p21$^{Waf1/Cip1}$ Expression by Curcumin in U-87MG Human Glioma Cells: Role of Early Growth Response-1 Expression

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Abstract

Curcumin, a natural compound, is a well-known chemopreventive agent with potent anticarcinogenic activity in a wide variety of tumor cells. Curcumin inhibits cancer cell proliferation in part by suppressing cyclin D1 and inducing expression of the cyclin-dependent kinase inhibitor p21$^{Waf1/Cip1}$. Both p53-dependent and p53-independent mechanisms regulate p21$^{Waf1/Cip1}$ expression, but the mechanism by which curcumin regulates p21$^{Waf1/Cip1}$ expression remains unknown. Here, we report that transcription of the *p21$^{Waf1/Cip1}$* gene is activated by early growth response-1 (Egr-1) independently of p53 in response to curcumin treatment in U-87MG human glioblastoma cells. Egr-1 is a transcription factor that helps regulate differentiation, growth, and apoptosis in many cell types. Egr-1 expression is induced by curcumin through extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK), but not the p38, mitogen-activated protein kinase (MAPK) pathways, which mediate the transactivation of Elk-1. Transient expression of Egr-1 enhanced curcumin-induced p21$^{Waf1/Cip1}$ promoter activity, whereas suppression of Egr-1 expression by small interfering RNA abrogated the ability of curcumin to induce p21$^{Waf1/Cip1}$ promoter activity. In addition, stable knockdown of Egr-1 expression in U-87MG cells suppressed curcumin-induced p21 expression. Our results indicate that ERK and JNK MAPK/Elk-1/Egr-1 signal cascade is required for p53-independent transcriptional activation of p21$^{Waf1/Cip1}$ in response to curcumin in U-87MG human glioblastoma cells. [Cancer Res 2008;68(5):1369–77]

Introduction

The p21$^{Waf1/Cip1}$ protein (hereafter, p21) is a well-characterized cell cycle regulator that inhibits the activity of cyclin/cyclin-dependent kinase (cdk)2 complexes (1). It inhibits cell cycle progression through the transactivation of p53 when cells are exposed to DNA-damaging agents, such as doxorubicin and γ-irradiation (2). In addition to playing a role in DNA damage responses, p21 also plays a crucial role in differentiation, senescence, and apoptosis.

Although p21 expression was initially identified as being p53-dependent, a variety of transcription factors, including SP1/SP3, Smads, AP2, p150(Sal2), the vertebrate homologue of the *Drosophila melanogaster* homeotic transcription factor Spalt, sterol regulatory element-binding protein–1a, STATs, ets-related transcription factor E1AF, AP2, CAAT/enhancer binding protein α/β, Ets-1, and hepatocyte nuclear factor-4α, bind to specific *cis*-acting elements in the p21 promoter to activate transcription of the gene via a p53-independent mechanism (3–12). In addition, the region between −58 and −51 bp from the p21 transcription initiation site contains consensus early growth response-1 (Egr-1) binding sequences (EBS), which play a major role in regulating p21 transcription in response to resveratrol treatment in K562 cells (13) and to tamoxifen in MDA-MB-361 breast cancer cells (14).

Egr-1, also known as *nerve growth factor I-A, zif268, krox24,* and *Tis8,* is an immediate early-response gene induced by stress, injury, mitogens, and differentiation (15). Egr-1 regulates expression of genes involved in the control of growth and apoptosis by *trans*-activating p21, p53, PTEN, transforming growth factor β1, fibronectin, and Gadd45 (13–17). Significantly reduced Egr-1 expression has been observed during tumor formation in various mammalian cells and tissues (18). On the other hand, ectopic expression of Egr-1 inhibits cell proliferation and soft agar growth of NIH3T3 cells transformed with v-*sis*, suggesting that Egr-1 functions as a tumor suppressor (19).

Curcumin (diferuloylmethane) is a natural compound with potent antioxidant, antiinflammatory, anticarcinogenic, and chemopreventive activities in a variety of cancer cell types (20). Curcumin-induced suppression of cellular proliferation is mediated at least in part by blocking cell cycle progression at G_1. This arrest may result from the down-regulation of cyclins D1 (21) and E (22), as well as up-regulation of cell cycle inhibitors such as p21 and p27 (22, 23) in multiple human tumor cell lines. Curcumin-induced down-regulation of cyclin D1 is caused by inhibition of nuclear factor-κB (NF-κB) activity through the suppression of IκB kinase (IKK) and activation of Akt (22) because the promoter of cyclin D1 is regulated by NF-κB (24). However, the molecular mechanism underlying the curcumin-induced up-regulation of p21 remains unanswered.

In this study, we assessed the mechanism by which curcumin stimulates transcriptional activation of p21 in U-87MG cells. We show for the first time that the induction of p21 by curcumin is mediated by transactivation of Egr-1 independently of p53. We also show that curcumin activates the extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) pathways, which activate the transcription factor Elk-1. This activation leads to up-regulation of Egr-1, which results in the transcriptional activation of the p21

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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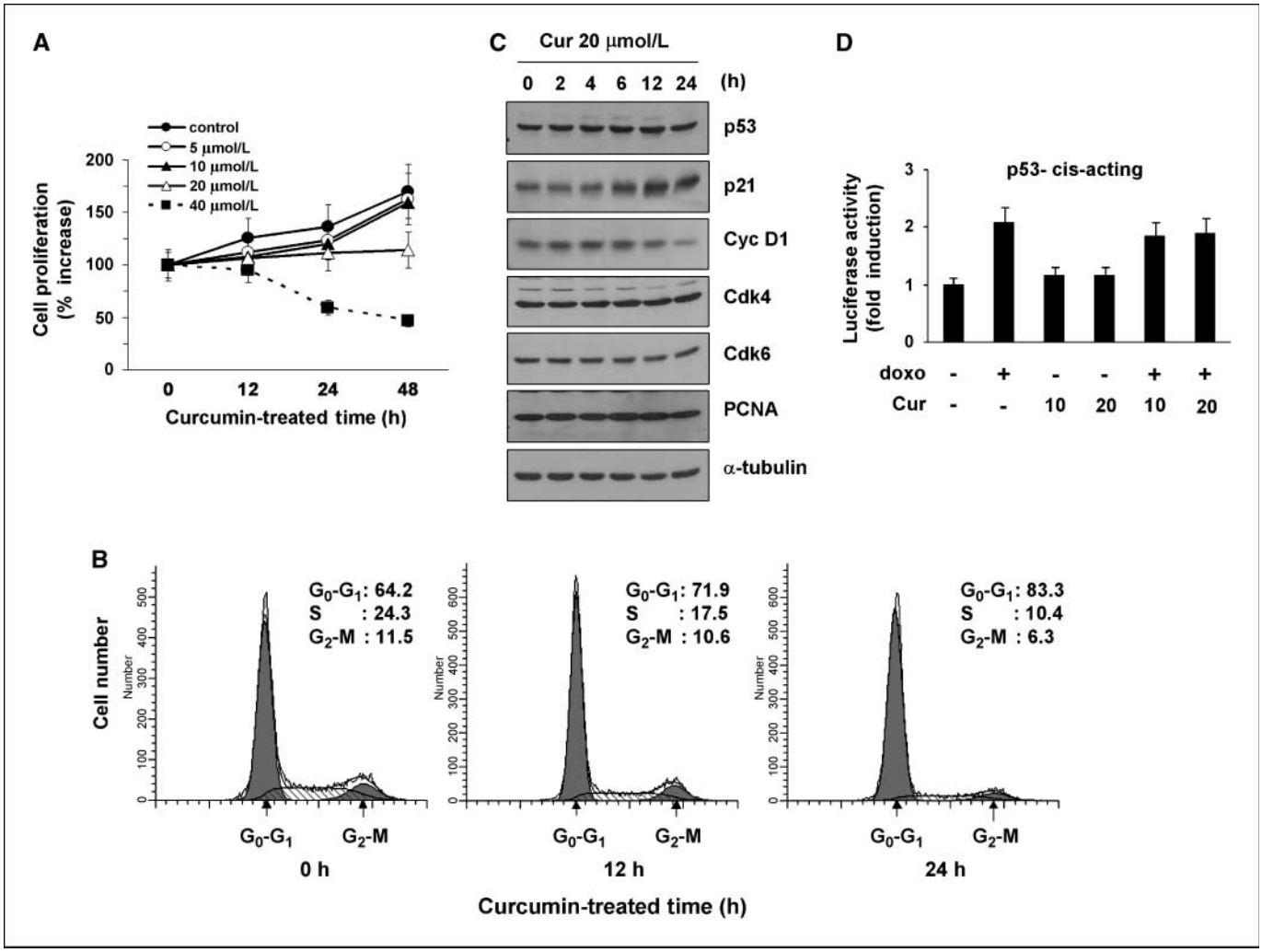

Figure 1. Curcumin arrests the cell cycle at G_1. *A,* U-87MG cells were seeded onto 96-well culture plates and treated with various concentrations of curcumin for the indicated lengths of time. Cellular proliferation was measured using Cell Counting kit-8. *Points,* mean of one experiment performed in triplicate; *bars,* SD. Similar results were obtained from two other independent experiments. *B,* U-87MG cells were treated with 20 μmol/L curcumin. After the indicated times (12 h and 24 h), the cells were harvested, fixed with ethanol, and stained with propidium iodide. The DNA content was analyzed using flow cytometry. The percentages of the cell population at each phase of the cell cycle are indicated in each histogram. Similar results were obtained from three independent experiments. *C,* exponentially growing U-87MG cells were cultured in the absence or presence of 20 μmol/L curcumin for the indicated lengths of time. At the indicated time points, cells were collected and analyzed for protein expression using Western blotting. The same blot was reprobed with anti–α-tubulin antibody as an internal control. Each blot represents three separate experiments. *D,* U-87MG cells were transfected with 0.5 μg 5× p53-Luc *cis*-reporting plasmid containing five repeats of the p53 binding site, along with 50 ng pRL-null vector. After 24 h, the cells were treated with doxorubicin (1 μg/mL), curcumin (10 or 20 μmol/L), or a combination for an additional 8 h. Firefly luciferase activity was normalized to the *Renilla* activity. *Columns,* mean of three independent experiments performed in triplicate; *bars,* SD.

promoter. Our results indicate that Egr-1 is required for p53-independent transcriptional activation of p21.

Materials and Methods

Cell culture and reagents. Human U-87MG glioblastoma cells and rat C6glioma cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone). Curcumin was purchased from Sigma Co. Antibodies against phospho-ERK1/2 MAPK (Thr^{202}/Tyr^{204}), phospho-JNK1/2 (Thr^{183}/Tyr^{185}), phospho-p38 MAPK (Thr^{180}/Tyr^{182}), and phosphor-Elk-1 (Ser^{383}) were obtained from Cell Signaling Technology. Antibodies against Egr-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ERK2 were obtained from Santa Cruz Biotechnology. The firefly and *Renilla* Dual-Glo Luciferase Assay System was purchased from Promega.

Construction and mutagenesis of the $p21^{Waf1/Cip1}$ promoter. The construction of p21-Luc(−150/+38), a $p21^{Waf1/Cip1}$ promoter fragment that spans nt −150 to +39 and lacks the p53 binding site, and a mutant construct, p21-Luc(−150/+38)mtEgr1, which contains a mutated EBS, has been described elsewhere (14).

Plasmids. The luciferase reporter plasmid pGL-2.4, which contains 2.4 kb of the human p21 promoter, was provided by Dr. Jae-Yong Lee (Department of Biochemistry, College of Medicine, Hanlym University, Korea). Full-length [Pegr1-Luc(−780/+1)] and serial deletion mutant reporter constructs of the Egr-1 promoter expressing firefly luciferase were provided by Dr. H. Eibel (Department of Orthopedic Surgery, University of Tubingen Medical Center, Germany) and are described elsewhere (25). The plasmid pRL-null, which encodes *Renilla* luciferase, was purchased from Promega, whereas the p53 *cis*-acting reporter plasmid p53-Luc was purchased from Stratagene. Plasmids expressing dominant-negative (dn) JNK1 (pSRα/HA-JNK T183A/Y185F) and kinase-dead (kd) p38 kinase (pCDNA3/flag-p38 T180A/Y182F) were donated by Dr. D. S. Min (Department of Molecular Biology, College of Natural Science, Pusan National University, Korea).

Cell proliferation assay. U-87MG cells were seeded onto 96-well plates (2×10^3 cells per well) and treated with various concentrations of curcumin for the various lengths of time. Proliferation was measured using a Cell Counting kit-8 (Dojindo Molecular Technologies) with the water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] as a substrate.

Cell cycle analysis. Cellular DNA content was analyzed by flow cytometry as described previously. Briefly, U-87MG cells were collected after 12 or 24 h of exposure to 20 μmol/L curcumin, fixed in 70% ethanol, washed twice with PBS, and stained with a 50 μg/mL propidium iodide solution containing 0.1% Triton X-100, 0.1 mmol/L EDTA, and 50 μg/mL RNase A. Fluorescence was measured and analyzed using a FACSCalibur Flow Cytometer (Becton Dickinson Immunocytometry Systems).

Western blot analysis. Cells were lysed in a buffer consisting of 20 mmol/L HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 150 mmol/L NaCl, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. The protein extracts (20 μg each) were separated by 10% SDS-PAGE and transferred to nitrocellulose filters. The blots were incubated with the corresponding primary antibodies and developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Northern blot analysis. For each sample, 10 μg total RNA was electrophoresed on a formaldehyde/agarose gel and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech). Northern blotting was performed with a [γ-^{32}P]dCTP-labeled Egr-1 cDNA probe followed by hybridization with a GAPDH cDNA probe.

Transient transfection and promoter reporter assay. U-87MG cells were seeded onto 12-well plates and transfected with 0.5 μg p21 promoter (14) or Egr-1 promoter construct (26) using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. To monitor the transfection efficiency, a pRL-null plasmid (50 ng) encoding *Renilla* luciferase was included in all transfections. Where indicated, a mammalian expression vector encoding dn-MEK1, dn-JNK1, or kd-p38 kinase was also included. At 24 h posttransfection, the cells were serum starved by culturing them for 12 h in 0.5% serum and then treated with curcumin. After 6 to 12 h, the levels of firefly and *Renilla* luciferase activity were measured sequentially from a single sample using the Dual-Glo Luciferase Assay System. Luminescence was measured with a luminometer (Centro LB960; Berthold Tech).

Expression of Egr-1 small interfering RNA. The small interfering RNA (siRNA) expression plasmid targeted to Egr-1 mRNA (pSilencer/siEgr1#1) is described elsewhere (26). To generate another Egr-1 siRNA expression plasmid (pSilencer/siEgr1#2), we used siRNA Target Finder, a Web-based algorithm maintained by Ambion. The oligonucleotide sequences corresponding to nucleotides 1630 to 1638 (5′-GTTACTACCTCTTATC-CAT-3′) downstream of the transcription start site of Egr-1 (Supplementary Fig. S3) were selected and cloned into the pSilencer 2.1-U6 siRNA expression vector according to the manufacturer's protocol. A mixture of double-stranded RNA nucleotides targeting different regions of Egr-1 mRNA and a negative control siRNA were obtained from Dharmacon Research (Thermo Fisher Scientific; Supplementary Fig. S3). For transient expression, U-87MG cells were transfected with pSilencer/scramble or pSilencer/siEgr1 plasmids using Lipofectamine 2000 (Invitrogen Life Technologies), or transfected with siRNA oligonucleotides using DharmaFECT reagent according to the manufacturer's protocol (Dharmacon). For the generation of stable cells

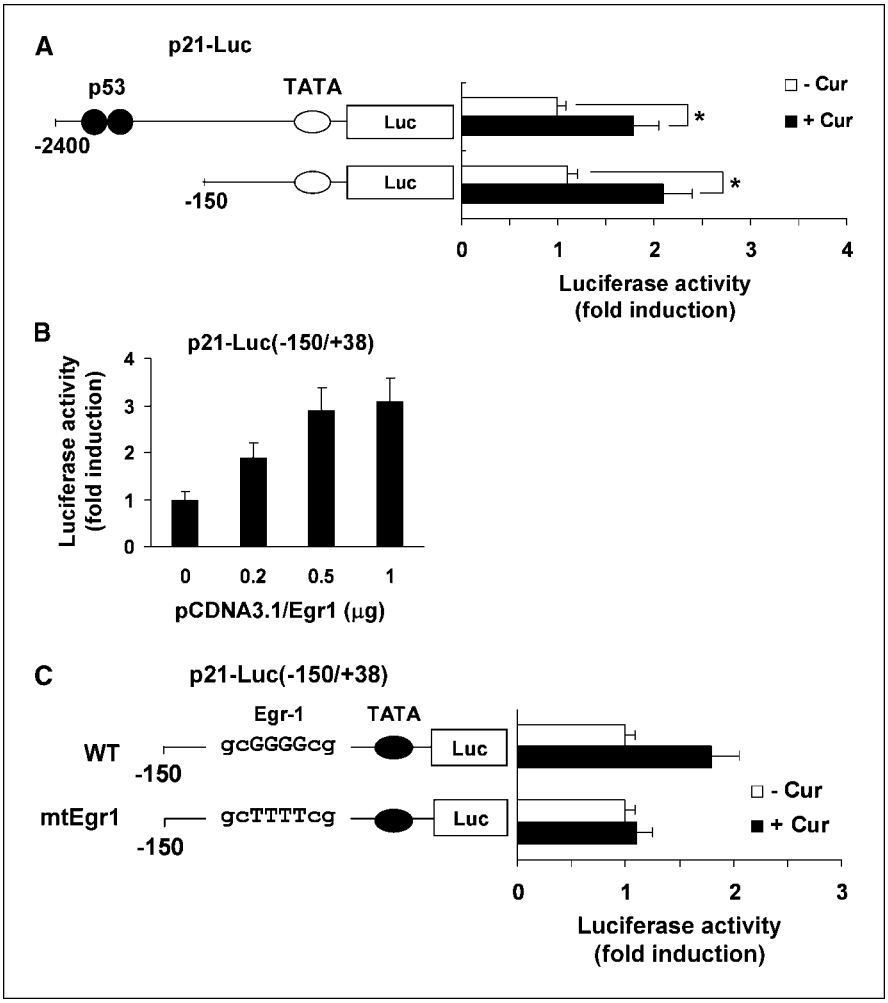

Figure 2. Curcumin stimulates p21 promoter activity via Egr-1. *A,* U-87MG cells were cotransfected with 0.5 μg p21-Luc (−2,400/+1 or −150/+38) plasmid and various concentrations of an Egr-1 expression plasmid (pcDNA3.1zeo/Egr1). After 24 h, the cells were either left untreated or were treated with 10 μmol/L curcumin for 8 h and then analyzed for luciferase activity. The statistical significance of the assay was evaluated using Student's *t* test (*, $P < 0.01$ compared with vector-transfected control cells). *B,* U-87MG cells were cotransfected with 0.5 μg p21 promoter-reporter plasmid p21-Luc(−150/+38) with increasing amounts of Egr-1 expression plasmid pcDNA3.1zeo/Egr1 as indicated. *C,* U-87MG cells were transfected with p21-Luc(−150/+38; wild-type; *WT*) or with the mutant construct mtEgr1. After 24 h, the cells were either left untreated or treated with 10 μmol/L curcumin for 8 h and then analyzed for luciferase activity. Firefly luciferase activity was normalized to the *Renilla* activity. *Columns,* mean of three independent experiments performed in triplicate; *bars,* SD.

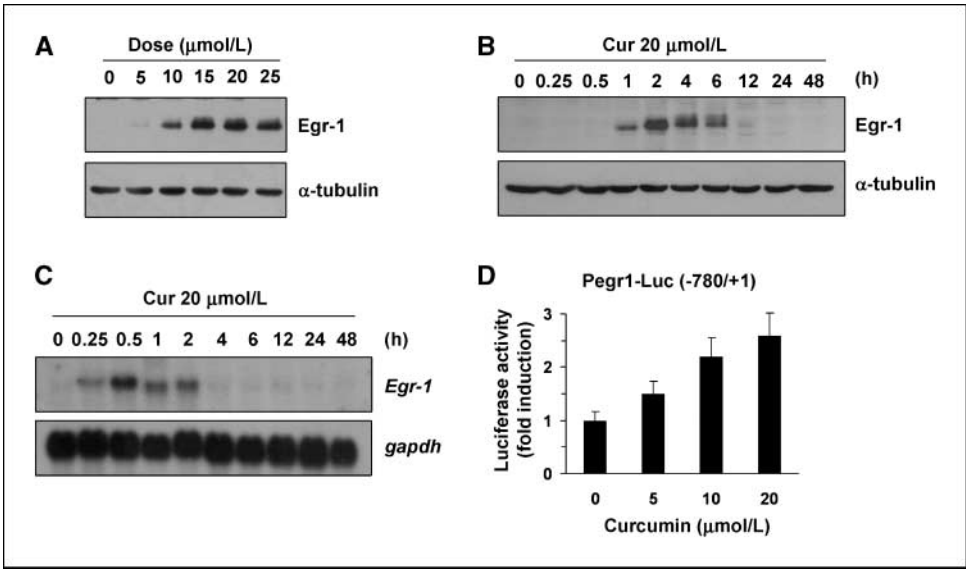

Figure 3. Induction of Egr-1 expression by curcumin. *A* and *B,* serum-starved U-87MG cells (grown with 0.5% serum for 24 h) were treated with the indicated concentrations of curcumin for 2 h (*A*) or with 20 μmol/L curcumin for various lengths of time (*B*). The level of Egr-1 was measured in whole cell lysates (15 μg per lane) by Western blotting with rabbit anti–Egr-1 antibodies. *Bottom,* same blot probed with anti–α-tubulin antibodies as a loading control. *C,* total RNA (10 μg) was isolated from cells treated as in *B*, electrophoresed on a 1% agarose gel, and capillary transferred to a nylon filter. The blot was hybridized with a ^{32}P-labeled Egr-1 cDNA probe. GAPDH mRNA expression was measured as a control to verify the amount of RNA in each lane. Each blot is representative of at least three separate experiments. *D,* subconfluent U-87MG cells grown in 12-well plates were cotransfected with 0.5 μg full-length Egr-1 promoter construct Pegr1-Luc(−780/+1) and 50 ng pRL-null vector. Twenty-four hours after transfection, the cells were treated with varying concentrations of curcumin (5, 10, or 20 μmol/L) for 8 h. Firefly luciferase activity was normalized to the *Renilla* activity. *Columns,* mean of three independent experiments performed in triplicate; *bars,* SD.

expressing Egr-1 siRNA, U-87MG glioblastoma cells were transfected with empty pSilencer/neo or pSilencer/siEgr1#2 using Nucleofactor (AMAXA, Inc.). Stable transfectants were selected using 400 μg/mL G418 2 days after transfection. After 2 weeks, silencing of Egr-1 expression in response to curcumin was determined by Western blotting.

Statistical analysis. Each experiment was performed at least thrice. The data were plotted as the mean ± SD. Student's *t* test was used for all comparisons. A *P* value of <0.05 was considered statistically significant.

Results

Curcumin arrests the cell cycle at G_1 in U-87MG glioblastoma cells. To understand the mechanism by which curcumin inhibits cell growth, we initially tested its antiproliferative effect on U-87MG cells using a Cell Counting kit-8. Exponentially growing cells were exposed to various concentrations of curcumin for 12, 24, and 48 h, and their proliferation was monitored. A significant decrease in proliferation was observed in cells treated for 48 h with 20 μmol/L curcumin compared with untreated control cells (Fig. 1*A*). Exposure to 40 μmol/L curcumin produced an even stronger effect, probably by promoting cell death (27). Thus, curcumin has an antiproliferative effect on U-87MG glioma cells.

Then, we created a cell cycle profile to examine the mechanism by which curcumin inhibits cellular proliferation. We used a curcumin concentration of 20 μmol/L for these experiments because curcumin at this concentration significantly reduced proliferation without producing cytotoxic effects. As shown in Fig. 1*B*, an effect on cell cycle progression was clearly observed within 12 h of treatment, with an increased number of cells in G_1. With prolonged exposure (24 h), a progressive accumulation of cells in G_1 (83.3%, compared with baseline of 64.2%) was observed, with a concomitant decline in the number of cells in S (10.4%, compared with baseline of 24.3%) or in G_2-M phase (6.3%, compared with a baseline of 11.5%). These data clearly suggest that curcumin inhibits proliferation of U-87MG glioblastoma cells by inducing G_1 arrest.

We examined the effect of curcumin on several G_1 regulatory proteins (Fig. 1*C*). Time-response analysis showed that after 6 h of curcumin exposure, the level of p21 increased significantly, whereas the level of cyclin D1 decreased. In contrast, levels of p53, Cdk4, Cdk6, proliferating cell nuclear antigen (PCNA), and α-tubulin were largely unchanged. Curcumin treatment increased the abundance of the p21 protein in a concentration-dependent manner. Treatment with 10 μmol/L curcumin resulted in an ~2-fold increase in the p21 level relative to that in untreated cells, whereas a near-maximum increase of ~2.9-fold was observed after stimulation with 20 μmol/L curcumin (Supplementary Fig. S1). Moreover, no *cis*-activation of p53 by curcumin was detected, whereas doxorubicin, a positive control, stimulated the activity of the reporter by ~2.2-fold (Fig. 1*D*). These data suggest that curcumin arrests the cell cycle at G_1 via p53-independent induction of p21, with a concomitant reduction in cyclin D1.

Curcumin stimulates p21 promoter activity through a p53-independent mechanism. Because p21 expression preceded the suppression of cyclin D1 expression in our experiment (Fig. 1*C*), and curcumin-induced down-regulation of cyclin D1 was previously shown to be mediated by inhibition of NF-κB activity through the suppression of IKK and Akt activation (28), we focused on the mechanism by which curcumin induces p21 accumulation. To this end, we examined whether curcumin increased p21 levels through transcriptional activation of the p21 promoter. The full-length p21 promoter-reporter construct with 2.4 kb 5′-flanking sequence (−2400/+1) was transfected into U-87MG cells, and the level of luciferase activity was measured. As shown in Fig. 2*A*, curcumin increased the transcriptional activity of the full-length promoter by 1.8-fold, which was statistically significant ($P < 0.05$; $n = 9$). To rule out a role of p53 in curcumin-induced p21 promoter activation, we

used p21-Luc(−150/+38), a deletion construct lacking the distal p53 consensus binding site. Curcumin also activated this construct (~2.5-fold; $P < 0.05$; $n = 9$), despite the lack of a p53 binding site. These data show that curcumin-induced accumulation of p21 is mediated via transcriptional activation of the p21 gene, independently of p53, and that the proximal region (i.e., within −150 bp of the transcription start site) is necessary for curcumin-induced activation of p21.

Egr-1 is required for curcumin-induced activation of the p21 promoter. Egr-1 is a Cys2/His2-type zinc-finger transcription factor that regulates cell growth, differentiation, and development (15). We and others have shown that activation of p21 gene transcription by resveratrol in K562 erythroleukemic cells (13) and by tamoxifen in MDA-MB-361 breast cancer cells (14) is mediated by Egr-1 *trans*-activation through a putative consensus EBS in the −58 to −51 region of the p21 promoter. To determine if Egr-1 is involved in p53-independent activation of the p21 promoter by curcumin in U-87MG cells, p21-Luc(−150/+38), which lacks the p53 binding site, was cotransfected into the cells with an Egr-1 expression vector (pCDNA3.1/Egr1mycHis). As shown in Fig. 2*B*, p21 promoter activity increased as the amount of transfected Egr-1 increased, suggesting that Egr-1 elevates *trans*-activation of the p21 promoter independently of p53.

To evaluate the functional role of EBS in mediating curcumin-induced p21 promoter activity, we made point mutations in the core EBS sequence (*gggg* to *tttt*); these mutations completely eliminated the response to curcumin (Fig. 2*C*). These results show that p21 is transcriptionally regulated by Egr-1 in response to curcumin, and that the EBS in the proximal region of the gene is necessary for the curcumin responsiveness of U-87MG cells.

Curcumin up-regulates Egr-1 expression at the transcriptional level. We evaluated whether Egr-1 is induced by curcumin treatment in U-87MG cells. Serum-starved U-87MG cells were treated with various concentrations of curcumin for 2 h, after which the amount of Egr-1 was measured by Western blotting. We found that the amount of Egr-1 increased markedly with curcumin treatment, and that the effect was concentration-dependent, with a near maximum at 15 μmol/L (Fig. 3*A*). A time course study showed that the level of Egr-1 increased within 1 h of initiation of curcumin treatment, reached a peak at ~2 h, dropped considerably by 6 h, and subsequently returned to a basal level (Fig. 3*B*).

To establish whether curcumin induces Egr-1 transcription, we used Northern blotting to measure Egr-1 mRNA levels in curcumin-treated U-87MG cells. Egr-1 transcription was rapidly induced within 15 min and reached a maximum within 30 min; the Egr-1 mRNA level returned to its basal value after 2 h exposure, whereas the GAPDH mRNA level remained constant the entire time (Fig. 3*C*). Then, we examined whether curcumin influences Egr-1 expression by directly affecting transcription. Egr-1 promoter activity was analyzed in U-87MG cells using the construct Pegr1-Luc(−780/+1), which contains 780 bp 5′-flanking sequence and the TATA motif of the Egr-1 promoter (26). Increasing the concentration of curcumin increased Egr-1 promoter activity (Fig. 3*D*), suggesting that the curcumin-induced accumulation of Egr-1 mRNA occurs primarily through transcriptional activation. In addition, Egr-1 and p21 proteins also accumulated in human U-251MG and rat C6 glioblastoma cells (Supplementary Fig. S2), suggesting that curcumin-induced p21 expression via Egr-1 is not restricted to U-87MG cells.

ERK and JNK MAPKs are required for curcumin-induced Elk-1 activation. In many cell systems, activation of MAPK signaling induces Egr-1 promoter activity (14, 29–33). To determine whether curcumin can activate MAPK signaling, we treated serum-starved U-87MG cells with curcumin for various lengths of time and measured the activation status of three major MAPK pathways using phospho-specific antibodies. In accordance with previous observations (34), the levels of phosphorylated ERK1/2, JNK1/2, and p38 MAPK increased in a time-dependent manner in response to curcumin treatment (Fig. 4*A*). The total amount of these proteins did not change, however, demonstrating an actual increase in phosphorylation status, which is indicative of increased activity.

Elk-1, a member of the Ets family of transcription factors (35), plays a crucial role in growth factor-induced Egr-1 transcription by forming a complex with serum response factor on the serum response element of the Egr-1 promoter (36). Elk-1 is phosphorylated and *trans*-activated by the ERK, JNK, and p38 MAPKs (37).

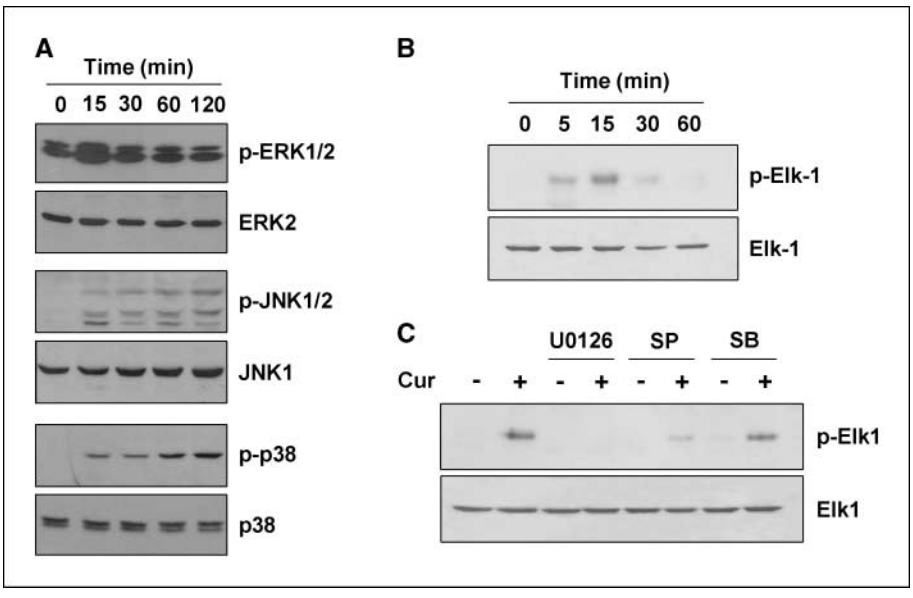

Figure 4. Stimulation of multiple MAPKs by curcumin. *A* and *B*, U-87MG cells were serum-starved (grown in 0.5% serum) for 24 h and then treated with 20 μmol/L curcumin for various lengths of time. Western blotting was performed using total protein extracts and antibodies against phospho-ERK1/2 (Thr^{202}/Tyr^{204}), phospho-JNK1/2 (Thr^{183}/Tyr^{185}), phospho-p38 kinase (Thr^{180}/Tyr^{182}; *A*), and phospho-Elk-1 (Ser^{383}; *B*). Blots were then probed with antibodies against total proteins as an internal control. Each blot is representative of at least three separate experiments. *C*, serum-starved U-87MG cells were treated with U0126, SP600125, or SB203580 for 30 min, and then either left unstimulated or stimulated with curcumin for 2 h. Total cell lysates were prepared and subjected to Western blotting with anti–phospho-Elk-1 (Ser^{383}) antibody. The same blot was reprobed with anti–Elk-1 antibody as an internal control. Each blot is representative of at least three separate experiments.

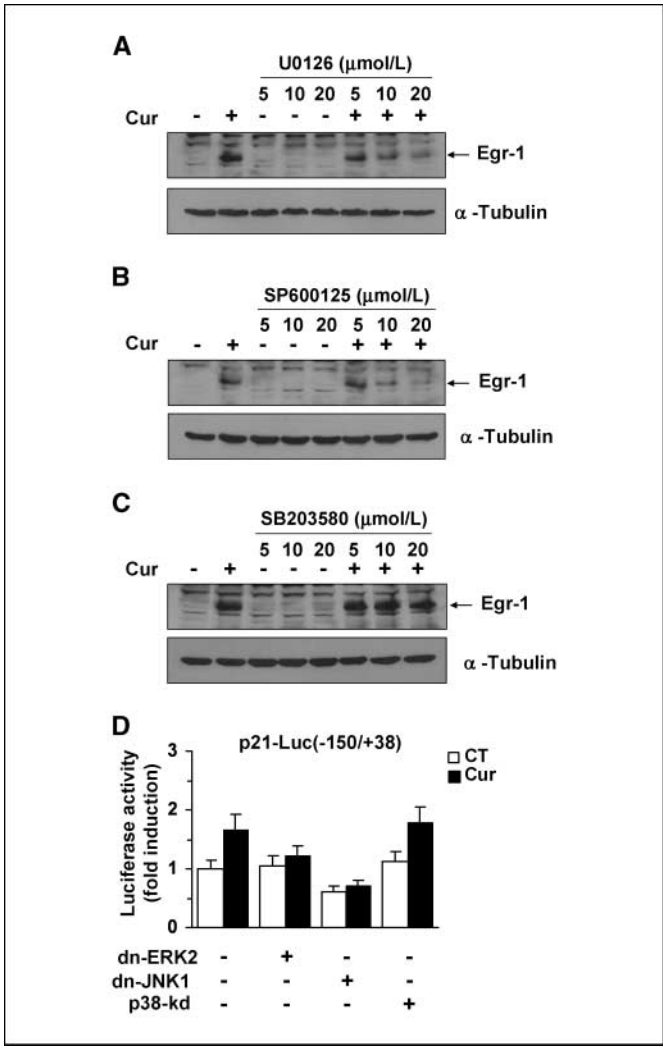

Figure 5. Induction of Egr-1 expression by curcumin via the ERK and JNK pathways. *A* to *C,* serum-starved U-87MG cells were treated with various concentrations of U0126 (*A*), SP600125 (*B*), or SB203580 (*C*) for 30 min, and then either left unstimulated or stimulated with curcumin for 2 h. Total cell lysates were prepared and subjected to Western blotting with anti–Egr-1 antibody. The same blot was reprobed with anti–α-tubulin antibody as an internal control. Each blot is representative of at least three separate experiments. *D,* U-87MG cells were cotransfected with 0.5 μg p21-Luc(−150/+38) and 50 ng pRL-null vector, along with 0.2 μg dn-ERK2, dn-JNK1, or kd-p38 MAPK, as indicated. Twenty-four hours after transfection, the cells were either left untreated or treated with 10 μmol/L curcumin for 8 h. Firefly luciferase activity was normalized to the *Renilla* activity. *Points,* mean of three independent experiments performed in triplicate; *bars,* SD.

Therefore, we tested the ability of curcumin to stimulate the phosphorylation of Elk-1. As shown in Fig. 4*B*, curcumin treatment increased the amount of phosphorylated Elk-1, but not the total amount of Elk-1, in the nuclear fraction of U-87MG cells. The increase in phosphorylated Elk-1 was evident within 5 min, continued for 15 min, and subsided by 60 min. Moreover, pretreatment with the MAP/ERK kinase inhibitor U0126 and the JNK inhibitor SP600125 inhibited induction of Elk-1 phosphorylation by curcumin, whereas pretreatment with the p38 inhibitor SB203580 did not (Fig. 4*C*). These data suggest that ERK and JNK signaling pathways are required for curcumin-induced Elk-1 activation.

ERK and JNK MAPKs are required for curcumin-induced p21 expression via Egr-1. We next examined whether the ERK and JNK kinase pathways play a role in curcumin-induced Egr-1 expression. In accord with the Elk-1 phosphorylation results, pretreatment with U0126 (Fig. 5*A*) and SP600125 (Fig. 5*B*), but not SB203580 (Fig. 5*C*), blocked the curcumin-induced accumulation of Egr-1 in a dose-dependent manner. To investigate the possible contribution of the ERK and JNK pathways in curcumin induction of p21, U-87MG cells were cotransfected with the p21 promoter reporter and mutant MAPK constructs. As shown in Fig. 5*D*, expression of dn-ERK2 or dn-JNK1, but not kd-p38, strongly inhibited curcumin-induced activation of the p21 promoter. These data suggest that stimulation of ERK and JNK pathways is functionally linked to p21 expression by curcumin. Thus, the ERK and JNK MAPK pathways seem to be required for curcumin-induced p21 expression via Egr-1 induction.

Silencing of Egr-1 expression blocks curcumin-induced p21 expression. To determine whether the silencing of Egr-1 expression is correlated with the reduction of p21 expression, RNA interference was used. As shown in Fig. 6*A*, transient transfection of two different Egr-1 siRNA plasmids (pSilencer/siEgr1#1 and pSilencer/siEgr1#2; Supplementary Fig. S3) clearly attenuated curcumin-induced accumulation of Egr-1 (2 h after stimulation) and p21 proteins (12 h after stimulation). Similarly, a mixture of four siRNA oligonucleotides that target different regions of Egr-1 mRNA also attenuated the ability of curcumin to induce the expression of both Egr-1 and p21 (Fig. 6*B*). In addition, curcumin-induced p21 promoter activity was substantially inhibited by transient transfection of Egr-1 siRNA plasmid (pSilencer/siEgr1#2) in U-87MG cells (Supplementary Fig. S4).

To further corroborate the role of Egr-1 in the induction of p21 by curcumin, we established stable cell lines expressing Egr-1 siRNA (U87/siEgr1). Stable expression of Egr-1 siRNA on knockdown of the Egr-1 protein level was evaluated after curcumin treatment in serum-starved cells (Supplementary Fig. S5). In exponentially growing U87/siEgr1 cells, the silencing of Egr-1 was also observed (Fig. 6*C, top*). Under these conditions, the ability of curcumin to induce p21 expression was substantially attenuated (Fig. 6*C, second panel*). Similarly, stable expression of Egr-1 siRNA in rat C6 glioma cells suppressed curcumin-induced p21 expression (Supplementary Fig. S6). These data strongly implicate Egr-1 as the transcription factor responsible for curcumin-induced up-regulation of p21 expression. Moreover, compared with control U87/vec cells, U87/siEgr-1 cells displayed resistance to curcumin-induced G_1 arrest (Fig. 6*D*).

Collectively, these results lead us to conclude that the antiproliferative effects of curcumin in U-87MG glioblastoma cells are mediated at least in part by the induction of p21 via a p53-independent mechanism involving activation of the ERK and JNK MAPK/Elk-1/Egr-1 cascade.

Discussion

In this study, we found that curcumin induces cell cycle arrest at G_1 in U-87MG human glioblastoma cells by promoting the accumulation of the cdk inhibitor p21 with a concomitant decrease in the level of cyclin D1. We focused on the regulatory mechanism of p21 induction by curcumin. Our data provide multiple lines of evidence that the induction of p21 by curcumin in U-87MG cells is mediated by p53-independent transactivation of Egr-1. Differential activation of the ERK and JNK MAPK pathways by curcumin promotes phosphorylation of Elk-1, which activates Egr-1 expression, and Egr-1 binds directly to the proximal region of the p21

promoter to stimulate p21 transcription independently of p53. The requirement for Egr-1 is supported by the fact that human U-87MG cells expressing Egr-1 siRNA are refractory to curcumin-induced p21 expression.

p21 plays an important role in cell cycle arrest, senescence, differentiation, and apoptosis, and its expression is modulated by a wide variety of external stimuli, including radiation, DNA damage, and growth factors (38, 39). It has become apparent that at low concentrations, p21 promotes the association and activation of D-type cyclin kinase, whereas at higher concentrations, it inhibits Cdk activity, resulting in the suppression of cell cycle progression. A major transcriptional activator of p21 is the tumor suppressor p53, which binds specific sites within the p21 promoter (2). However, several lines of evidence suggest that the transcriptional activity of the p21 promoter can be induced in a variety of cell types by several transcription factors other than p53 (3–12). Here, we observed that curcumin induced the transcriptional activation of p21 in U-87MG human glioblastoma cells without significantly affecting the expression and *cis*-acting activity of p53, which suggests that curcumin-induced p21 expression is independent of p53. To test this possibility, we analyzed a response element within the p21 promoter. In U-87MG cells transiently transfected with a mutant p21 promoter lacking the p53 binding site, curcumin still induced luciferase expression, indicating that the p53 site does not affect curcumin-induced activation of the p21 promoter.

In contrast, transient expression of Egr-1 in the above system enhanced both the basal and curcumin-induced levels of

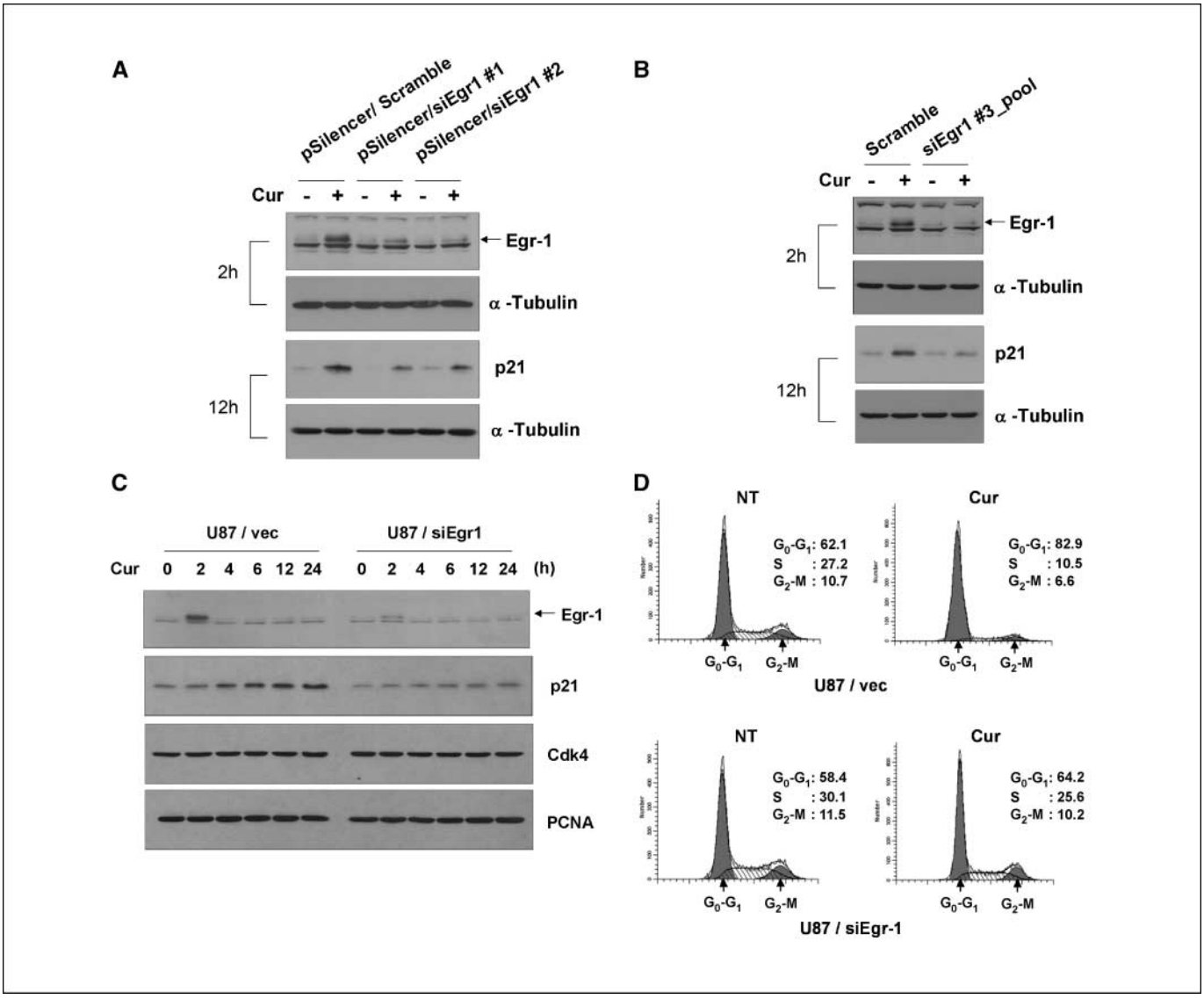

Figure 6. Effect of silencing of Egr-1 on the expression of p21. *A,* U-87MG cells were transiently transfected with pSilencer/scramble or pSilencer/siEgr1 plasmids encoding siRNA against Egr-1 mRNA. *B,* U-87MG cells were transfected with a negative control siRNA (scramble) or a mixture of four double-stranded Egr-1 siRNAs (siEgr1#3_pool). After 48 h, cells were either left untreated or treated with 20 μmol/L curcumin for 2 h (for Egr-1 expression) or for 12 h (for p21 expression), as indicated. *C,* exponentially growing U87/vec and U87/siEgr-1 cells were cultured in 20 μmol/L curcumin for increasing lengths of time. At the indicated time points, the cells were collected and analyzed for Egr-1 or p21 expression using Western blotting. The same blot was reprobed with anti-CDK4 or PCNA antibody. Each blot shown is representative of at least three separate experiments. *D,* exponentially growing U87/vec and U87/siEgr-1 cells were treated with 20 μmol/L curcumin. After 24 h, the cells were harvested, fixed with ethanol, and stained with propidium iodide. The DNA content was analyzed using flow cytometry. The percentages of the cell population at each phase of the cell cycle are indicated in each histogram. Similar results were obtained from three independent experiments.

p21-Luc(−150/+38) reporter activity. The putative consensus EBS is in the region from −58 to −51 within the p21 promoter (13). Site-directed mutagenesis of the EBS completely eliminated curcumin-induced p21 promoter activity, as did transient expression of siRNA targeted to Egr-1 mRNA. More importantly, human U-87MG cells stably expressing the Egr-1 siRNA showed attenuation of p21 expression in response to curcumin treatment. These results imply that Egr-1 plays a critical and p53-independent role in curcumin-induced activation of the p21 promoter. This view is supported by previous studies from our laboratory and others showing that Egr-1 is involved in tamoxifen-induced up-regulation of p21 in MDA-MB-361 breast cancer cells (14) and in resveratrol-induced up-regulation of p21 in K562 leukemia cells (13).

MAPK pathways play an important role in signal transduction from the cell surface to the nucleus in response to a wide variety of extracellular signals, and the expression of these pathways may activate gene transcription (40). ERK, JNK, and p38 are the best-known of the five major MAPK subfamilies in mammals, and Elk-1 is a direct target of all three (37). We found that ERK1/2, JNK1/2, and p38 MAPK were rapidly and transiently activated after curcumin treatment. Our results are in agreement with a previous report that curcumin can activate the ERK, JNK, and p38 MAPK signaling pathways in U-87MG cells (34). In U-87MG cells, although the level of p38 phosphorylation was increased by curcumin treatment, pretreatment with the p38 inhibitor SB203580 did not prevent curcumin-induced Elk-1 phosphorylation or Egr-1 expression. Moreover, transient expression of kd-p38 did not block curcumin-induced p21 promoter activity. In contrast, pretreatment with MEK1 inhibitor U0126 or JNK inhibitor SP600125 strongly abrogated the curcumin-induced Elk-1 phosphorylation and Egr-1 expression. The transient expression of dominant-negative mutants of ERK2 and JNK1 prevented curcumin-induced p21 promoter activity. These results suggest that ERK and JNK pathways, but not p38 MAPK, are involved in curcumin-induced up-regulation of p21 in U-87MG cells.

In general, the ERK MAPK pathway is associated with mitogenic signaling and represents an excellent target for anticancer therapy in many human cancers (41), whereas the JNK MAPK pathway induces apoptosis. For example, JNK-dependent apoptosis is suppressed by activation of the ERK signal, whereas JNK activation, in response to tumor necrosis factor and ceramide, negatively regulates ERK1/2 by uncoupling ERK1/2 from MEK (42). In arsenic trioxide–treated A431 cells, the ERK pathway induces activation of the p21 promoter, whereas JNK suppresses p21 expression (43). In addition, ERK1/2 contributes to p53 activation by phosphorylating p53 on Ser^{15} (44), whereas JNK targets p53 for ubiquitin-mediated proteasomal degradation in unstressed cells (45), suggesting that ERK and JNK may have opposite effects on p21 expression. However, we did not observe these opposing functions, but rather found that both U0126 and SP600125 inhibited Elk-1 phosphorylation, which resulted in the inhibition of curcumin-induced Egr-1 expression. In curcumin-induced apoptosis, the inhibition of Akt and activation of ERK, but not JNK or p38, induce autophagy in U-87MG cells (34). In contrast, JNK, but not p38 or ERK, signaling is involved in curcumin-induced apoptosis in HCT116 human colon cancer cells (46). Thus, the specific roles of the ERK and JNK pathways likely depend on cell-type–specific physiologic conditions. Indeed, the JNK and ERK pathways seem to be involved in both apoptosis and proliferation (47).

In summary, we have presented here the first evidence that curcumin promotes p21 transcription independently of p53 via ERK and JNK MAPK/Elk-1/Egr-1 signaling in U-87MG human glioblastoma cells. Our results support previous findings that Egr-1 is required for p53-independent transcriptional activation of p21. Egr-1 is thus a promising target for future developments in the treatment or prevention of human gliomas.

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