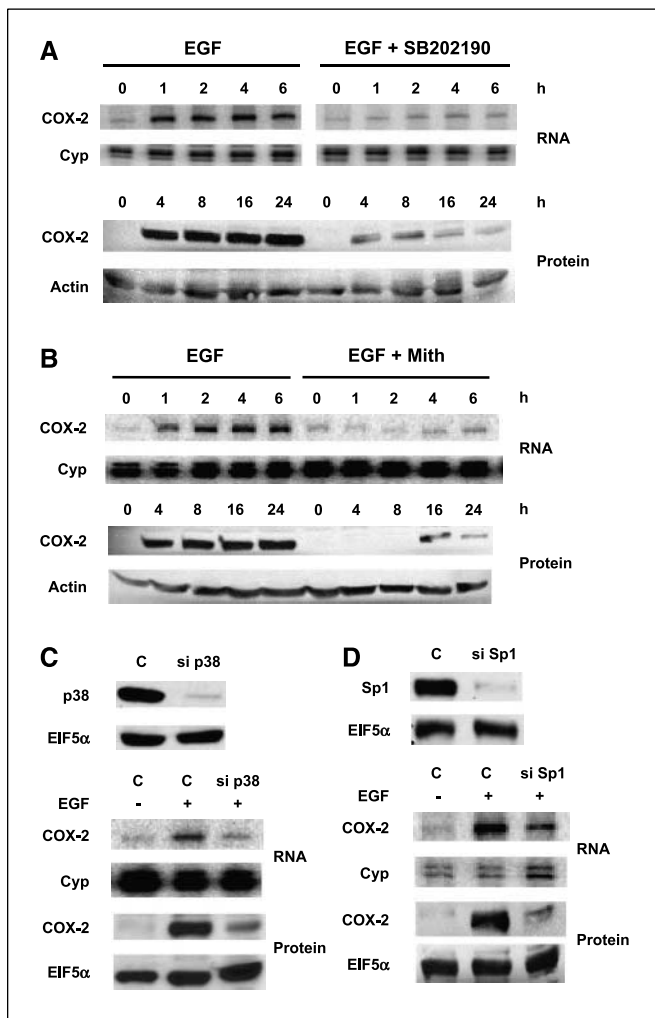
**Characterization of COX-2 induction by EGF at the protein and mRNA levels.** To further characterize induction of COX-2 in glioma cells, we did more detailed studies on the SF767 line that displayed low basal levels but very robust EGF-dependent induction of COX-2 expression. EGF dose-response studies done in SF767 indicated that doses as low as 10 ng/mL still induced COX-2 protein to a significant extent (data not shown). Time course studies were also done in SF767 cells. These studies showed that COX-2 protein was induced within 1 h after EGF stimulation reaching its peak at approximately the 4 h time point and maintained these peak levels for at least 24 h after stimulation with ligand (Fig. 1C, left). RNase protection assays confirmed that COX-2 mRNA was similarly induced (Fig. 1C, right). We and others have used the specific quinazoline-based EGFR inhibitor PD153035 to inhibit EGFR kinase activity (20, 24). We found that EGF-dependent induction of COX-2 at both the protein and mRNA levels was completely blocked by PD153035 (Fig. 1C). These results indicate that activation of EGFR signaling is responsible for the observed COX-2 induction.

**Involvement of p38-MAPK and Sp1 and/or its family members in EGF-induced COX-2 expression.** EGFR activation can result in enhanced signaling of many downstream kinase cascades. Accordingly, EGF treatment of the SF767 glioma cell line activates many of these pathways, including the p38-MAPK, MAP/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK, c-Src, and PI3K pathways (data not shown). To determine the role these kinases have on EGF-induced COX-2 expression, pharmacologic inhibition of these pathways were done. In the SF767 cells, we consistently found that the p38-MAPK inhibitor SB202190 was the strongest suppressor of EGF-dependent COX-2 induction. This inhibitor could suppress EGF-induced COX-2 expression both at the mRNA and protein levels (Fig. 2A and data not shown). The COX-2 promoter is under strict control through a tight regulatory network and is known to contain many trans-acting elements that bind a variety of transcription factors, including Sp1, NF- $\kappa$ B, CAAT/enhancer binding protein, cyclic AMP-responsive element binding protein, NFAT, and AP-1 that regulate the promoter activity in many cell types under various conditions (for review, see ref. 25). To test whether some of these transcription factors are involved in EGF-induced COX-2 expression in glioma cells, we used chemical inhibitors against Sp1 and related family members, including Sp3 (mithramycin), NF- $\kappa$ B (SN50), AP-1 (JNK inhibitor SP600125), and NFAT (cyclosporin A). Of these inhibitors, only mithramycin was capable of suppressing EGF-induced COX-2 expression, doing so at both the mRNA and protein levels (Fig. 2B and data not shown).

To confirm the role of p38-MAPK and Sp1 on EGF-dependent COX-2 induction, their expression was specifically inhibited by siRNA. In Fig. 2C (top), after transfection of a p38-MAPK-specific siRNA into SF767, expression of this kinase was reduced by >90%. When these cells were then assessed for COX-2 induction after treatment with EGF, the degree of induction was significantly reduced at both the mRNA and protein levels (Fig. 2C, bottom). Similarly, after transfection of siRNA against Sp1 into SF767, expression of this factor was reduced by >90% (Fig. 2D, top).



**Figure 1.** EGF-induced expression of COX-2 in glioma cell lines. *A*, immunoblots of lysates from six different glioma cell lines (U87, SF188, U343, U1242, SF767, and SF763). Cells were treated with EGF (100 ng/mL) for 8 h before harvest/lysis as described in Materials and Methods. *B*, immunoblots of lysates from LN229/puro and LN229/EGFR. Cells were treated with EGF (100 ng/mL) for the indicated times (0–8 h) before harvest. *C*, immunoblots of lysate from SF767 glioma cells treated with 100 ng/mL of EGF for increasing lengths of time (0–24 h) with and without the addition of the EGFR inhibitor PD153035 (PD; 2  $\mu$ Mol/L, treatment starting 2 h before addition of EGF). *D*, RPAs were done to quantitatively determine expression of COX-2 mRNA. Cells were treated with 100 ng/mL of EGF for increasing lengths of time (0–24 h) with or without PD153035 (2  $\mu$ Mol/L, treatment starting 2 h before addition of EGF). Actin and cyclophilin (*Cyp*) were used as normalization controls for immunoblots and RPA, respectively. In (*C*) and (*D*), control cells were treated with vehicle only (V, DMSO). Displayed results are representative of two to three independent experiments in each case.



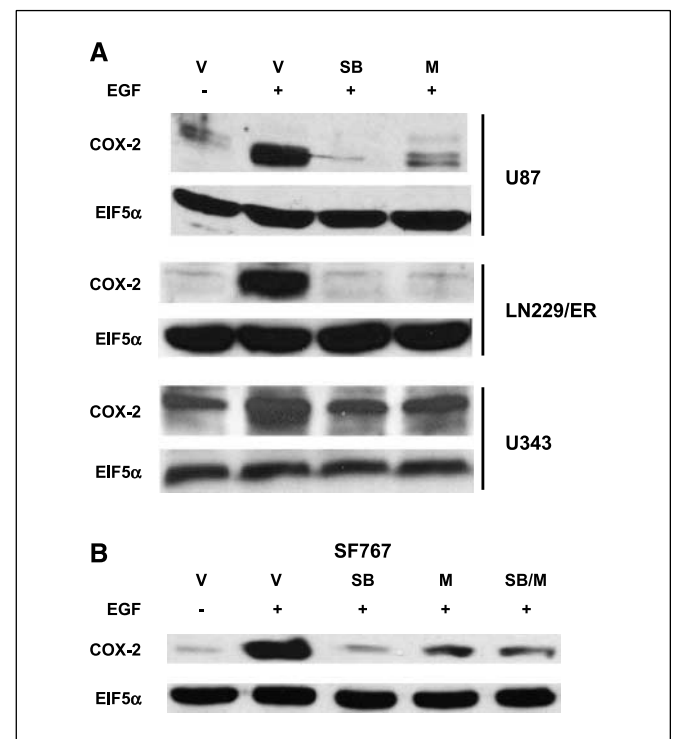
**Figure 2.** Downstream factors involved in EGF-induced expression of COX-2 mRNA and protein. *A* and *B*, RPAs and immunoblots were done on total RNA or protein lysates isolated from SF767 glioma cells stimulated with EGF (100 ng/mL) for the indicated times (0–6 h). Before addition of ligand, cells were either treated or not treated with (A) SB202190 (20  $\mu$ mol/L) or (B) mithramycin (Mith, 1  $\mu$ mol/L) for 1 h. SF767 cells were transfected with control siRNA (C) or siRNA targeting either (C) p38-MAPK (si p38) or (D) Sp1 (si Sp1). Expression assessed ~2 d after siRNA transfection shows efficient suppression p38-MAPK and Sp1 (C and D, top). To assess effect on COX-2 expression, RPAs and immunoblots were done on RNA or protein isolated from cells cultured with EGF (100 ng/mL) for 2 or 8 h, respectively, starting ~2 d after transfection of siRNA targeting p38-MAPK and Sp1, as indicated (C and D, bottom). Cyclophilin was used as the normalization control for the RPA. Actin or EIF5 $\alpha$ , as indicated, was used as normalization controls for the immunoblots. Displayed results are representative of two to three independent experiments in each case.

In these cells, COX-2 induction after treatment with EGF was again found to be significantly reduced at both the mRNA and protein levels (Fig. 2D, bottom). Overall, these results strongly suggest that the p38-MAPK pathway and the Sp family of transcription factors are critical for EGF-induced COX-2 expression.

**Role of p38-MAPK and Sp1/Sp3 on COX-2 expression in other glioma cell lines.** Our data above clearly indicates that p38-MAPK and Sp1 activities are required for induction of COX-2 expression in the SF767 glioma cell line. However, it remained possible that this regulation of COX-2 expression is not generally applicable to other glioma cell lines. To determine whether these factors are also important in other glioma cell lines, U87, U343, and LN229/EGFR, cells previously shown to display significant EGF-

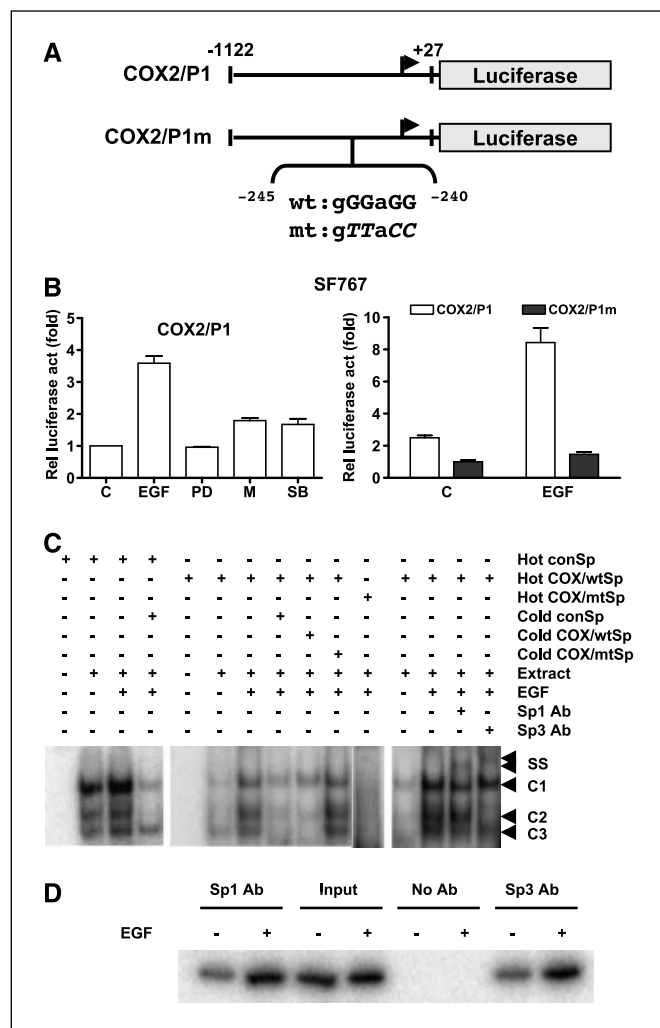
induced COX-2 expression (Fig. 1A), were assessed. Inhibition of p38-MAPK and Sp1/Sp3 was accomplished as described previously by treating with SB202190 and mithramycin, respectively. In each case, EGF-induced expression of COX-2 was significantly suppressed with this inhibition (Fig. 3A). Next, we were interested in determining the effectiveness of combining p38-MAPK and Sp1/Sp3 inhibition on suppression of EGF-dependent COX-2 expression. In this experiment, relatively low doses of each inhibitor (10  $\mu$ mol/L SB202190, 0.1  $\mu$ mol/L mithramycin) were used either alone or together on EGF-treated SF767 glioma cells. At these concentrations, each inhibitor alone was able to suppress EGF-dependent COX-2 expression to a significant extent although the p38-MAPK inhibitor was somewhat more effective. The addition of both inhibitors seemed more effective than with mithramycin alone but not with SB202190 alone, suggesting that p38-MAPK may affect COX-2 expression by non-Sp1/Sp3-dependent mechanisms, whereas Sp1/Sp3 transactivation is downstream and therefore largely dependent on p38-MAPK (Fig. 3B). In fact, initial experiments examining the role of the COX-2 3' untranslated region in response to EGF suggest that p38-MAPK-dependent increase in mRNA stability does have a minor role in regulating COX-2 expression (data not shown).

**COX-2 promoter analysis by luciferase reporter assay.** Our data presented above show that p38-MAPK and the Sp family of transcription factors is involved in EGF-induced COX-2 expression. Due to the role of the Sp factors in COX-2 induction, we



**Figure 3.** Role of p38-MAPK and Sp1 on EGF-induced COX-2 expression in other glioma cell lines. Immunoblotting was done on lysates from the indicated glioma cell lines after either no stimulation or stimulation with EGF (100 ng/mL) for 8 h (in A) or 16 h (in B). *A*, cells were treated with vehicle only (DMSO), SB202190 (SB, 20  $\mu$ mol/L), or mithramycin (M, 1  $\mu$ mol/L) for 1 h before addition of ligand. *B*, cells were treated with vehicle only (DMSO), SB202190 (10  $\mu$ mol/L), mithramycin (0.1  $\mu$ mol/L), or both SB202190 and mithramycin for 1 h before addition of ligand. Representative blots of two to three independent experiments detected with antibodies against COX-2 and EIF5 $\alpha$  are shown in each case above.

postulated that transcriptional activation was likely to be an important mechanism contributing to EGF-induced COX-2 expression. Therefore, to confirm whether this is the case, a series of experiments studying the COX-2 promoter were initiated. For



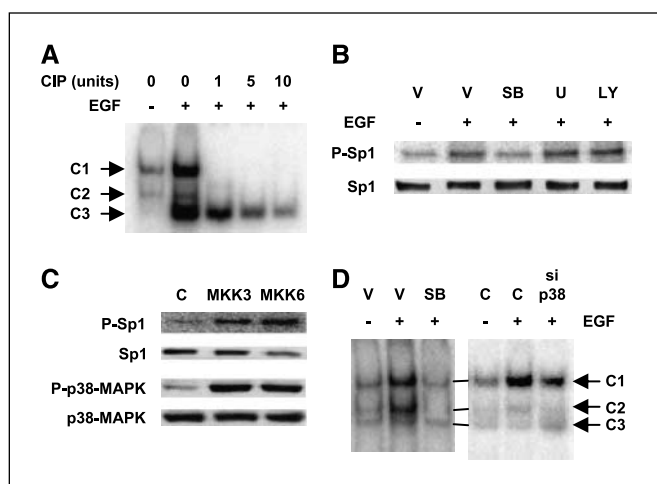
**Figure 4.** COX-2 promoter analysis. **A**, COX-2 promoter constructs used. The constructs include COX2/P1 (-1,122 to +27 from transcriptional start) and COX2/P1m (-1,122 to +27 with the indicated mutation at the Sp1 site at -245 to -240). **B**, SF767 cells were infected with retroviruses containing luciferase driven by the COX-2 promoter regions as described in Materials and Methods. Two independently generated pools of cells were used for each COX-2 reporter construct. Three determinations were done in triplicate for each pool of cells. *Columns*, relative levels of luciferase normalized to total protein in lysate with the control (no EGF or drug), which has been set at an arbitrary level of 1; *bars*, 1 SE. *Left*, luciferase activity from SF767 cells containing COX2/P1 treated with DMSO only (EGF), PD153035 (2  $\mu$ Mol/L), mithramycin (10  $\mu$ Mol/L), or SB202190 (20  $\mu$ Mol/L) for 1 h and then stimulated with EGF (100 ng/mL) for 5 h. *Right*, luciferase activity from SF767 cells containing COX2/P1 and COX2/P1m stimulated with EGF (100 ng/mL) for 5 h. **C**, representative gels of EMSA from three independent experiments using nuclear extracts from SF767 cells treated with EGF for 30 min. The labeled oligonucleotide used contains either the consensus Sp1 site (*conSp*) or sequence comprising the Sp1 site (*COX/wtSp* or *COX/mtSp*) of the human COX-2 promoter. A 40-fold molar excess of unlabeled oligonucleotide is used in lanes where a cold competitor is used. Three complexes are found in the binding reaction (*arrows*, C1, C2, and C3). For the supershift lanes, nuclear extracts were preincubated with 0.2  $\mu$ g of antibody against Sp1 or Sp3 10 min before the binding reaction (see Materials and Methods). *Arrows*, SS, supershifted complexes. **D**, ChIP assays were done on chromatin isolated from SF767 cells treated with EGF for 30 min. Input control was established by amplification of an equivalent amount of DNA that had not been previously subject to immunoprecipitation. Negative control for antibody specificity was established by precipitation with normal rabbit serum. A representative gel of the ChIP assay from three independent experiments.

direct promoter analysis, luciferase reporter constructs containing COX-2 promoter sequences were stably introduced into the SF767 cells. These constructs included the human COX-2 promoter (-1,122 to +27) relative to the transcriptional start; COX2/P1 or this same promoter (-1,122 to +27) with point mutations of the putative Sp1 consensus sequence (COX2/P1m) driving luciferase expression (Fig. 4A). We observed that EGF increased luciferase activity by ~3.5-fold with COX2/P1 5 h after ligand stimulation (Fig. 4B, left). As expected, this induction was completely blocked by the EGFR inhibitor PD153035 (Fig. 4B, left). Inhibition of p38-MAPK or Sp1 with SB202190 or mithramycin, respectively, also significantly reduced induction of luciferase by EGF (Fig. 4B, left). This result implied that p38-MAPK and the Sp factors contribute to transcriptional activation of the COX-2 promoter. Although up to three putative Sp1 sites have been identified within the human COX-2 promoter, a functional site has been confirmed between -245 to -240 bases from the transcriptional start site (26, 27). We sought to determine what role this site play in EGF-induced promoter activation. Although mutation of this Sp1 site decreased the basal luciferase activity to some extent, EGF-induced luciferase activity was nearly abolished with this mutant construct (Fig. 4B, right). This result further confirms that the Sp family of factors is mediating activation of the COX-2 gene promoter after EGF stimulation.

**EGF stimulates Sp1/Sp3 binding to the COX-2 promoter.** To provide further evidence that the Sp factors regulate COX-2 promoter activity after EGF stimulation, we did EMSA using an Sp1 consensus site oligonucleotide and a wild-type oligonucleotide corresponding to the -253/-232 region of the human COX-2 promoter (COX/wtSp) with its corresponding mutant oligonucleotide containing alterations at the Sp1 sites (COX/mtSp). Basal levels of nuclear protein binding to the Sp1 consensus site and COX/wtSp oligonucleotides were detectable (Fig. 4C, lanes 1 and 2, 5 and 6). Three major complexes termed C1, C2, and C3 are formed consistent with what others have found (Fig. 4C; ref. 27). EGF can increase the formation of all three complexes on both labeled Sp1 consensus site and COX/wtSp probes (Fig. 4C, lanes 2 and 3, 6 and 7). The Sp1 consensus site gel-shifted complexes were competitively blocked by unlabeled Sp1 consensus site (Fig. 4C, lanes 3 and 4). Similarly, the COX/wtSp gel-shifted complexes were also competitively blocked by both unlabeled Sp1 consensus site and COX/wtSp oligonucleotides but not by unlabeled COX/mtSp (Fig. 4C, lanes 7-9). As we would expect, no gel-shifted complexes were seen with the use of labeled COX/mtSp (Fig. 4C, lane 11). Preincubation of nuclear extract with either anti-Sp1 or Sp3 antibodies reduced the formation of either the C1 complex or the C1 (to a lesser extent) and C2 complexes, respectively, and led to detection of slower migrating bands representing the supershifted complexes (Fig. 4C, lanes 14 and 15). These results suggest that C1 contains both Sp1 and Sp3, whereas complex C2 is mainly composed of Sp3. The C3 complex does not seem to contain either Sp1 or Sp3. Overall, these results show that EGF can induce binding of Sp1 and Sp3 factors to the Sp binding site found in the COX-2 promoter.

Our results above show EGF-induced association of Sp1 and Sp3 transcription factors with the COX-2 promoter at the putative Sp1 site (-245/-240) *in vitro* by EMSA. However, to show that this interaction with the COX-2 promoter is actually found *in vivo*, we did a ChIP assay using anti-Sp1 and Sp3 antibodies. Our results show basal association of both Sp1 and Sp3 to this site with an increase in binding of ~2.5-fold 30 min after stimulation with EGF (Fig. 4D).

**Involvement of p38-MAPK in the phosphorylation and binding activity of Sp1.** Phosphorylation of Sp1 and Sp3 has been previously shown to increase their DNA binding activity (for review, see ref. 28). To determine whether this is also the case in our system, we did EMSA using calf intestinal alkaline phosphatase (CIP)-treated nuclear extract. Our results indicate that dephosphorylation of nuclear proteins by treating extract with CIP abolishes the binding of Sp1 and Sp3 to their consensus sequence (Fig. 5A). The above results imply that EGF-induced Sp1/Sp3 binding to the COX-2 promoter requires their phosphorylation. Therefore, we hypothesized that EGF stimulation of its receptor activates kinase cascade(s) that result in increased phosphorylation and binding activity of Sp1/Sp3. To test this hypothesis, SF767 cells were metabolically labeled with [<sup>32</sup>P]P<sub>i</sub> before treatment with EGF and lysates were immunoprecipitated with antibody directed against Sp1. The immunoprecipitated products were resolved by SDS-PAGE and transferred to membrane. The membrane was first subjected to autoradio-



**Figure 5.** Role of the p38-MAPK pathway on Sp1 phosphorylation and binding to the Sp1 site. **A**, representative gel of EMSA from two independent experiments using nuclear extracts from SF767 cells treated with EGF for 30 min. Nuclear extract was pretreated with increasing amounts CIP, as indicated, for 10 min before the binding reaction. *Arrows*, the three expected gel-shifted complexes (C1–C3). **B**, SF767 cells were metabolically labeled with [<sup>32</sup>P]P<sub>i</sub> as described in Materials and Methods. Cells were treated with vehicle (DMSO), SB202190 (20 μmol/L), U0126 (20 μmol/L), or LY294002 (25 μmol/L) for 1 h and then stimulated with EGF (100 ng/mL) for 30 min. Labeled Sp1 was immunoprecipitated using an anti-Sp1 antibody, resolved by 7.5% SDS-PAGE, transferred to PVDF membrane, and exposed to phosphor imaging screen overnight. A representative image of the phosphorylated Sp1 (*P-Sp1*) from two independent experiments. After exposure to the phosphor imaging screen, immunoblotting for Sp1 was done with similar levels of total Sp1 present in each lane. **C**, SF767 cells were transiently transfected with pcDNA3 (C), pcDNA3/MKK3be (*MKK3*), and pcDNA3/MKK6be (*MKK6*). Thirty-six hours posttransfection, cells were subjected to either *in vivo* labeling with [<sup>32</sup>P]P<sub>i</sub> for 4 h or lysis without labeling in sample buffer. *In vivo* labeled cells were immunoprecipitated with anti-Sp1 antibody, resolved by 7.5% SDS-PAGE, transferred to PVDF membrane, and exposed to phosphor imaging screen overnight. A representative image of the phosphorylated Sp1 from two independent experiments. After exposure to the phosphor imaging screen, immunoblotting for Sp1 was done with similar levels of total Sp1 present in each lane. For the unlabeled lysate, immunoblots were probed with antibody against phosphorylated and total p38-MAPK. Representative blots from two independent experiments. **D**, representative gels of EMSA from two independent experiments using nuclear extracts from SF767 cells treated with EGF for 30 min are shown in each case. The labeled probe contains the wild-type Sp1 site from the human COX-2 promoter (*COX2/wtSp*). *Arrows*, the three expected gel-shifted complexes (C1–C3). *Left*, SB201290 (20 μmol/L), when used, was added 1 h before treatment with EGF. *Right*, SF767 were transfected with either control siRNA or siRNA targeting p38-MAPK (*si p38*). Lysates were harvested 4 h after treatment with EGF.



**Figure 6.** PGE2 stimulates expression of VEGF in glioma cell lines. RPAs were done on total RNA isolated from LN229/puro (*left*) and SF767 (*right*) glioma cells stimulated with PGE2 (concentrations as noted) for the indicated times (0–6 h). The VEGF riboprobe is described in Materials and Methods. Cyclophilin was used as a normalization control. The displayed results are representative of two independent experiments.

graphy and then, to show that the lanes contained equal amounts of Sp1 protein, probed with anti-Sp1 antibody and detected by a standard chemiluminescence protocol. Our results show that EGF stimulation moderately increased Sp1 phosphorylation. The EGF-induced increase in phosphorylation is blocked by the p38-MAPK inhibitor SB202190 but not by the MEK and PI3K inhibitors, U0126 and LY294002, respectively (Fig. 5B). To further confirm the involvement of p38-MAPK in the phosphorylation of Sp1, we transiently transfected SF767 cells with vectors encoding constitutively active MKK3 and MKK6, and then metabolically labeled these cells with [<sup>32</sup>P]P<sub>i</sub> for 4 h. The lysates were used to perform the same analysis as described above. This experiment shows that overexpression of constitutively active MKK3 and MKK6 leads to increased phosphorylation of both Sp1 and p38-MAPK in comparison with control confirming that activation of p38-MAPK results in phosphorylation of Sp1 (Fig. 5C). Based on these data, we hypothesized that the increased binding activity of Sp1/Sp3 to the COX-2 promoter after EGF stimulation is due to phosphorylation of these factors that result from activation of the p38-MAPK pathway. Therefore, we predict that either chemically inhibiting this kinase with SB202190 or suppressing its expression levels with siRNA will reduce association of the Sp factors to the promoter element at –245/–240 after stimulation with EGF. In fact, this is exactly what we observed with SF767 either treated with SB202190 or transfected with a p38-MAPK-specific siRNA displaying significantly decreased EGF-induced formation of Sp1/Sp3 complexes by EMSA (Fig. 5D).

#### PGE2 induces expression of VEGF mRNA in glioma cell lines.

EGF treatment will induce numerous downstream responses in addition to increased expression of COX-2. Because PGE2 is the predominant product of COX-2 activity, treatment of glioma cells with this prostaglandin would allow us to determine the consequence of COX-2 activation in isolation from other EGF-dependent effects. The LN229/puro and SF767 glioma cell lines were treated with either 5 or 1 μmol/L, respectively, of PGE2 and expression of VEGF mRNA was determined over time. In each case, VEGF mRNA expression was increased by 1 h posttreatment and persisted up to 6 h (Fig. 6). These results suggest that elevated COX-2 expression contributes to the microvascular proliferation typically seen in glioblastoma multiformes through induction of VEGF expression.

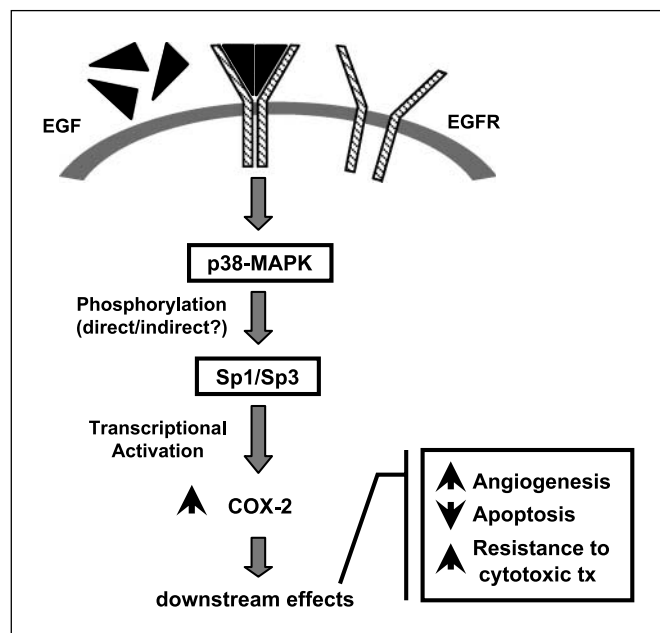
#### Discussion

In this article, we find that EGFR activation increases COX-2 expression in human glioma cell lines. The p38-MAPK pathway, through its activation of the Sp1/Sp3 transcription factors,



seems to be particularly important in this process. We present several lines of evidence that support the role of the Sp1/Sp3 factors in EGF-induced transcriptional activation of COX-2. These include demonstrating that treatment with the Sp1/Sp3 inhibitor mithramycin and transfection of an Sp1-specific siRNA can suppress EGF-induced expression of endogenous COX-2 and/or exogenous reporter gene constructs containing the COX-2 promoter as well as demonstrating that EGF-induced binding of Sp1/Sp3 to the COX-2 promoter occur both *in vitro* by EMSA and *in vivo* by ChIP assay. We further show that Sp1 phosphorylation not only correlates with its activation but is also increased with activation of the p38-MAPK pathway through overexpression of activated MKK3 and MKK6. Therefore, our work shows a link between EGFR overactivity and COX-2 expression and defines a molecular mechanism for this association.

EGFR alterations are some of the most frequent changes seen in malignant glial tumors and seem to correlate with increasing tumor grade (17). Given the importance of EGFR in the pathology of glioblastoma multiformes, we hypothesize that COX-2 contributes to some of the downstream consequences of excessive signaling through this receptor. There is certainly precedence for COX-2 expression playing a significant role in the pathology of other malignancies, most prominently in colon cancer. In fact, a double-blind, placebo-controlled study found that the COX-2 inhibitor celecoxib can significantly reduce the extent of disease in patients with familial adenomatous polyposis, a condition that leads to colorectal cancers in 100% of cases (29). For glial tumors, we find that treatment of glioma cell lines with PGE<sub>2</sub>, the predominant product of COX-2, increases expression of VEGF, which may be contributing to the neovascularization seen in malignant gliomas (Fig. 6).



**Figure 7.** Working model for p38-MAPK and Sp1/Sp3-dependent induction of COX-2 in human glioma cells. Our current model for EGFR-dependent induction of COX-2 expression. Signaling through p38-MAPK is a strong inducer of COX-2 through phosphorylation and activation of Sp1/Sp3 transcription factors and increased activity of the COX-2 promoter.

To date, links between EGFR and transcriptional activation of COX-2 have not been described in malignant gliomas. Matsura et al. (30) showed that transforming growth factor  $\alpha$  can increase COX-2 expression through activation of EGFR in normal human epidermal keratinocytes by both ERK1/2-dependent activation of COX-2 transcription and p38-MAPK-dependent stabilization of COX-2 mRNA. Huh et al. (31) also similarly found that exposure of articular chondrocytes to EGF results in ERK1/2- and p38-MAPK-dependent increase in COX-2 expression although they did not distinguish whether this increase was due to increased transcription or greater message stability. Finally, EGF-induced transcriptional activation of COX-2 in the human epidermoid carcinoma A431 was found to result from increased activator protein 1 (AP-1; c-Jun) activity although some question exist with regard to whether this involves receptor signaling to JNK (32). Our own data suggest that the EGFR to JNK pathway is not influencing COX-2 expression in SF767 glioma cells because inhibition of JNK by SP600125 did not alter EGF-induced accumulation of COX-2 mRNA or protein (data not shown).

The binding activity of Sp1 can be regulated by its phosphorylation (for review, see ref. 28). Chupreta et al. (33) reported that the phosphorylation of Sp1 by ERK2 is involved in EGF-induced expression of *gastrin* gene. Others have also found that phosphorylation of Sp1 can be mediated either directly or indirectly by various kinases, including DNA-PK, PI3K, PKA, and PKC $\zeta$ , leading to enhanced binding activity and increases in the expression of its target genes (34–37). Moon et al. (38) found that Sp1 is involved in p38-MAPK-dependent activation of the *p21WAF1* gene promoter but did not show whether the p38-MAPK is involved in its phosphorylation. In our current study, we find that the p38-MAPK pathway regulates the phosphorylation and binding activity of Sp1, leading to activation of the COX-2 promoter. It remains unclear whether p38-MAPK directly or indirectly phosphorylates Sp1. D'Addario et al. (39) found that p38-MAPK can physically interact with Sp1, suggesting the possibility that p38-MAPK may directly phosphorylate Sp1.

Our results add to the wealth of knowledge on the regulation of COX-2 expression. In our model, we believe that p38-MAPK is predominantly inducing transcription of COX-2 mRNA through either direct or indirect phosphorylation and subsequent activation of the Sp1/Sp3 transcription factors (Fig. 7). As a stress-activated kinase, it is consistent that p38-MAPK would increase expression of COX-2, an enzyme that promotes inflammation through increased production of prostaglandins (for review, see ref. 40). Most previous reports about the role of p38-MAPK in COX-2 regulation has been about its ability to promote increased stability of COX-2 mRNA (41, 42). It remains possible that this mechanism is still partially responsible for the EGF-induced COX-2 expression in our glioma cells in light of the ability of the p38-MAPK inhibitor SB202190 to further reduce COX-2 expression in combination with mithramycin (Fig. 3B). However, clearly, our data suggest that transcriptional activation is the main mechanism for this induction. Although potential transcriptional regulation of the COX-2 promoter by p38-MAPK has been previously reported, the predominant molecular mechanism for this activation had been unclear (43–45). Our report is the first to provide a molecular mechanism linking EGFR through p38-MAPK to Sp1/Sp3 activation and transcriptional induction of COX-2.

Because alterations in EGFR are so prominent in glioblastoma multiformes, inhibition of this receptor has been explored as a potential therapy for these tumors. Mellinghoff et al. (46) recently

showed that malignant gliomas that express a constitutively active, mutant form of EGFR may be more likely to respond to such therapies. However, overall results from these trials remain poor and observed responses are generally short-lived (47, 48). We speculate that monotherapy with pure EGFR inhibitors may not be effective at producing sustained responses in malignant gliomas because these tumors develop alternative means of activating critical downstream signaling pathways that mediates the aggressive behavior of these tumors. COX-2 expression is one such potential effector of tumor aggressiveness that may become resistant to EGFR inhibitor. Many researchers are now advocating the use of combination therapy with these molecularly targeted agents. In fact, preclinical evidence suggest that joint inhibition of EGFR and COX-2 may be more effective than either alone in providing tumor responses in head and neck squamous cell carcinoma models (49). In addition, this therapeutic combination improves radiosensitization of human lung cancer cells compared with separate inhibition of either EGFR or COX-2 alone (50). Such approaches may be appropriate to fully realize the potential of EGFR inhibition in high-grade gliomas.

In summary, we have shown that EGFR can regulate the expression of COX-2 in malignant gliomas by multiple mechanisms. In particular, we have identified signaling to p38-MAPK as a novel, critical step for Sp1/3-dependent transcriptional activation of COX-2. As we develop a better understanding of the various factors that mediate COX-2 activation by EGFR, new molecular targets for inhibition may be identified. We hope to explore the utility of inhibiting these other targets either alone or in combination with more established molecular therapies, including EGFR inhibition in malignant glioma models, with the ultimate goal of improving the therapy for patients with this disease.

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