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The Cyclooxygenase Inhibitor Sulindac Sulfide Inhibits EP4 Expression and Suppresses the Growth of Glioblastoma Cells

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Abstract   EP4 expression in human glioblastoma cells correlates with growth on soft agar. The cyclooxygenase inhibitor sulindac sulfide first altered specificity protein-1 (Sp-1) and early growth response gene-1 expression, then increased the expression of nonsteroidal anti-inflammatory drug-activated gene 1 and activating transcription factor 3, and then decreased EP4 expression. EP4 suppression was dependent on blocking the Sp-1 binding sites in the *human EP4* promoter. Mutation in the Sp-1 sites in EP4 altered the promoter activity and abolished sulindac sulfide effects. The inhibitory effect of sulindac sulfide on EP4 expression was reversed by PD98059, a mitogen-activated protein/extracellular signal–regulated kinase kinase-1/extracellular signal–regulated kinase inhibitor. Sp-1 phosphorylation was dependent on sulindac sulfide–induced Erk activation. Chromatin immunoprecipitation assay confirmed that Sp-1 phosphorylation decreases Sp-1 binding to DNA and leads to the suppression of EP4. Inhibition of cell growth on soft agar assay was found to be a highly complex process and seems to require not only the inhibition of cyclooxygenase activity but also increased expression of nonsteroidal anti-inflammatory drug-activated gene 1 and activating transcription factor 3 and suppression of EP4 expression. Our data suggest that the suppression of EP4 expression by sulindac sulfide represents a new mechanism for understanding the tumor suppressor activity.

Glioblastomas are the most common primary malignant brain tumors. Despite advancements in cancer therapies including surgery, radiation therapy, and chemotherapy, the median survival time is 12 to 15 months for patients newly diagnosed with glioblastoma (1). Shono et al. (2) reported that high cyclooxygenase-2 (COX-2) expression is associated with clinically more aggressive gliomas and is a strong predictor of poor survival. Thus, the activation of EP2 and EP4 is a potential modulator of glioblastoma progression. The overexpression of COX-2 is observed in several human cancers and increased COX-2 expression is associated with a poor prognosis in colon (3), breast (4), ovarian (5), and pancreatic cancers

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(6). The overproduction of prostaglandin E_2 (PGE_2) promotes tumor growth by binding to receptors designated EP1, EP2, EP3, and EP4. The EP receptor signaling pathways control cell proliferation, invasion, apoptosis, and angiogenesis. Deletion of the EP2 and EP4 receptors in mice causes a reduction of tumor growth in colorectal and breast cancers (7). EP2 and/or EP4 expression is upregulated compared with normal tissues in colorectal (7) and breast (8) cancers, and EP4 mRNA expression is upregulated in human astrocytoma cells (9). Raza et al. (10) reported that the EP4 expression in surgically resected glioblastoma tissues is also upregulated compared with the expression in tissues from anaplastic astrocytomas.

Nonsteroidal anti-inflammatory drugs (NSAID) exert their effects by inhibiting COX activity, thereby reducing the levels of prostaglandins. Some NSAIDs have chemopreventive effects against the development of human cancers, including glioblastomas (11–13). COX inhibitors may be useful, but the recently discovered toxic side effects of the selective COX-2 inhibitors seem to eliminate their clinical use. Sulindac, the prodrug for sulindac sulfide, a nonselective COX inhibitor, is well documented as an effective drug in preventing the development of intestinal polyps in experimental animals and of tumors in humans, but little information is available of prevention of glioblastoma by sulindac. The chemopreventive effect of sulindac sulfide and other NSAIDs, in general, seems to be complex and not exclusively dependent on inhibition of COX activity. Drugs such as sulindac sulfide, as well as other chemopreventive chemicals, increase the expression of

the tumor suppressor genes *NAG-1* (14–16) and *ATF3* (17) and is mediated by the expression of the transcription factor Egr-1, which alters the expression of a number of genes (18). Egr-1 sites frequently overlap with Sp-1 binding sites, and interplay between Egr-1 and Sp-1 exists. Sulindac sulfide, like tolfenamic acid, independent of new protein synthesis, directly activates the epithelial-specific transcription factor ESE-1 and facilitates the translocation from the nucleus, thereby increasing the expression of Egr-1 (19). Thus, sulindac sulfide may have two targets in the colorectal cancer cell, COX inhibition and ESE-1 translocation; however, the relative contribution of non-COX targets such as ESE-1 in cancer prevention is not known.

EP2 and EP4 receptors are critical proteins mediating prostaglandin responses. Drugs that inhibit or suppress EP2/EP4 expression could profoundly influence cancer development.

The regulation of EP2 and EP4 has not been extensively investigated. Peroxisome proliferator–activated receptor-γ (PPARγ) ligands inhibit the expression of EP2, whereas PPARγ ligands increase the expression of EP4 in human lung tumor cells (20, 21). The PPARγ ligand troglitazone also increases Egr-1 expression by a mechanism independent of PPARγ activation and subsequently increases NAG-1 expression. Recently, we characterized a functional Egr-1/Sp-1 site in the *human EP4* promoter and showed that troglitazone first increased the expression of the EP4 receptor, then suppressed EP4 expression (22). The troglitazone-mediated increase in expression was dependent on Egr-1, whereas the reduction in expression was dependent on phosphorylation of Sp-1 protein. Because sulindac sulfide alters Egr-1 expression, we suspected that sulindac sulfide may decrease the expression of EP4, and this decrease in EP4 expression could be important in mediating

Fig. 1. EP4 expression in T98G cells. *A,* EP4 protein levels as determined by Western blot analysis. *B,* EP4 mRNA levels as determined by quantitative real-time RT-PCR. T98G cells were treated with vehicle, indomethacin (25 μmol/L), sulindac sulfide (30 μmol/L), sulindac (30 μmol/L), sc-560 (25 μmol/L), sc-58125 (25 μmol/L), or acetaminophen (100 μmol/L) for 48 h. β-Actin serves as a loading control. *Columns,* mean values related to control; *bars,* SD. *C,* Western blot analysis shows dose-dependent expression of EP4 after treatment with sulindac sulfide for 48 h. *Lanes 1* to *4,* sulindac sulfide–treated cells at 0 to 30 μmol/L, analyzed by densitometry. β-Actin is a control for the amount of protein loaded and is used to normalize density. *Columns,* mean of three independent treatments; *bars,* SD.
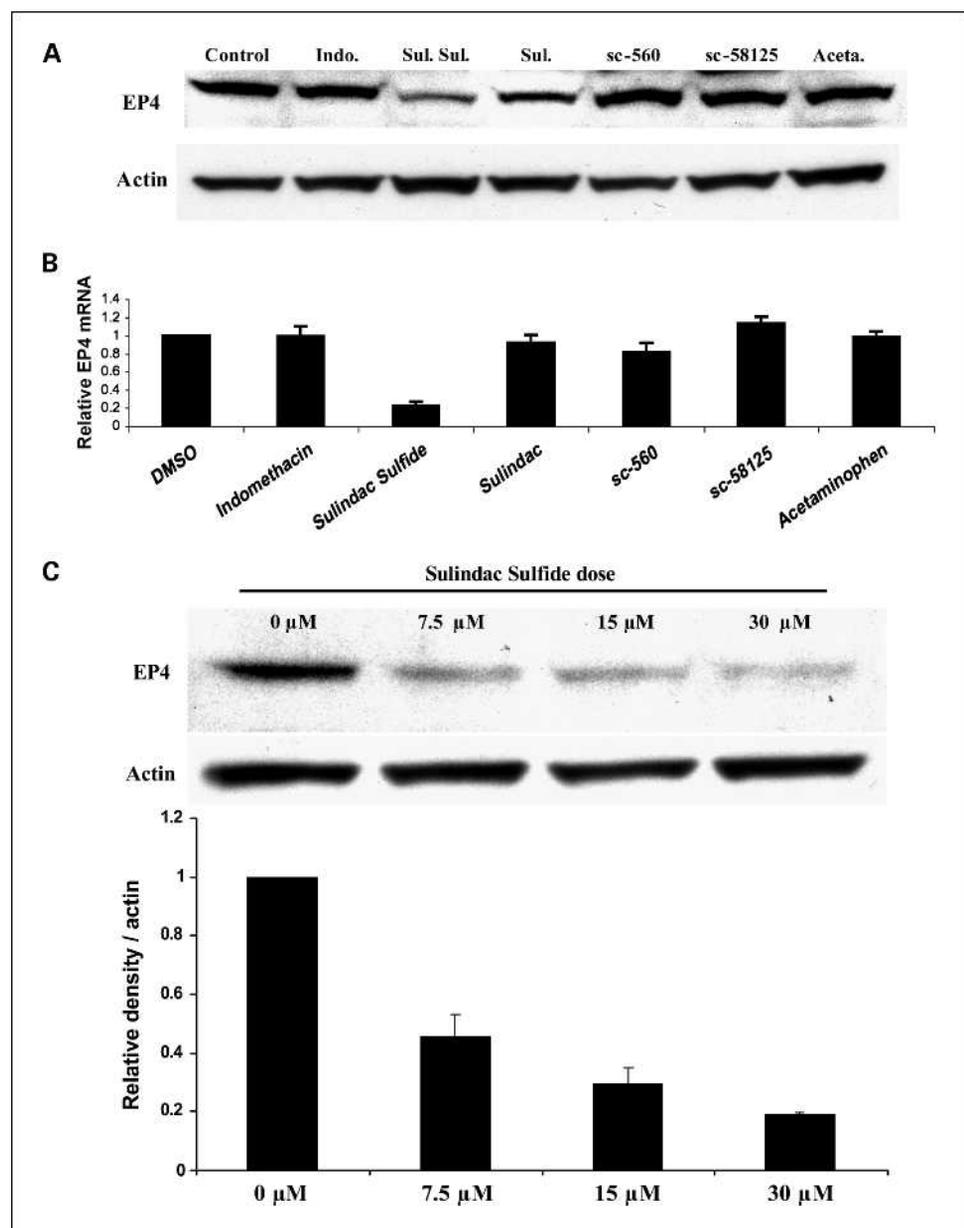
the antitumor activity of sulindac sulfide. The response to sulindac sulfide is complex and may be dependent on both COX inhibition and changes in the expression of Egr-1 and altered EP4 expression. Because the expression of EP4 is upregulated in glioblastoma (10), glioblastoma cells maybe a useful model system to investigate the complex mechanism for sulindac sulfide–induced chemopreventive activity.

In this report, we used glioblastoma cells expressing COX to investigate this problem. Sulindac sulfide reduced EP4 expression in human glioblastomas via changes in the Egr-1/Sp-1 pathway and inhibited the growth of glioblastoma cells on soft agar. Growth inhibition was investigated in relation to inhibition of COX activity, alteration in the expression of NAG-1 and ATF3, and suppression of EP4 expression.

Materials and Methods

Cell lines and reagents

Human glioblastoma T98G, U118, and U87 cells and low-grade glioma Hs683 cells were purchased from the American Type Culture Collection. All cells were grown in Eagle's MEM with 1 mmol/L MEM sodium pyruvate solution (Life Technologies, Inc.), 2 mmol/L L-glutamine (Life Technologies), 10 μg/mL gentamicin (Life Technologies), and 10% fetal bovine serum. Indomethacin, sulindac, sc-560 (COX-1 inhibitor), sc-58125 (COX-2 inhibitor), anti-EP4 antibody, anti–COX-1 antibody, and anti–COX-2 antibody were purchased from Cayman Chemical Co., Inc. Sulindac sulfide and acetaminophen were purchased from Sigma-Aldrich. The mitogen-activated protein/extracellular signal–regulated kinase (Erk) kinase (MEK)-1/Erk inhibitor PD98059 was purchased from EMD Biosciences and the anti–phospho-Erk mitogen-activated protein kinase (Thr202/Tyr204), anti–Egr-1, and anti–phospho-threonine antibodies were purchased from Cell Signaling Technology. Anti–Sp-1 (sc-59), anti–Sp-3 (sc-644), anti–Sp-4 (sc-645), anti-Erk1 (sc-93), anti-Erk2 (sc-154), anti–ATF-3 (sc-188), and anti-actin (sc-1615) antibodies were purchased from Santa Cruz Biotechnology. Anti–NAG-1 antibody was reported previously in our laboratory (23).

Western blot analysis

After reaching 70% to 80% confluence, T98G cells were starved overnight with serum-free medium, then treated at the indicated concentrations and time periods with different drugs in the absence of serum. Total cell lysates were isolated in radioimmunoprecipitation assay buffer (1× PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L Na$_3$VO$_4$, 1 mmol/L NaF, 1 μmol/L okadaic acid, 10 mmol/L β-glycerolphosphate, and Complete Mini protease inhibitor cocktail tablets from Roche). Quantitation of protein was done by bicinchoninic acid assay (Pierce), and 30 μg of total proteins were separated by SDS-PAGE 4% to 12% Bis-Tris gel (Invitrogen) and transferred onto a nitrocellulose membrane (Invitrogen). The blots were blocked for 1 h with 5% skim milk in TBS containing 0.05% Tween 20 (TBS-T; Sigma-Aldrich) and probed overnight at 4°C with 5% skim milk in TBS-T with each primary antibody. After washing with TBS-T, the blots were incubated with the appropriate horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature with 5% skim milk in TBS-T and washed several times in TBS-T. Proteins were detected by the enhanced chemiluminescence system (Amersham Biosciences).

Immunoprecipitation

One milligram of nuclear extracts was prepared with the Nuclear Extract kit (Active Motif) according to the manufacturer's protocol. The nuclear extract was precleaned with 60 μL of protein A/G PLUS-agarose (sc-2003, Santa Cruz Biotechnology) by incubating for 1 h at 4°C with rotator. After pelleting agarose beads, the supernatant
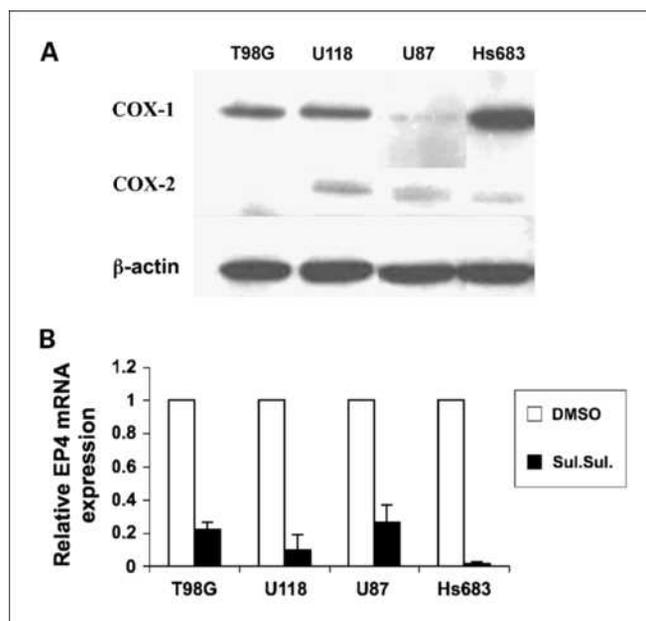

Fig. 2. COX and EP4 expression in several glioma cells. *A*, basal COX-1 and COX-2 protein levels in T98G, U118, U87, and Hs683 cells as determined by Western blot analysis. β-Actin is a control for the amount of protein loaded. *B*, EP4 mRNA levels as determined by quantitative real-time RT-PCR. T98G, U118, U87, and Hs683 cells were treated with vehicle or sulindac sulfide (30 μmol/L) for 48 h. *Columns*, mean values related to control; *bars*, SD.

was transferred to a new tube and immunoprecipitated with 5 μg of anti–Sp-1 antibody (sc-59 AC, Santa Cruz Biotechnology) for 3 h at 4°C. For a negative control, normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) was added to the sample instead of anti–Sp-1 antibody. After removal of the supernatant and washing the beads four times with radioimmunoprecipitation assay buffer, the beads were resuspended in 30 μL of lysis buffer [1× LDS (Invitrogen), 1× sample reducing agent (Invitrogen), 1% SDS, and 10 mmol/L DTT] and boiled for 5 min at 100°C. The samples were separated by SDS-PAGE 4% to 12% Bis-Tris gel and transferred onto a nitrocellulose membrane. The immunoprecipitated proteins were analyzed by immunoblotting using anti–phospho-threonine antibody.

Real-time reverse transcription-PCR

Real-time reverse transcription-PCR (RT-PCR) assays using an ABI Prism 7700 (Applied Biosystems) were done as previously described by our laboratory (24). Real-time RT-PCR fluorescence detection was done in 96-well plates with Quantitect SYBR Green buffer (Qiagen). The sequences of PCR primers (Invitrogen) for human EP4 and actin were designed according to the published data (9) and described previously (22). The experiments were done in duplicate three times with individual time-matched vehicle-treated controls for each gene tested. Amplified product size was routinely checked by gel electrophoresis on a 1% agarose gel in the presence of 0.1 μg/mL ethidium bromide and then visualized under UV light to confirm that only one product was formed.

Constructions of plasmid

The *human EP4* promoter luciferase constructs have been described previously (22). The Sp-1–dependent reporter plasmid (pGAGC6), the Egr-1–dependent reporter plasmid (pEBS14), the Sp-1 expression plasmid, and the Egr-1 expression plasmid have been reported previously (16, 22, 23, 25). The mThr453/mThr739 Sp-1 expression plasmid, which has two mutations at residues Thr453 and Thr739, was produced using the PCR primers described previously (26). The EP4 cDNA, purchased from UMR cDNA Resource Center, was subcloned into pcDNA3.1.

Luciferase reporter assay

T98G cells were seeded in six-well plates at 2×10^5 per well in EMEM supplemented with 10% fatal bovine serum and grown to 50% to 60% confluence. The plasmid mixtures containing 2 μg of *EP4* promoter luciferase construct and 0.05 μg of pRL-null (Promega) were transfected using FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol. The cotransfection experiment was carried out using plasmid mixtures containing 1 μg of pEP4-3 luciferase construct, 1 μg of expression plasmid (Sp-1, Egr-1, or mutant Sp-1), and 0.05 μg of pRL-null. The pcDNA3.1 empty vector (Invitrogen) was used as a negative control for the expression plasmid. After 24-h transfection, the cells were treated with indicated concentrations of drugs or control (0.1% Me₂SO) for an additional 24 h. Finally, the cells were harvested in 1× luciferase lysis buffer (Promega) and luciferase activity was measured and normalized with the values of pRL-null luciferase activity using a dual luciferase assay kit (Promega).

Colony formation in soft agar assay

T98G cells were suspended at 2×10^3 in 1 mL of 0.35% agar solution containing MEM, 10% fetal bovine serum, 1 mmol/L MEM sodium pyruvate solution, 2 mmol/L L-glutamine, and the final concentration of sulindac sulfide, then layered on top of a 0.7% agar layer in six-well plates. For EP4 knockdown or overexpression, T98G cells were transfected with 100 nmol/L EP4 siRNA (M-005714-00, Dharmacon) or 5 μg of EP4 cDNA, and then the effect of EP4 knockdown or overexpression was confirmed by Western blot analysis. After 24-h transfection, the cells were trypsinized and resuspended in 0.35% agar solution. Plates were incubated for 2 wk at 37°C in a 5% CO₂ humidified atmosphere. Cell colonies were visualized following an overnight stain with 0.5 mL of *p*-iodonitrotetrazolium violet (Sigma-Aldrich) and examined microscopically. These were determined as mean colony number examined in 10 randomly chosen microscope fields.
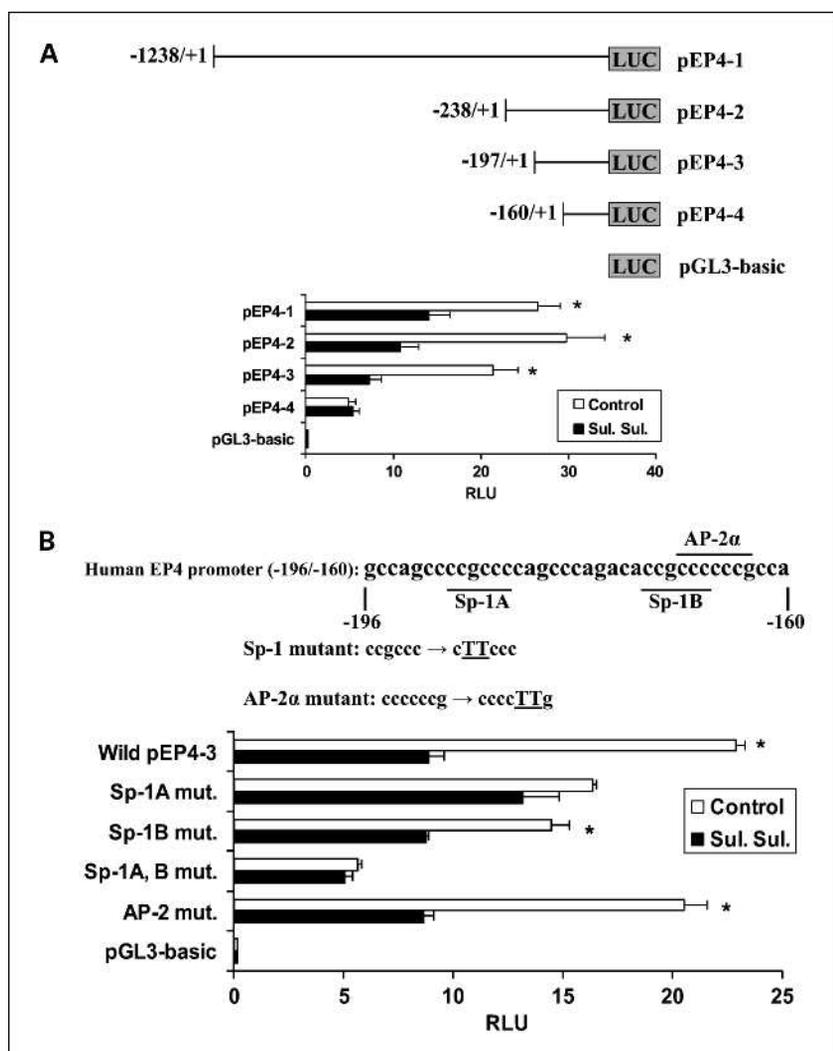
Chromatin immunoprecipitation assay

A chromatin immunoprecipitation assay was done using a chromatin immunoprecipitation assay kit (Upstate Biotechnology) according to the manufacturer's protocol. The details of the methods and conditions in the chromatin immunoprecipitation assay have been described previously (22). The immunoprecipitation was carried out using antibodies against Sp-1 (sc-59X, Santa Cruz Biotechnology), Egr-1 (sc-110X), or normal rabbit IgG overnight at 4°C. The 136-bp products were re-solved on a 2% agarose gel and visualized under UV light. PCR primers for a nonbinding region of the promoter were used for controls.

PGE₂ immunoassay

T98G cells (2×10^6) were placed in a 10-cm dish in complete medium and grown to full confluence. The cells were pretreated with different concentrations of sulindac sulfide as indicated for 5 min before the addition of arachidonic acid (100 μmol/L). After 1 h, cell culture supernatants were corrected, and the concentrations of PGE₂

Fig. 3. *EP4* promoter activity is induced by sulindac sulfide in T98G cells. *A,* the indicated promoter regions were fused to the luciferase reporter gene (*LUC*). Each construct was cotransfected with pRL-null vector into T98G cells using FuGENE 6, and the cells were treated with control (*white columns*) or 30 μmol/L sulindac sulfide (*black columns*) for 24 h. Promoter activities were measured by luciferase activity. *B,* point mutation effects on *EP4* promoter activity. The construction of *EP4* promoter vectors with mutations has been described previously. Wild type (*Wild pEP4-3*), Sp-1 site mutant reporters, or AP-2α site mutant reporter and pRL-null were cotransfected into T98G cells and treated as described in *A*. The point mutations are underlined. Transfection efficiency for luciferase activity was normalized to Renilla luciferase (pRL-null vector) activity. *X axis,* relative luciferase units (*RLU*; firefly luciferase/Renilla luciferase). *Columns,* mean of three independent transfections; *bars,* SD. *, $P < 0.01$, compared with control.
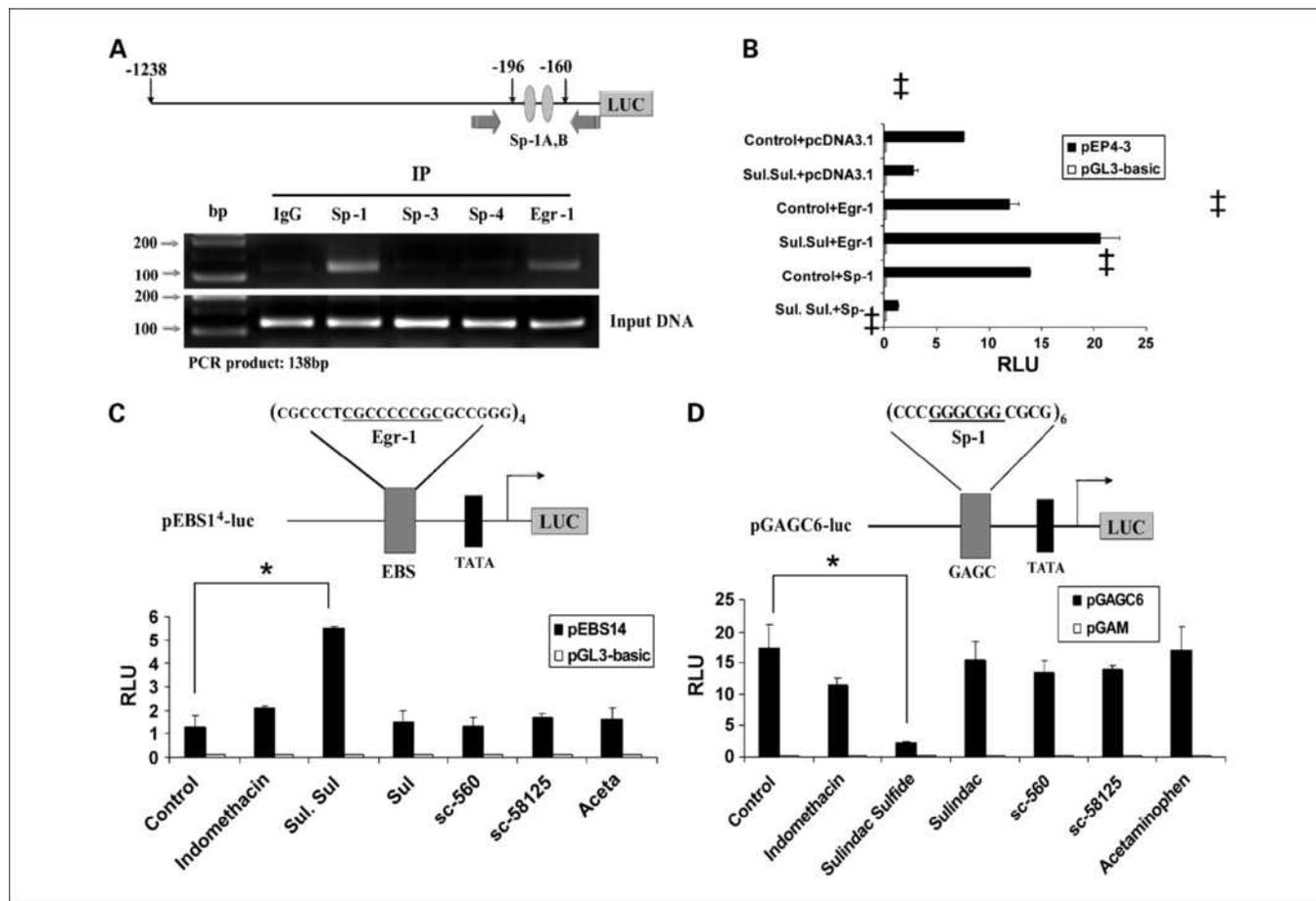
Fig. 4. Effects of Sp-1 or Egr-1 binding on the human *EP4* promoter. *A,* the Sp-1 binding region amplified by the specific PCR primers in chromatin immunoprecipitation (*IP*) assays is indicated. *Large arrows,* a primer pair of *EP4* genes. Antibodies specific for Sp-1, Sp-3, Sp-4, or Egr-1 were used to immunoprecipitate chromatin. PCR was done with the primer pair of *EP4* genes. Input corresponded to PCR containing 0.5% of the total amount of chromatin used in immunoprecipitation reactions. *B,* wild-type pEP4-3 (*black columns*) or pGL3-basic (*white columns*) vector and pRL-null were cotransfected with pDNA3.1, Sp-1, or Egr-1 overexpression plasmid, and T98G cells were treated with control or 30 µmol/L sulindac sulfide for 24 h. Promoter activities were measured by luciferase activity. *X axis,* relative luciferase units (firefly luciferase/Renilla luciferase). ‡, $P < 0.01$, compared with control + pcDNA3.1. *C,* the Egr-1 reporter plasmid pEBS1[4]-luc (*black columns*) or pGL3-basic (*white columns*) vector was cotransfected with pRL-null vector and the cells were treated with vehicle, indomethacin (25 µmol/L), sulindac sulfide (30 µmol/L), sulindac (30 µmol/L), sc-560 (25 µmol/L), sc-58125 (25 µmol/L), or acetaminophen (100 µmol/L) for 24 h. Promoter activities were measured by luciferase activity. *Y axis,* relative luciferase units (firefly luciferase/Renilla luciferase). *, $P < 0.01$. *D,* the Sp-1 reporter plasmid pGAGC6-luc (*black columns*) or pGAM-basic (*white columns*) vector was cotransfected with pRL-null vector and treated as described in *C*. Promoter activities were measured by luciferase activity. *Y axis,* relative luciferase units (firefly luciferase/Renilla luciferase). Transfection efficiency for luciferase activity was normalized to Renilla luciferase (pRL-null vector) activity. *Columns,* mean of three independent transfections; *bars,* SD. *, $P < 0.01$.

were measured using a PGE$_2$ EIA kit (Cayman) according to the manufacturer's protocol. Briefly, cell culture supernatants were incubated with PGE$_2$ acetyl cholinesterase tracer and PGE$_2$ antibody in 96-well plates for 18 h at 4°C. After rinsing, the plates were incubated with Ellman's reagent for 1 h and then read at 405-nm wavelength by a microplate reader.

Statistical analysis

Statistical differences between experimental groups were evaluated by the two-tailed unpaired Student *t* test.

Results

Sulindac sulfide suppresses EP4 expression in T98G cells

T98G cells were treated with indomethacin (25 µmol/L), sulindac sulfide (30 µmol/L), sulindac (30 µmol/L), sc-560 (COX-1 specific inhibitor; 25 µmol/L), sc-58125 (COX-2 specific inhibitor; 25 µmol/L), acetaminophen (100 µmol/L), or vehicle for 48 hours. As shown in Fig. 1A and B, only sulindac

sulfide at these concentrations significantly suppressed EP4 expression at the protein and mRNA levels. The inhibition of EP4 expression by sulindac sulfide was concentration dependent (Fig. 1C) and no changes to cell viability was observed by MTA assay (Supplementary Fig. 1). As shown in Fig. 2, the suppression of EP4 expression by sulindac sulfide was also examined in other glioblastoma cell lines (U87 and U118) and low-grade glioma cell line (HS683) that have different patterns of COX-1 and COX-2 expression. T98G cells express COX-1, but not COX-2 (Fig. 2A). Treatment with 30 µmol/L sulindac sulfide suppressed the expression of EP4 mRNA in several glioma cell lines (Fig. 2B). Thus, inhibition of EP4 expression by sulindac sulfide seems to be a general response in glioblastoma cells.

Sulindac sulfide suppresses EP4 promoter activity

To determine whether EP4 suppression by sulindac sulfide occurred at the transcriptional level and to examine the *cis*-acting

elements in the *human EP4* promoter, luciferase assays were done with *human EP4* promoter constructs in T98G cells. As shown in Fig. 3A, a significant decrease in luciferase activities by sulindac sulfide treatment was observed with pEP4-1 to pEP4-3 constructs. However, sulindac sulfide treatment did not decrease luciferase activity after transfection of the pEP4-4 construct. This result suggests that the response element to sulindac sulfide could be located in the region between −197 and −160 of the *EP4* promoter. In this region, the promoter contains two Sp-1 binding sites (Sp-1A and Sp-1B) and one AP-2α binding site overlapping with the Sp-1B site (26). We generated point mutations in the Sp-1 (ccgccc→cTTccc) and AP-2α (cccccg→ccccTTg) binding sites of the pEP4-3 construct. Mutations in the AP-2α site did not alter the luciferase activities after sulindac sulfide treatment compared with the wild pEP4-3 construct. In contrast, point mutations in both Sp-1A and Sp-1B binding sites together caused a dramatic reduction in the sulindac sulfide inhibition of luciferase activity compared with the wild pEP4-3 construct, indicating that the two Sp-1 sites are important for transcription of the *human EP4* gene (Fig. 3B).

Effects of Sp-1 or Egr-1 binding on the human EP4 promoter

Proteins in the Sp family are known to bind to the GC-rich box. In addition, Egr-1 binding sites frequently overlap with the Sp-1 binding site. To determine which Sp protein binds and if Egr-1 binds to the Sp-1/Egr-1 binding sites in the *human EP4* promoter, we carried out a chromatin immunoprecipitation assay experiment using primers that amplify the response element containing two Sp-1 binding sites. As shown in Fig. 4A, only Sp-1 and Egr-1 were bound to the response element. Immunoprecipitation using anti-IgG, anti–Sp-3, or anti–Sp-4 failed to produce PCR products. The pEP4-3 luciferase construct and Egr-1 or Sp-1 expression plasmid were co-

transfected into T98G cells. Interestingly, transfection of the Egr-1 expression plasmid increased EP4 luciferase activity, and this effect was further enhanced by sulindac sulfide treatment. In contrast, Sp-1 overexpression increased EP4 luciferase activity, but sulindac sulfide treatment markedly decreased the activity as compared with control (Fig. 4B). To further confirm the effect of Egr-1 and Sp-1 binding at the transcriptional level, the Egr-1 reporter plasmid that contains four conventional Egr-1 binding sites (pEBS14-luc) or the Sp-1 reporter plasmid that contains six conventional Sp-1 binding sites (pGAGC6-luc) was transfected into T98G cells and subsequently treated with several drugs as indicated. As shown in Fig. 4C and D, only sulindac sulfide significantly increased luciferase activity after transient transfection of pEBS14-luc or the Egr-1 binding sites, whereas sulindac sulfide decreased luciferase activity after transfection with pGAGC6-luc or Sp-1 binding sites. These data support the hypothesis that Egr-1 and Sp-1 proteins have opposite effects on *human EP4* promoter after sulindac sulfide treatment.

Location of the putative Egr-1/Sp-1 binding site in the EP4 promoter

pEP4-3 or pEP4-4 plasmids were transfected with Egr-1 expression plasmid into T98G cells and then treated with sulindac sulfide. The pcDNA3.1 plasmid was used as a control of Egr-1 expression plasmid. As shown in Fig. 5A, after transfection of pEP4-4, sulindac sulfide failed to increase EP4 luciferase activity relative to control. However, after transfection of pEP-4-3, Egr-1 expression further increased luciferase activity, suggesting that a putative Egr-1 binding site is located in the region between −197 and −160. This result coincided with the result of the chromatin immunoprecipitation assay experiment (Fig. 4A). We next transfected several mutant Sp-1 or AP-2α luciferase constructs with Egr-1 expression plasmid into

Fig. 5. Location of the putative Egr-1 binding site in the *EP4* promoter. *A*, each construct and pRL-null were cotransfected into T98G cells with pcDNA3.1 (*white columns*) or Egr-1 overexpression plasmids (*black columns*), and the cells were treated with 30 μmol/L sulindac sulfide for 24 h. Promoter activities were measured by luciferase activity. *B*, wild type (*Wild pEP4-3*), Sp-1 site mutant reporters, or AP-2α site mutant reporter and pRL-null were cotransfected with pDNA3.1 (*white columns*) or Egr-1 overexpression plasmid (*black columns*) and treated as described in *A*. Transfection efficiency for luciferase activity was normalized to Renilla luciferase (pRL-null vector) activity. *X axis*, relative luciferase units (firefly luciferase/Renilla luciferase). *Columns*, mean of three independent transfections; *bars*, SD. *, $P < 0.01$, compared with pcDNA3.1. *C*, proposed binding site of Egr-1.

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T98G cells and subsequently measured EP4 luciferase activity after sulindac sulfide treatment. As shown in Fig. 5B, the luciferase activity of mutant Sp-1A and Sp-1A,B constructs failed to increase after Egr-1 expression. This result suggests that the putative Egr-1 binding site overlaps with the Sp-1A binding site (Fig. 5C).

Time-dependent changes in EP4 expression after sulindac sulfide treatment

Egr-1 and Sp-1 proteins are induced after sulindac sulfide treatment at different times. As shown in Fig. 6A, Egr-1 expression was induced about 3.5-fold after 4 hours of treatment with sulindac sulfide and then declined. In contrast, Sp-1 protein expression increased at 8 and 16 hours after treatment and then also declined. Sp-3 and Sp-4 expression did not change. EP4 protein first increased at 4 and 8 hours after sulindac sulfide treatment and then declined to below initial levels at 24 and 48 hours (Fig. 6B). We propose that the increase in Egr-1 expression mediates the increase in EP4 protein expression, whereas Sp-1 may be responsible for the decrease in expression. This hypothesis is in agreement with results obtained with Egr-1 or Sp-1 expression on *EP4* promoter luciferase assays.

MEK-1/Erk inhibition and changes in EP4 expression by Egr-1 and Sp-1

Phosphorylation of threonine residues in Sp-1 by activated Erk is involved in the troglitazone-induced EP4 suppression (22). Erk activation could be involved in sulindac sulfide–induced changes in EP4 expression mediated by Egr-1 and Sp-1. The phosphorylation of Erk protein after sulindac sulfide treatment was first confirmed by Western blot analysis. As shown in Fig. 7A, sulindac sulfide treatment markedly and rapidly induced Erk phosphorylation. We pretreated T98G cells with the MEK-1/Erk inhibitor PD98059 for 1 hour before the addition of sulindac sulfide. The MEK-1/Erk inhibitor reduced the phosphorylation of Erk1/2 and the Egr-1 or Sp-1 expression induced by sulindac sulfide (Fig. 7B). Next, the cells were pretreated with PD98059 for 1 hour followed by 8 or 48 hours of sulindac sulfide treatment. As shown in Fig. 7C, the increase in EP4 protein observed at 8 hours of treatment with sulindac sulfide was significantly inhibited by PD98059. Furthermore, the suppression of EP4 protein observed at 48 hours of treatment with sulindac sulfide was reduced by cotreatment with the Erk inhibitor. Thus, an increase in EP4 protein expression is followed by a decrease in expression after sulindac sulfide treatment. Both the increase and the decrease in expression are mediated by Egr-1 and Sp-1, respectively, and are also dependent on the Erk pathway.

Sp-1 phosphorylation and EP4 suppression

Sp-1 is phosphorylated at two specific threonine residues (Thr453 and Thr739) and at other sites on the *EP4* promoter by Erk (27). Phosphorylated threonine residues in Sp-1 after sulindac sulfide treatment were measured by immunoprecipitation of Sp-1 followed by Western blot analysis using the phospho-threonine-specific antibody. The phosphorylated threonine residues in Sp-1 were detected after treatment with sulindac sulfide and were reduced by 1-hour pretreatment with 30 μmol/L PD98059 (Fig. 8A). Moreover, we cotransfected pEP4-3 and the mThr453/mThr739 Sp-1 expression
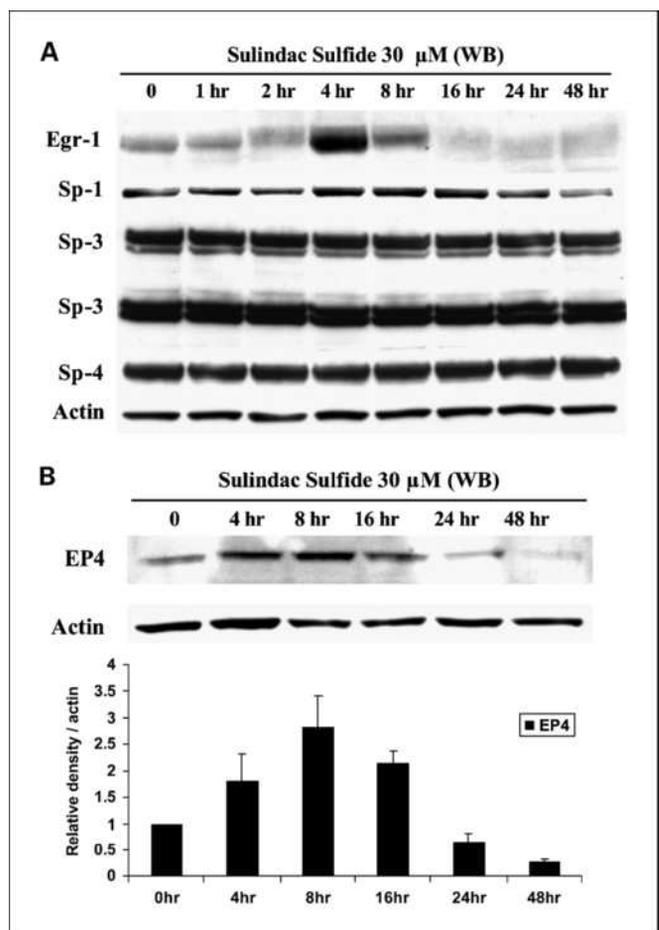

Fig. 6. Time-dependent changes in Sp family, Egr-1, and EP4 expression after sulindac sulfide treatment of T98G cells. *A*, Western blot analysis shows protein induction after treatment with 30 μmol/L sulindac sulfide for each indicated time. *B*, Western blot analysis shows time-dependent expression of EP4 after treatment with 30 μmol/L sulindac sulfide. *Lanes 1* to *6*, sulindac sulfide–treated cells at 0 to 48 h, analyzed by densitometry. β-Actin serves as a loading control and is used to normalize density. *Columns*, mean of three independent treatments; *bars*, SD.

plasmid, which has two mutations at residues Thr453 and Thr739, or the wild Sp-1 expression plasmid into T98G cells, and then treated the cells with sulindac sulfide. EP4 suppression was observed with Sp-1 expression, whereas less inhibition was observed with transient transfection of the mutated Sp-1 expression plasmid (Fig. 8B). Taken together, these data indicate that threonine phosphorylation of Sp-1 induced by activated Erk is required for EP4 suppression by sulindac sulfide.

Changes in the DNA binding of Egr-1 and Sp-1 influence the expression of EP4

As shown in Fig. 6A, Egr-1 and Sp-1 proteins were induced at different time points by sulindac sulfide. We suspected that DNA binding of Egr-1 and Sp-1 to the *human EP4* promoter may be dependent on phosphorylation. We used the chromatin immunoprecipitation assay to measure DNA binding. To confirm that binding of Egr-1 and Sp-1 to DNA required MEK-1/Erk phosphorylation, we pretreated the cells with PD98059 for 1 hour and subsequently added sulindac sulfide for 4 hours (the peak for Egr-1 expression) or 16 hours (the

peak for Sp-1 expression). The increase in Egr-1 binding and the decrease in Sp-1 binding were attenuated by PD98059 (Fig. 9). Thus, the DNA binding of Egr-1 and Sp-1 to the *EP4* promoter requires MEK-1/Erk phosphorylation and correlates with an increase in EP4 expression followed by a decrease in EP4 expression. Thus, Sp-1 is essential for the expression of EP4 in T98G cells, and the decrease of Sp-1 binding as a result of phosphorylation by Erk after sulindac sulfide treatment leads to the suppression of EP4 expression.

Soft agar growth, inhibition of PGE$_2$, and changes in protein expression

Soft agar assay was used to estimate the antitumorigenic activity of sulindac sulfide. Higher concentrations of drugs are necessary to inhibit the growth of T98G cells on soft agar because the cells require complete medium with 10% fetal bovine serum for colony formation. In addition to the suppression of EP4 expression, the expression of the tumor suppression proteins NAG-1 and ATF3 can be increased by sulindac sulfide via the MEK-1/Erk/Egr-1 pathway in human colorectal cancer HCT-116 cells (16, 28). Changes in NAG-1 and ATF3 expression after drug treatment were measured to confirm that the observations in HCT-116 cells would be observed also in T98G cells. The expressions of both proteins were markedly induced after 48 hours of treatment with 30 μmol/L sulindac sulfide in T98G cells in serum-free conditions (Fig. 10A). T98G cells were then treated with sulindac sulfide at several concen-

trations under soft agar assay conditions to determine the dose-response relationships for the inhibition of cell growth on soft agar. In addition, we determined the relationship between sulindac sulfide concentration and inhibition of COX activity, induction of NAG-1/ATF3, and suppression of EP4. Indomethacin was also used as negative control because at the concentration used, it does not alter Egr-1 and hence does not alter NAG-1 or ATF3 expression. As shown in Fig. 10B, tumor growth of the cells on soft agar was inhibited in a concentration-dependent manner, with ~25% suppression at 60 μmol/L and ~80% suppression at 120 μmol/L concentration. Sulindac sulfide also inhibited prostaglandin formation, but was considerably less effective for inhibiting COX activity in the presence of serum. At the highest concentration tested, 120 μmol/L, only 40% inhibition of prostaglandin formation was detected. Sulindac sulfide increased the expression of NAG-1 and ATF3, with an ~2-fold increase at 60 μmol/L and a 6-fold increase at 120 μmol/L concentrations. In contrast, sulindac sulfide (Fig. 10C) first increased the expression and then suppressed the expression of EP4 as observed previously. A 50% inhibition was observed at 120 μmol/L. Incubation with indomethacin at doses up to 120 μmol/L did not alter colony formation (data not shown).

Soft agar growth and EP4 expression in T98G cells

T98G cells were transiently transfected with EP4 cDNA expression vector or siRNA to correspondingly increase or

Fig. 7. Egr-1, Sp-1, and EP4 expressions are dependent on the MEK-1/Erk pathway. *A*, Western blot analysis shows time-dependent expression of EP4 after treatment of T98G cells with 30 μmol/L sulindac sulfide. *Lanes 1 to 10*, sulindac sulfide–treated cells at 0 to 48 h, analyzed by densitometry. *B to D*, PD98059 or vehicle was added 1 h before T98G cells were treated with 30 μmol/L sulindac sulfide (*lanes 3* and *4*) or control (*lanes 1* and *2*) for 48 h (*B* and *D*) or 8 h (*C*). The indicated protein expressions were detected by Western blot analysis. β-Actin serves as a loading control and is used to normalize density. *Columns*, mean of three independent treatments; *bars*, SD. *, $P < 0.01$, compared with control. ‡, $P < 0.01$, compared with sulindac sulfide.
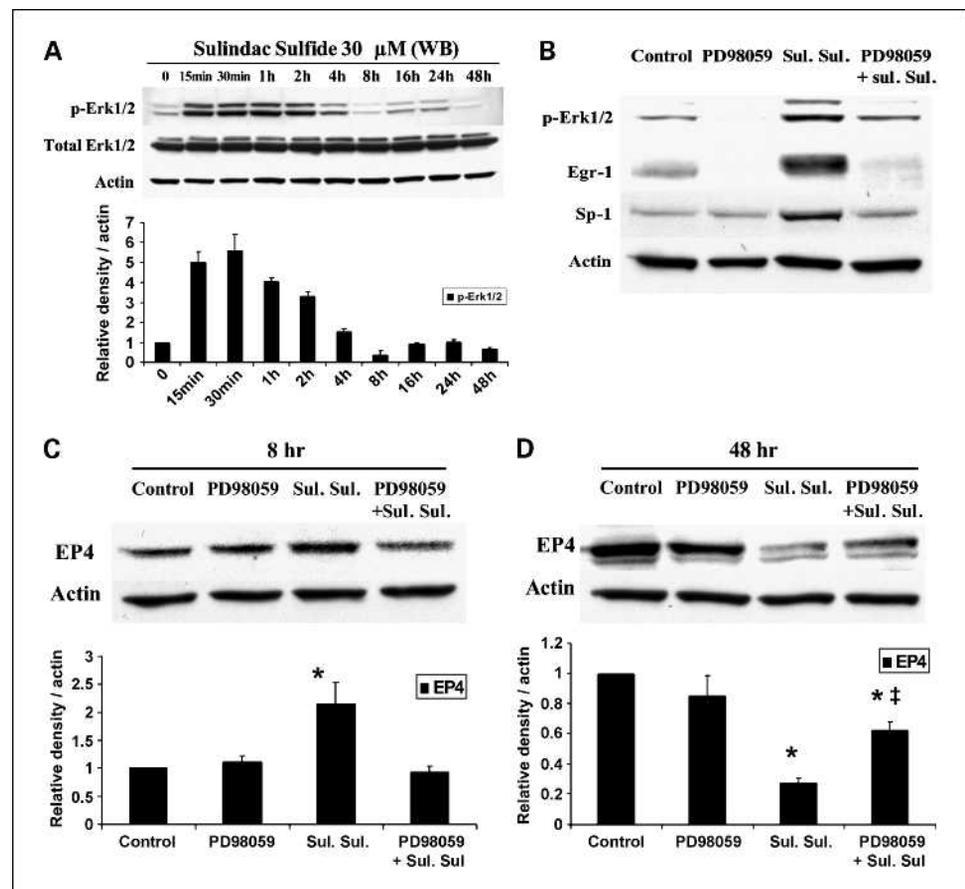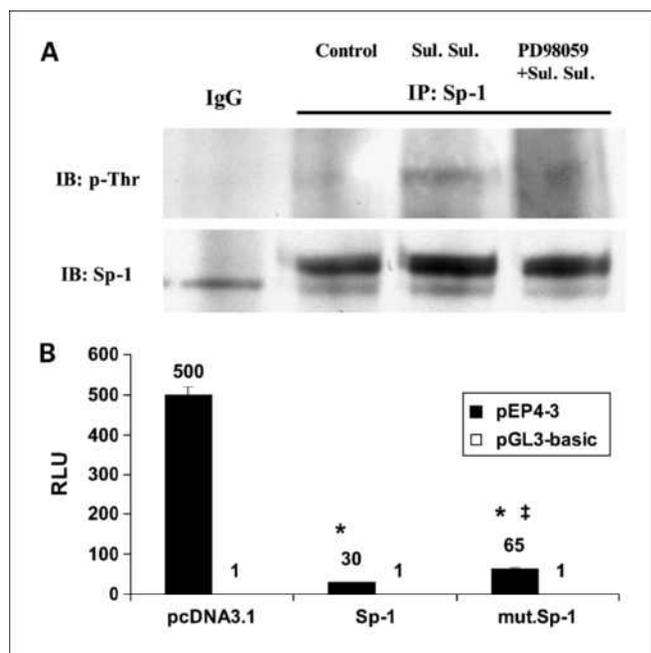
Fig. 8. Phosphorylation of Sp-1 is required for EP4 suppression by sulindac sulfide. *A*, T98G cells were pretreated with or without 30 μmol/L PD98059 for 1 h before addition of sulindac sulfide or control for 24 h. The immunoprecipitated proteins were subjected to Western blot analysis with anti–phospho-threonine (*p-Thr*) or anti–Sp-1 antibody. *B*, the pEP4-3 (*black columns*) or pGL3-basic (*white columns*) and pcDNA3.1, wild Sp-1, or mThr[453]/mThr[739] Sp-1 expression plasmid were cotransfected into T98G cells for 24 h, then treated with sulindac sulfide for 48 h. The mean is given above the column. *Columns,* mean relative luciferase units from three independent transfections; *bars,* SD. *, $P < 0.01$, compared with pcDNA3.1. ‡, $P < 0.01$, compared with wild Sp-1.

decrease EP4 expression. As shown in Fig. 10D, EP4 overexpression increased growth whereas suppression of EP4 by EP4 siRNA decreased growth. Thus, alteration in EP4 expression alters the growth of T98G cells on soft agar. Treatment with sulindac sulfide also inhibited soft agar growth

and antagonized the increase in growth observed after transfection of EP4 receptor into the cell. Furthermore, transfection with EP4 siRNA and treatment with sulindac sulfide inhibited colony formation to a greater extent than either sulindac sulfide or EP4 siRNA alone. Taken together, these findings indicate that the expression level of EP4 has significant effect on soft agar growth. Thus, sulindac sulfide decreases cell growth, in part, by suppressing the expression of EP4 in T98G cells.

Discussion

In this report, we show for the first time that changes in the expression of EP4 influence the growth of glioblastoma cells and that sulindac sulfide suppresses EP4 expression by altering the expression of Egr-1/Sp-1 transcription factors. Further, we present evidence for a complex mechanism for the inhibition of glioblastoma cell growth by the COX inhibitor and chemopreventive drug sulindac sulfide. Treatment of glioblastoma cells, T98G, with sulindac sulfide inhibits the growth of these cells on soft agar, and this is dependent on the concentration of the drug. In addition to COX inhibition, sulindac sulfide treatment alters the Egr-1/Sp-1 pathways, resulting in increased expression of NAG-1 and ATF3 and reduced expression of the EP4 receptor. Treatment with another COX inhibitor, indomethacin, at concentrations that do not alter the Egr-1/Sp-1 pathway did not alter soft agar growth. Previously, we have reported that Egr-1 protein expression is induced by sulindac sulfide and some other COX inhibitors. The transcription factor Egr-1 binds to the Egr-1 binding site presented in the *NAG-1* (18) and *ATF3* (28) promoters and results in the increased expression of these two proteins. Data from cells in culture (23) and studies of NAG-1 transgenic mice expressing human NAG-1 (29) support the hypothesis that NAG-1 acts to suppress the development of intestinal tumors. Because sulindac sulfide and other NSAIDs alter the Egr-1/Sp-1 pathways and because of our recent studies showing the suppression of EP4 expression by troglitazone, we suspected that sulindac sulfide and other COX inhibitors
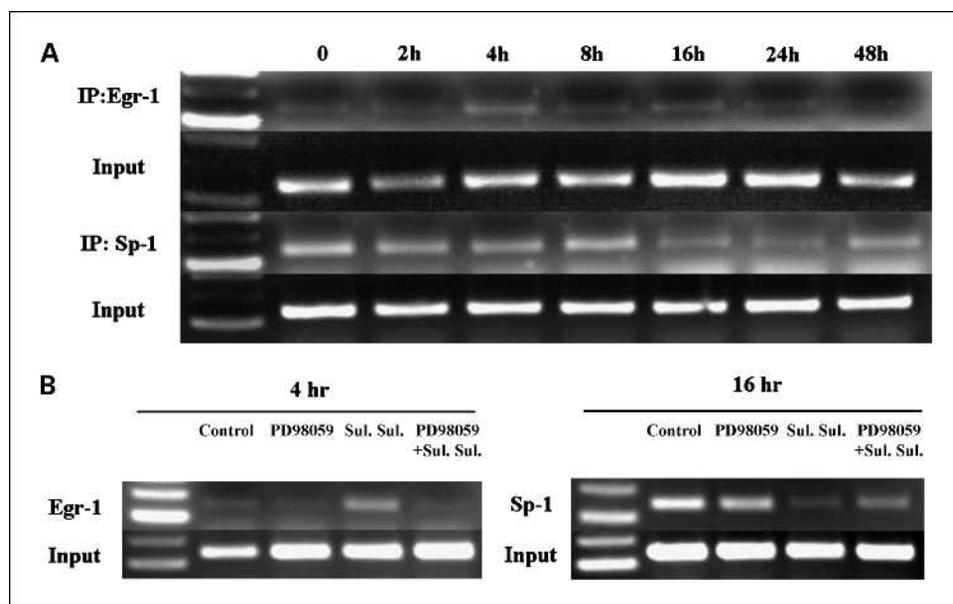

Fig. 9. DNA binding changes of Egr-1 and Sp-1. T98G cells were plated in 10-cm plates and grown to 60% to 70% confluency. The cells were pretreated with or without PD98059 before addition of sulindac sulfide or control for the indicated hours, The cells were treated with sulindac sulfide or control for the indicated hours and then fixed with 1% formaldehyde. Three microliters of each purified sample were used as a template for PCR amplification. Normal rabbit IgG was used as a control. PCR primers to a nonbinding region were used as a control.
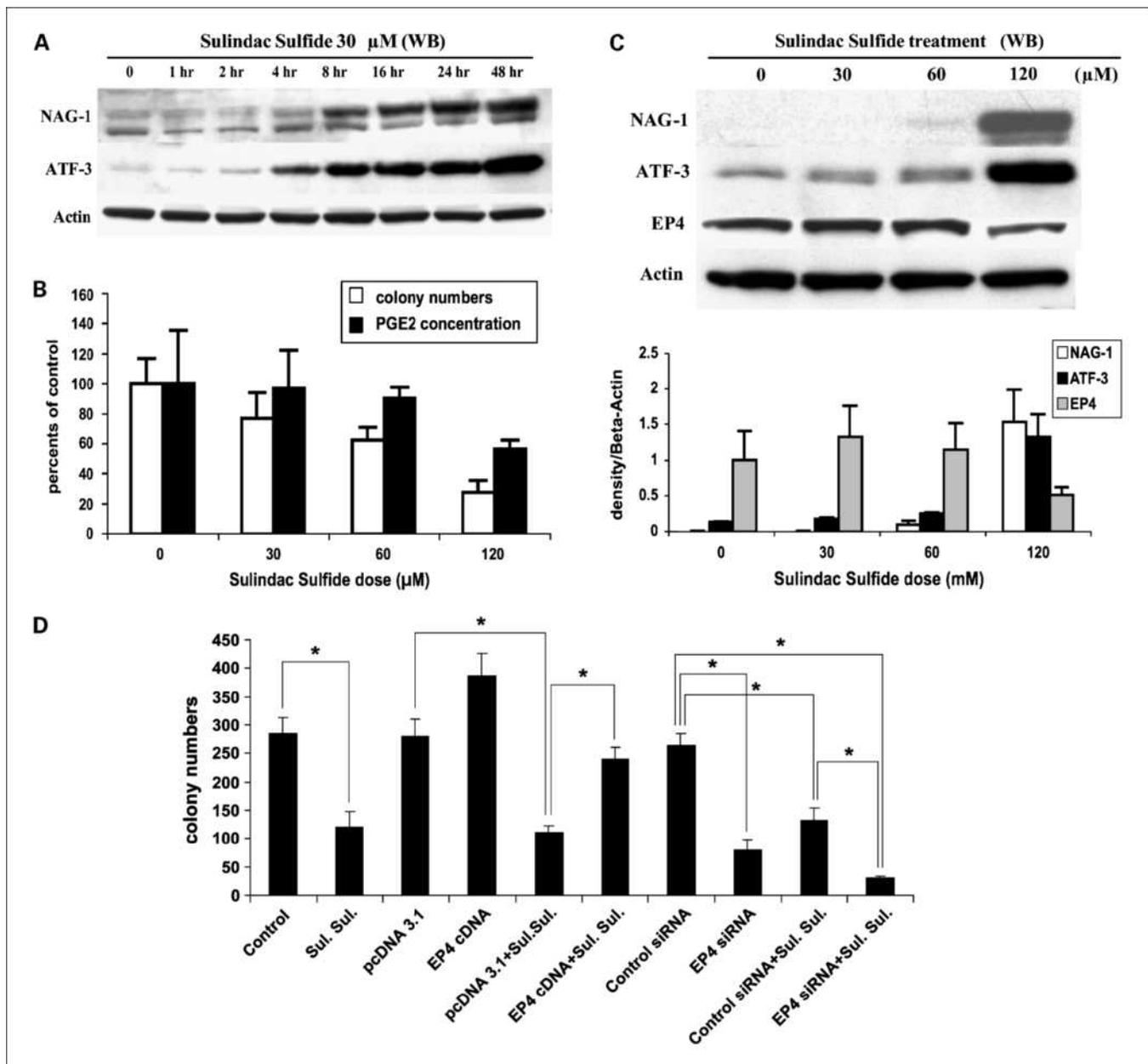
Fig. 10. Association between cell growth, inhibition of PGE$_2$, and changes in protein expressions in sulindac sulfide treatment. *A,* Western blot analysis shows NAG-1 and ATF3 protein induction after treatment with 30 μmol/L sulindac sulfide for each indicated time. *B, white columns,* percent of cell number with control treatment. Cells were grown in soft agar containing indicated concentrations of sulindac sulfide. *Black columns,* percent of PGE$_2$ concentration with control treatment. Cells were grown in growth media with the indicated concentration of sulindac sulfide, and then the media were corrected and analyzed by ELISA. *Columns,* mean; *bars,* SD. *, $P < 0.01$, compared with control. *C,* Western blot analysis shows dose-dependent expression of NAG-1, ATF3, and EP4. *Lanes 1* to *4,* cells treated with sulindac sulfide at 0 to 120 μmol/L for 48 h and analyzed by densitometry. β-Actin serves as a loading control and is used to normalize density. *D,* alteration of EP4 expression with EP4 siRNA or EP4 overexpression plasmid affects soft agar growth. T98G cells were transiently transfected with pcDNA3.1, EP4 cDNA, control siRNA, or EP4 siRNA for 24 h, then resuspended and grown for 2 wk in soft agar containing control or 120 μmol/L sulindac sulfide. Colonies grown under the indicated condition were stained with *p*-iodonitrotetrazolium violet. *Columns,* mean colony number of two different experiments, each done in triplicate; *bars,* SD. *, $P < 0.01$.

would also reduce the expression of EP4 in glioblastoma cells. Of the COX inhibitors examined, sulindac sulfide was the most effective in inhibiting EP4 expression, a finding in agreement with results showing that it is the most potent COX inhibitor for increasing the expression of Egr-1 and its downstream targets, NAG-1 and ATF3 (29, 30).

Sulindac sulfide suppresses EP4 expression by increasing the phosphorylation of transcription factor Sp-1. The *human EP4* promoter region contains two Sp-1 sites (31) that overlap with an Egr-1 site (22), and mutations of these sites in luciferase promoter studies confirmed that Sp-1/Egr-1 sites are important in regulating EP4 expression and responding to sulindac sulfide treatment. In addition, the chromatin immunoprecipitation assay and expression studies with Egr-1 and Sp-1 proteins confirmed that the Egr-1 sites are involved in the increased expression of EP4, whereas Sp-1 sites are

important for sulindac sulfide suppression of EP4 expression. We also investigated the involvement of Sp-3 in EP4 suppression by sulindac sulfide because Sp-3 is known to bind to GC-rich sites and inhibit Sp-1–induced transcription. Sp-3 protein expression was not changed by sulindac sulfide treatment. Additionally, the chromatin immunoprecipitation assay revealed that Sp-3 did not bind to the troglitazone response element and that transfection of Sp-3 siRNA had no effect on EP4 suppression (data not shown). These data indicate that Sp-3 is not involved in sulindac sulfide–induced EP4 suppression.

Erk activation plays a role in the induction and suppression of EP4. Incubation with an Erk kinase inhibitor prevents the changes in NAG-1, ATF3, and EP4 expression by sulindac sulfide. Sp-1 protein is phosphorylated by several kinases including Erk. Five phosphorylation sites (Ser59, Ser131, Thr453, Thr579, and Thr739) have been identified in Sp-1 (27, 32, 33), and threonine phosphorylation at Thr453 and Thr739 occurs in response to Erk activation (27). Phosphorylation of Sp-1 decreases DNA binding activity and transcriptional activation of target genes. In this study, we detected phosphorylated threonine residues in Sp-1 activated by sulindac sulfide–induced Erk. The chromatin immunoprecipitation assay experiment revealed that sulindac sulfide decreases DNA binding activity of responsible Sp-1 binding sites in the *human EP4* promoter (34). Taken together, these data suggest that phosphorylation of Sp-1 is critical and results in a decrease in Sp-1 DNA binding, and hence, suppression of transcriptional activation of target genes such as EP4 is observed.

Sp-1 is a member of a family of zinc finger transcription factors and binds to the GC-rich sequences, and Sp-1 sites in promoters can overlap with Egr-1 sites. Sp-1 plays important roles in a wide range of cellular processes including cell cycle regulation, hormonal activation, apoptosis, and angiogenesis (35). Egr-1, a transcription factor, is also involved in cell growth and differentiation. Egr-1 has been proposed as a tumor suppressor and regulates the expression of the NAG-1/MIC/GDF15 protein, which is reported to suppress intestinal tumor growth (29). Egr-1 also regulates the expression of ATF3, a transcription factor involved in controlling cell growth, apoptosis, and tumor invasion (28, 36). Sulindac sulfide increases the expression of Sp-1 and Egr-1 and alters the phosphorylation of these proteins via Erk (16, 37). As a consequence of these actions, NAG-1 and ATF3 are induced, followed by suppression of EP4 expression.

With the glioblastoma cells, the expression of EP4 seems to correlate with the grade of the tumor (22). The growth of T98G cells on soft agar, a measure of tumorigenicity, is either increased or decreased by a corresponding change in the expression levels of EP4 in these cells. Sulindac sulfide suppresses growth on soft agar, in part, by reducing EP4 expression. Expression of EP4 attenuates the response to sulindac,

whereas inhibition of EP4 expression by siRNA enhances the sulindac sulfide inhibition of cell growth. Examination of the dose-response relationship between growth on soft agar and inhibition of prostaglandin synthesis, induction of NAG-1/ATF3, and suppression of EP4 expression reveals complex relationships. One unexpected observation was that the inhibition (~40%) of prostaglandin formation was observed only at the highest drug concentration, and indomethacin, at up to 120 μmol/L, did not inhibit cell growth. An increase in NAG-1 and ATF3 expression was observed at lower concentrations, whereas inhibition of EP4 expression was observed at the highest drug concentration, resulting in a 75% inhibition of growth. Thus, the inhibition of tumor growth by sulindac sulfide is very complex and involves a number of signal transduction pathways. After treatment with sulindac sulfide, an increase in the expression of NAG-1, ATF3, and EP4 was observed. Although NAG-1 and ATF3 expressions remained high, the level of EP4 declined. The net effect of the changes in protein expression is the suppression of cell growth. Stimulation of the Sp-1/Egr-1 pathways and the subsequent changes in the expression of NAG-1, ATF3, and EP-4 seem to be very important in growth suppression in these cells.

Thus, we propose that one explanation for the effect on chemoprevention activity of sulindac sulfide, in contrast to other COX inhibitors such as indomethacin, is that the drug has two targets, COX inhibition and the ESE-1/EGR-1 pathway. Sulindac sulfide inhibits COX activity and hence reduces the levels of prostaglandins, attenuating the down signaling events that result in tumor growth. In addition, sulindac sulfide activates the ESE-1/EGR-1 pathway, which increases the expression of the antitumorigenic proteins NAG-1 and ATF3 and downregulates the expression of the EP4 receptor. Because EP4 is required for PGE_2 activity, changes in the level of expression should have dramatic effect on prostaglandin biological activity mediated by EP4. The suppression of EP4 expression by sulindac sulfide is a novel mechanism to help explain the antitumorigenic property and suggests a new target for the development of drugs for the prevention or treatment of cancer. Because the side effects of specific COX-2 inhibitors seem to preclude their use as cancer prevention drugs, new approaches are necessary. Suppression of EP4 and the induction of NAG-1/ATF3 expression are likely candidates.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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The Cyclooxygenase Inhibitor Sulindac Sulfide Inhibits EP4 Expression and Suppresses the Growth of Glioblastoma Cells

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