The Transcription Factor EGR-1 Directly Transactivates the Fibronectin Gene and Enhances Attachment of Human Glioblastoma Cell Line U251*

Received for publication, November 10, 1999, and in revised form, March 16, 2000
Published, JBC Papers in Press, April 26, 2000, DOI 10.1074/jbc.M900546199

Chaoting Liu‡§, Jin Yao¶, Dan Mercola§§, and Eileen Adamson**

From the ‡Sidney Kimmel Cancer Center, San Diego, California 92121, the ¶Department of Immunology, Scripps Research Institute and the **Burnham Institute, La Jolla, California 92037, and the †Cancer Center, University of California at San Diego, La Jolla, California 92039

EGR-1, a transcription factor with important functions in the regulation of growth and differentiation, is highly expressed in brain. Previous studies have shown that EGR-1 suppresses the transformed phenotype. However, the expression and role of EGR-1 in human glioblastoma cells are not yet determined. In this study, we found that the basal expression of the EGR-1 protein is undetectable, but is inducible in four human glioblastoma cell lines. To determine EGR-1 functions, we re-expressed EGR-1 in human glioblastoma U251 cells and found that the secretion of transforming growth factor-β (TGF-β1), plasminogen activator inhibitor-1 (PAI-1), and fibronectin (FN) was greatly enhanced. Addition of anti-TGF-β antibodies completely inhibited the secretion of PAI-1, but had little effect on secretion of FN, indicating that PAI-1 is under the control of EGR-1-induced TGF-β1. An examination of the promoter of the FN gene revealed two EGR-1-binding sites between positions −75 and −52 and positions −4 and +14 that specifically bind EGR-1 in gel mobility shift experiments. Utilizing wild-type and mutant FN promoter/luciferase reporter genes, we demonstrated that EGR-1 positively regulates the activity of the FN gene. In addition, cell adhesion and migration were greatly increased in the EGR-1-expressing cells, and adhesion was reversed by addition of RGD-containing peptides. These results suggest that EGR-1 may regulate cell interaction with the extracellular matrix by coordinated induction of TGF-β1, FN, and PAI-1 in human glioblastoma cells.

Egr-1 (also known as NGFI-A (1), TIS8, Krox-24, and Zif268) (33) is a member of the immediate-early gene family that encodes a nuclear phosphoprotein. EGR-1 contains three zinc finger motifs that bind and regulate transcription through GC-rich elements (GCEs)1 with a consensus sequence of 5’-GCG(T/A)-9(G)-GGCG-3’ (2–4). The promoter regions of many genes, including several growth factors and cytokines, are regulated by EGR-1 (for a review, see Ref. 5). The Egr-1 gene is rapidly and transiently induced by growth factors and other signals and is functionally implicated in cell proliferation and in differentiation processes (6–8).

Egr-1 is broadly expressed during development and in the tissues of adults of many species. It can be found in epithelial tissues, heart, thymus, and central and peripheral nervous systems. The basal expression of the EGR-1 protein in adult rat and mouse brain is high (for a review, see Ref. 9). At a functional level, several in vitro and in vivo studies initially characterized Egr-1 as having a role in the control of cell growth, proliferation, differentiation, and development. However, whether EGR-1 is a participant in disparate activities or is a more central factor regulating coordinate expression of a characteristic phenotype is unclear. Our previous studies have shown that stable re-expression of EGR-1 inhibits transformation in model cells and in several human tumor cell lines (10, 11, 38, 39). The re-expression of Egr-1 in fibrosarcoma HT-1080, glioblastoma U251, and U373 cells leads to decreased DNA synthesis, growth, and tumorigenicity (39). The mechanism of the EGR-1 suppressive function has been studied in detail in fibrosarcoma HT-1080 cells (40–42). The EGR-1 protein specifically binds the GCE sites of the human TGF-β1 promoter, transactivates the TGF-β1 gene, and enhances the expression and secretion of functional TGF-β1 in the Egr-1-expressing fibrosarcoma cell line, leading to inhibition of cell proliferation and restoration of anchorage-dependent growth (40).

TGF-β1 is the prototype of a large family of cytokines that control cell proliferation, differentiation, adhesion, and extracellular matrix metabolism. TGF-β1 has been shown to induce fibrotenin (FN) expression at both the mRNA and protein levels (12, 24) and promotes net matrix deposition by increasing the expression of specific ECM components such as FN and collagen and by up-regulating the expression of inhibitors of ECM proteases such as plasminogen activator inhibitor-1 (PAI-1) (13, 26). FN plays an important role in organizing the extracellular matrix and facilitates cell adhesion, migration, wound healing, and tumor metastasis (14, 15). We previously showed that the level of FN secretion dramatically increases in human fibrosarcoma HT-1080 cell lines transfected with the Egr-1 gene.Surprisingly, we found that EGR-1 directly binds to GC-rich elements in the FN promoter (42). Although a function
transcriptional effect of EGR-1 on transcriptional activity was not shown, the increased secretion of FN was found to enhance attachment in an RGD-dependent manner, and attachment was further enhanced by the TGF-β1-dependent secretion of PAI-1. Thus, the increased expression of endogenous FN in human fibrosarcoma HT-1080 cells cooperates with increased expression of TGF-β1 to suppress the transformed phenotype and to suppress tumor growth in vivo (39, 42).

To determine whether EGR-1 plays a more general role in the control of cell growth, we examined the basal level of EGR-1 protein expression in four glioblastoma cell lines and re-expressed Egr-1 in the human glioblastoma cell line U251. We report here that the basal expression of EGR-1 was found to be undetectable in the four lines examined. Re-expression of EGR-1 leads to the increased expression of TGF-β1 and PAI-1 and, in addition, enhances the secretion of FN. We show that the regulation of the FN gene by EGR-1 is direct by binding to two sites of the FN promoter and that these sites function to increase the transcriptional activity of the FN gene. The expression of EGR-1 in U251 cells results in reduction of cell growth rate (39) and promotion of cell adhesion as well as cell migration. These results indicate important regulatory roles of EGR-1 and FN that may be altered during tumor progression.

MATERIALS AND METHODS

Cell Culture and Retrovirus Packaging and Infection—Primary mouse embryo fibroblasts (MEFegr-1/− and MEFegr-1/+) derived from wild-type and knockout EGR-1 mouse embryos were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The human glioblastoma cell lines U251 and T98G were gifts from Dr. H. Oki (University of California at San Diego), and PA317 was a gift from Dr. H. Fakhrai. Cells were grown in DMEM supplemented with 5% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in an atmosphere of 10% CO2, 5% O2, and 95% N2. The U251 and U1 cells are transfected clones of U251 that stably express pCMV, an empty vector, or pCM-VeGr-1, which carries a full-length mouse Egr-1 cDNA, and were maintained in the presence of 400 μg/ml G418 (39). A retroviral vector, pLHCX (55), containing the full-length mouse Egr-1 cDNA was transfected into an amphotropic retrovirus producer cell line, PA317. High titer viral producer clones were grown in the presence of 100 μg/ml hygromycin B. The medium from the cultured clones containing the retrovirus was harvested from the confluent producer clones, filtered through a 0.45-μm pore size filter, and then incubated with U251 cells for 2 h in the presence of Polybrene (8 μg/ml). 48 h after retrovirus infection, cells were cultured in medium containing 100 μg/ml hygromycin B. After 2 weeks of selection, the hygromycin-resistant cell line was used for Western blot analysis, immunoprecipitation, and luciferase assay. The basal expression of EGR-1 before use in further analyses. The representative clones UX-13, UE-13, and UE-21 as well as UE-1, prepared as described previously (39), were used for further studies.

Protein Preparation and Western Blot Analysis—Cells were plated at a density of 4 × 105 cells/cm², incubated overnight, washed twice with PBS, and lysed by scraping from the plates with boiling lysis buffer (1% SDS, 1.0 mm sodium orthovanadate, and 10 mM Tris, pH 7.4). In cases where the cells were stimulated by mitogens, the cells were grown to confluence; made “quiescent” by serum deprivation overnight; and treated with 20% fetal bovine serum (FBS), 100 ng/ml phorbol 12-myristate 13-acetate (PMA), or 40 μM UV-C for 2 h before lysis. After boiling for an additional 5 min, the lysates were passed through a 26-gauge needle to shear DNA and centrifuged for 5 min to pellet insoluble material. Nuclear protein was obtained from cells as described previously (29). The protein concentrations were determined using Bio-Rad protein assay reagent. 50–100 μg of cellular proteins or 50 μg of nuclear protein were added to an equal volume of 2× sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromphenol blue, and 2% β-mercaptoethanol), boiled for 5 min, separated by 7% SDS-PAGE, and electrophoretically transferred onto Immobilon membranes (Millipore Corp., Bedford, MA, MA) and autoradiographed using enhanced chemiluminescence (ECL detection system, Amersham Pharmacia Biotech) according to the manufacturer’s instructions. To show equal loading of protein, the membranes were stripped and reprobed with anti-β-actin antibody (Sigma). The intensity of the bands was determined by image analysis using an Eastman Kodak Digital Science™ one-dimensional image analysis system.

TGF-β1 ELISA—Cells (1 × 10⁴) were plated in triplicate on 12-well plates, and 24 h later, the medium was replaced with serum-free medium and incubated for 24 h. The supernatant-free culture medium was then collected and centrifuged. One-half of the conditioned medium was used to determine active TGF-β1 secreted by cells using a TGF-β1 ELISA system (Promega, Madison, WI). The other half of the conditioned medium was used to determine the expression of TGF-β1 secreted by cells using a TGF-β1 ELISA system. The number of cells was determined by harvesting and counting (Coulter Counter, Coulter Corp., Hialeah, FL). In the case of U251, MEFegr-1/−, and MEFegr-1/− cells stimulated by mitogenic stimuli, the quiescent cells were treated with 100 ng/ml PA, 20% FBS, or 40 μM UV-C for 4 h, and media without serum were replaced and incubated for 24 h. After 24 h, the medium was collected for TGF-β1 ELISA.

Cell Labeling, Extracellular Matrix Preparation, and Immunoprecipitation—For the PAI-1 assay, 2 × 10⁵ cells were plated on six-well tissue culture plates in DMEM supplemented with 5% fetal bovine serum and incubated overnight. The medium was then removed, and the cells were subjected to cysteine/methionine-free DMEM in the absence or presence of 30 μg/ml monoclonal mouse anti-TGF-β1/2/3 anti- 
serum (Genzyme) for 48 h. Cells were then grown in cysteine/methionine-free medium. The next day, [35S]cysteine/methionine was added to 50 μCi/ml for 2 h. The media were collected and subjected to adsorption on gelatin-Sepharose beads (Amersham Pharmacia Biotech) in the presence of 0.5% Triton X-100 as described (28). The samples were resolved by 7% SDS-PAGE, and the gels were treated with Fluoro-Hancer™ autoradiography enhancer (Research Products International Corp., Mount Prospect, IL) for 30 min, followed by drying and autoradiography.

For the FN assay, 2 × 10⁵ cells were plated on six-well tissue culture plates. The cells were treated overnight without or with 30 μg/ml monoclonal mouse anti-TGF-β1/2/3 antibody in cysteine/methionine-free medium. The beads coated with FN were washed with PBS, and the cysteolic and nuclear proteins were extracted by subsequent washes with hypotonic buffer containing sodium deoxycholate. The remaining labeled extracellular matrix proteins were recovered by addition of electrophoresis buffer to the washed wells, followed by scraping. The samples were subjected to 10% SDS-PAGE, and the gels were treated with Fluoro-Hancer™ for 30 min, followed by drying and autoradiography.

Plasmid Constructs, Mutagenesis, Transient Transfection, and Luciferase Assay—The fragment containing the FN promoter region positions between −105 and +14 was cleaved from pHFN105CAT (a gift from Dr. K. Oda) with BglII and HindIII (43) and inserted into the BglII/HindIII sites of the pGL3 promoter vector (Promega), thereby replacing the SV40 promoter to generate pGLFN105 (see Fig. 6A). The fragments were excised from the recombinant plasmid and ligated into the pGL3 DNA vector system. The identity and integrity of the mutagenic primers independently into pGLFN105 using the Transform- 
ser site-directed mutagenesis kit from CLONTECH (Palo Alto, CA). Mutagenic primers for each specific site are as follows: FNAM, a mutation of GCE-A in the FN promoter, GGTCTCTCCTATACCCGCCCGC- 
CCCG; and FNBM, a mutation of GCE-B in the FN promoter, CTCC- 
CGACGCCCAATAGCGTGTG. The identity and integrity of the resulting plasmids, pGLFN105, pGLENFAM, and pGLFNBM, were verified by DNA sequence analysis.

For the transient transfection experiment assays, parental cells (U251) and EGR-1-expressing cloned cells (UE-1) were grown on six-well plates at a density of 2 × 10⁵ cells/well, and then grown in DMEM containing 5% FBS. After 32 h of incubation, the cells were harvested, and luciferase activity was measured with a luciferase assay kit (Promega).

Oligonucleotides and Electrophoretic Mobility Shift Assay—Nuclear protein extracts were prepared from a clone with maximum EGR-1 expression from (U251 cell line) as described (29). The protein concentrations in the nuclear extracts were determined using a Bio-Rad protein assay kit. Synthetic double-stranded oligonucleotides bearing sequences corresponding to either −75 to −52 base pairs or −4 to +14 base pairs of the human FN promoter, termed GCE-A and GCE-B, respectively (see Fig. 6A), were selected based on an analysis of the sequence of the human FN pro-
moter region for the presence of GCEs (Transcription Element Search software). The DNA sequences for the two oligonucleotides are as follows: GCE-A, 5’-GATCTCTTCTTCCCCGCGCCGCGG-3’; and GCE-B, 5’-GATCTCTCAGGCAGGCCCGCCCCGTG-3’. The prototypic EGR-1-binding sites were underlined. The GCE-A and GCE-B oligonucleotides were end-radiolabeled with [γ-32P]ATP using T4 polynucleotide kinase (Amersham Pharmacia Biotech) according to the supplier’s specification and used as probes A and B. Gel shift assays were performed as follows. Nuclear extracts (20 μg of protein) were incubated with radiolabeled DNA probe (1 × 10^6 cpm) for 20 min at 4 °C in a 20-μl reaction containing 25 mM HEPES, pH 7.9, 60 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 100 μg/ml spermine, 10% glycerol, and 100 μg/ml bovine serum albumin. Protein-DNA complexes were separated from free DNA probe by electrophoresis on 6% nondenaturing acrylamide gels in 0.5 × Tris borate/EDTA. The gels were dried and exposed to Kodak X-Omat x-ray film for autoradiography. For the competition experiments, excess unlabeled oligonucleotides for probes A and B or oligonucleotides containing two EGR-1-binding consensus sequences (GCE) and mutated EGR-1-binding sequences were incubated with the reaction mixture for 15 min at 4 °C before addition of radiolabeled probes A and B. Similarly, in the antibody supershift experiments, the specific antibodies against EGR-1 (38) were added to the binding reactions and incubated for 15 min before the appropriate radiolabeled probe was added.

Cell Adhesion Assay—Adhesion assays were performed essentially as described previously (42). Briefly, cells were trypsinized, resuspended in DMEM, and incubated at 37 °C for 2 h to recover from any stress or alteration caused by the harvesting procedure. The cells, at a density of 2 × 10^4 cells/well, were then added to 96-well flat-bottom ELISA plates (Sarstedt Inc., Newton, NC) that has been pretreated with 0.1% bovine serum albumin in PBS for 1 h to block nonspecific sites. In some cases, 10 μg/ml GRGDSP or ORGESP peptide (gifts from Dr. R. Pasqualini, Burnham Institute) was added to the wells. The cells were allowed to attach for 3 h at 37 °C in a 10% CO2 incubator. The wells were then washed gently with warm PBS to remove unattached cells. The number of cells attached to the wells was estimated by the tetracium-based colorimetric MTS/PMS assay (Promega) according to the manufacturer’s instruction.

Wound Healing Assay—Cell migration was measured by the in vitro wound healing assay. Cells (2.5 × 10^5/cm²) were plated on 60-mm dishes in DMEM containing 5% FBS overnight and then “wounded” by removing a thin line of cells with a yellow plastic tip. After washing twice with serum-free DMEM, the cells were incubated for 21 h in DMEM containing 5% FBS to allow the cells to migrate into the wound areas. The same areas were photographed before and after a 21-h period.

RESULTS

Basal EGR-1 Protein Expression in Glioblastoma Cell Lines—Several groups have reported that the basal levels of Egr-1 mRNA (31–33) and EGFR-1 protein (34) are readily detectable in normal mouse and rat cells and tissues. The EGR-1 protein appears to play important roles in normal brain development and brain injury responses following ischemia and nerve transection (35, 36). To understand the importance of Egr-1 in human normal brain and brain tumors, we examined four glioblastoma cell lines: U251, T98G, U-373MG, and U-87MG. Western blot analysis showed that the basal expression of the EGR-1 protein in these cell lines was undetectable, whereas the basal expression of EGR-1 in rat primary astrocytes was readily detectable (data not shown). The basis of this effect was explored in detail in these cell lines (Fig. 1). Steady-state EGR-1 expression was nearly undetectable in cells growing in complete medium containing 5–10% fetal bovine serum or in cells made quiescent by culturing in 0.5% serum for 24 h. Quiescent cells were treated with PMA, 20% fetal bovine serum, or 40 J/m² UV for 2 h since these stimuli are known to induce both Egr-1 mRNA and EGR-1 protein synthesis (33, 37). The EGR-1 protein was differentially induced by UV, PMA, and serum stimulation (Fig. 1). Moreover, the protein exhibits the characteristic broad band distribution centered at an apparent size of 85 kDa (5, 10, 11, 38–42), very similar to full-length normal EGR-1 (Fig. 1), suggesting that Egr-1 gene transcription and translation remain intact, but that steady-state expression is selectively reduced. This led us to investigate the functional effects of re-expression of Egr-1 in glioblastoma cells.

Glioblastoma Cell Line U251 Overexpressing EGR-1 Exhibits Elevated Secretion of TGF-β1—It is known that re-expressed EGR-1 inhibits proliferation and transformation of v-sis-transformed NIH/3T3 cells (38) and human tumor cell lines such as fibrosarcoma HT-1080, osteosarcoma Saos2, glioblastoma U251 and U373, and breast carcinoma ZR75-1 (39). In at least one case, it has been shown that EGR-1 directly activates the expression and secretion of active TGF-β1 (40). Several other proteins and activities that may be under the control of TGF-β1 are also increased, such as p21Waf1/Cip1 (41), fibronectin and PAI-1 (42), and focal adhesion kinase (41). Thus, TGF-β1 is an important marker of functional EGR-1. To examine whether the induced expression of the EGR-1 protein in the stimulation of U251 cells increased the secretion of TGF-β1, we tested the TGF-β1 secretion in culture medium from normal parental cell lines, quiescent cells, and PMA-, UV-, and fetal bovine serum-stimulated cells. As shown in Fig. 2A, TGF-β1 secretion was greatly increased up to 9.8-fold following stimulation with PMA and 4.2-fold following stimulation with UV. However, TGF-β1 secretion was increased only a little in U251 cells following stimulation with fetal bovine serum. In contrast to the induction of EGR-1 expression by stimulation, the induction of TGF-β1 seemed not to correlate completely. Since the induction of TGF-β1 by mitogenic stimuli such as PMA, stress, and UV irradiation through the AP-1 (Jun-Fos) complex has been reported broadly (56–58), we asked whether the increased TGF-β1 secretion observed here was specifically due to the induction of EGR-1 by stimulation in U251 cells. MEFEgr1+/− and MEFEgr1−/− fibroblasts, derived from wild-type and knockout EGR-1 mouse embryos, respectively, were used for measuring TGF-β1 secretion. As shown in Fig. 2B, MEFEgr1+/− and MEFEgr1−/− cells upon PMA stimulation com-

FIG. 1. Detection of EGR-1 protein expression in glioblastoma cell lines in culture by Western blotting. The human glioblastoma cell lines U251, T98G, U-87MG, and U-373MG were grown to confluence in conditioned medium, lysed (N) or made quiescent (Q) by serum deprivation, and treated with 20% FBS, 100 ng/ml PMA, or 40 J/m² UV for 2 h before lysis. The position of the EGR-1 protein (85 kDa) is indicated by arrows. Equivalent protein loading was confirmed by reprobing the same membranes with anti-β-actin antibodies as shown by arrows.
pared with unstimulated MEFEgr-1+/+). However, the secretion of TGF-β1 also increased up to 1.86-fold in MEFEgr-1−/− with PMA stimulation compared with no stimulation. Similar results were seen with UV-stimulated MEFEgr-1+/+ and MEFEgr-1−/− (Fig. 2B), indicating that the induction of TGF-β1 by stimulation of cells with mitogenic stimuli is not caused only by the increased expression of the EGR-1 protein, but is also caused by other factors such as c-Jun, c-Fos, etc.

To study the possibility that EGR-1 specifically regulates TGF-β1 expression in glioblastoma cells, we examined a clone of U251 cells previously prepared by calcium phosphate transfection with a full-length cDNA encoding mouse Egr-1 under the control of the cytomegalovirus promoter, UE-1 (39). In addition, to avoid any artifacts that may be associated with a single clone, we prepared additional EGR-1-expressing clones by an independent method: infection by a retrovirus encoding full-length Egr-1 cDNA under the control of the cytomegalovirus promoter or by a control retrovirus bearing an empty vector, leading to the control clone UX-13 (see “Materials and Methods”). To confirm that these clones appropriately expressed EGR-1, nuclear protein extracts from the clones were analyzed by Western blot analysis using antibodies against EGR-1. Fig. 3A shows the levels of EGR-1 in several clones (UE-1, UE-21, and UE-13) compared with the levels in the parental cell line (U251) and the empty vector-infected clone (UX-13). All three clones express an 85-kDa protein with broad band distribution characteristic of native EGR-1, which ranges in relative expression levels up to 8.5-fold. Thus, the selected clones exhibit graded levels of EGR-1.

Next, we determined whether TGF-β1 protein was also elevated in the EGR-1-expressing U251 clones. The TGF-β1 concentration in the conditioned medium from the same cloned lines was measured by the ELISA method. As shown in Fig. 3B, the EGR-1-expressing clones secreted two to three times more of the active form of TGF-β1 in the medium compared with parental cells (U251) or either of the empty vector control cell lines (UN or UX-13). Similarly, total TGF-β1 (sum of the active and latent forms following acid activation) was specifically increased (Fig. 3B). The secreted amounts of active form TGF-β1 and total TGF-β1 increased with the expression level of EGR-1, leading to very high levels that were maximum at >4000 pg/ml/10⁶ cells/24 h. Further increases in the expression of EGR-1 did not lead to a further increase in TGF-β1 production. These results indicate that EGR-1 re-expressed in U251 cells is fully functional in the regulation of a known target gene.
Expression of EGR-1 Increases the Production of the ECM Components FN and PAI-1—TGF-β1 has been reported to induce the production of extracellular matrix components such as FN and PAI-1 (24–26). To investigate whether increased secretion of TGF-β1 in EGR-1-expressing cells also increases the production of PAI-1 and FN, we first examined FN expression in these cell lines. Cell lines were metabolically labeled with [35S]cysteine/methionine for 2 h, and the labeled FN was adsorbed from the conditioned medium with gelatin-Sepharose beads, which are known to specifically bind FN (Amersham Pharmacia Biotech) (42). As shown in Fig. 4A, UE-21 and UE-1 cells that overexpressed EGR-1 exhibited increased FN expression to >6-fold compared with parental U251 cells and the empty vector control cell clones UN and UX-13. UE-13 expressed minimal basal levels of FN protein (Fig. 4A), consistent with the lower expression of EGR-1 (Fig. 2). Thus, the expression of EGR-1 is highly correlated with the secretion of FN ($R_{\text{Pearson}} = 0.981$) (Fig. 4C).

Secreted PAI-1 has been found to augment the attachment function of secreted FN (42). We therefore examined the expression of PAI-1 in these cells. The cells were metabolically labeled for 2 h. Extracellular matrix protein was extracted and subjected to electrophoresis and autoradiography. As shown in Fig. 4B, the expression of PAI-1 protein was increased ~2.8-fold in UE-1 cells and 1.4-fold in UE-21 cells compared with the parental cells (U251) and empty vector control cells (UN and UX-13). Again, UE-13 cells expressed minimal amounts of PAI-1 as well as FN, in keeping with the low level of EGR-1. EGR-1 is therefore also highly correlated with the expression of PAI-1 ($R_{\text{Pearson}} = 0.943$) (Fig. 4C). Thus, these studies show that re-expression of EGR-1 leads to increased production and secretion of FN and PAI-1.

Increased Expression of FN (but Not PAI-1) in EGR-1-expressing Cells Is Independent of the Enhanced Expression of TGF-β1—Both PAI-1 and FN have been reported to be under the regulation of TGF-β1 (24–26). Thus, the EGR-1-stimulated increase in TGF-β1 may be the basis of the elevated expression of FN and/or PAI-1. To test whether the increased production of PAI-1 or FN in EGR-1-expressing cells is due to the elevated amounts of TGF-β1 secretion, we used TGF-β-neutralizing antibodies to specifically block the expression of TGF-β1. As shown in Fig. 5, the expression of PAI-1 in EGR-1-expressing UE-1 cells was completely eliminated by treatment with anti-TGF-β1 antibody (Fig. 5, upper panel, compare lanes 2 and 4), whereas PAI-1 was readily detected in untreated UE-1 cells. In contrast, the expression of FN was only ~50% reduced by addition of anti-TGF-β antibodies in the EGR-1-expressing UE-1 cells (Fig. 5, lower panel, compare lanes 1 and 2 with lane 4). These results suggest that TGF-β1 is indeed required for expression of PAI-1. However, the results suggest that the enhanced expression of FN in EGR-1-expressing cells is not solely caused by increased expression of TGF-β1 and that additional factors account for the up-regulated expression of FN in EGR-1-expressing cells.

EGR-1 Is Able to Directly Activate FN Promoter Activity—The elevated levels of FN in cell lines stably overexpressing EGR-1 were not well regulated by TGF-β1, consistent with possible up-regulation of the FN promoter by another factor. One possible factor regulating the transactivation of the FN gene in U251 cells is EGR-1 itself. This is suggested by the presence of two sequences in the promoter region of the FN promoter that closely resemble EGR-1 consensus sequences or so-called GC-boxes. To determine whether EGR-1 can up-regulate FN promoter activity, we used a reporter construct containing the region spanning positions −105 to +14 of the human FN promoter, which contains GC-rich sequences (Fig. 6A). This construct was transiently transfected into the clone with a maximum of EGR-1 expression (UE-1) and parental cells (U251). As shown in Fig. 6B (black bars), the EGR-1-expressing UE-1 clone substantially activated the FN promoter-driven luciferase reporter by 5.5-fold over the same reporter in U251 control cells and by 27.5-fold over the reporter control vector pGL, an empty luciferase vector. When we used an empty luciferase vector without the FN promoter insertion as a negative control in both cell lines, luciferase activity was not significantly different in EGR-1-expressing UE-1 cells and parental U251 cells (Fig. 6B, white bars). This result confirms that EGR-1 does not augment the luciferase activity of the luciferase control vector (pGL vector). In contrast, the luciferase activity was increased ~5-fold in the FN promoter-driven reporter compared with the empty luciferase vector (pGL vector) in the parental U251 cells (Fig. 6B, U251, compare black and white bars), suggesting that some additional factor in U251 cells modestly activates the FN promoter. One candidate factor is Sp1. Sp1, a transcription factor, is commonly present in all mammalian cells and transactivates the human FN gene by binding to GC-rich elements (43).

If EGR-1 is indeed the major factor responsible for transactivation of the FN gene, it might be expected to exhibit a saturated dose response for transactivation of the FN/luciferase reporter, but not of the empty control vector. These constructs were cotransfected with expression vector for Egr-1 in U251 cells. Fig. 6C shows that Egr-1 can induce the expression...
Transactivation of the Fibronectin Gene by EGR-1

EGR-1 directly activates the FN promoter. A, schematic representation of the human FN promoter. The locations of two GCE sites, GC-rich boxes, and the transcription start site in the human FN promoter/luciferase constructs are shown. B, FN promoter activity in EGR-1-expressing cells and non-expressing cells. Human glioblastoma U251 cells and EGR-1-expressing UE-1 cells were transfected with 2 μg of pGL vector control construct (white bars) and pGLFN105 constructs (black bars), and luciferase activities were assayed 48 h after transfection. The experiment was performed in triplicate. Error bars indicate S.D. C, EGR-1 activates the FN promoter in a dose-dependent manner. A luciferase reporter plasmid (2 μg) was transfected into U251 cells along with increasing amounts of EGR-1 expression vector (0, 0.5, 1, 1.5, and 2 μg). Cells were lysed, and luciferase activity was assayed. The white bars correspond to cells cotransfected with the empty control vector. The activity expressed by the pGL control vector combined with the empty expression vector in U251 cells is taken as 1. The black bars correspond to the cells cotransfected with FN promoter constructs. The activity expressed by pGLFN105 combined with the empty expression vector in U251 cells is also taken as 1. The activities of other combinations are shown as relative values. The experiment was performed in triplicate. Error bars indicate S.D. D, functional analysis of mutated EGR-1-binding sites in the FN promoter. Plasmids containing 2 μg of wild-type FN promoter (−105 to +14 base pairs) or mutated derivatives were cotransfected into U251 cells with pCMVEgr-1 (1.5 μg), which expresses EGR-1 (see “Materials and Methods”). The relative luciferase activity is the luciferase activity of the FN promoter compared with the pGL control reporter. Error bars represent S.D. of five independent experiments. L.U., light units.

of the FN promoter-driven luciferase reporter. The enhancement of reporter activity was proportional to the quantity of the cotransfected Egr-1 expression vector, with a maximum activation of 27-fold. In contrast, there was no systematic activation of the control reporter. Moreover, there is evidence of a saturation effect consistent with a stoichiometric EGR-1/DNA binding process. Thus, these experiments show that EGR-1 has a potent activating effect on FN promoter activity that occurs in a dose-dependent and saturable manner.

To further test the regulatory role of EGR-1 in expression of the FN gene, we analyzed U251 cells transfected with plasmids containing the wild-type FN promoter and various mutated derivatives cloned upstream of the luciferase reporter constructs. Four plasmids were analyzed: pGL, an empty control luciferase reporter; pGLFN105, which contains the wild-type FN promoter between −105 and +14 base pairs; pGLFNAM, which contains a mutation in the GCE-A site; and pGLFNBM, which contains a mutation in the GCE-B site, all of which were confirmed by sequence analysis (see “Materials and Methods”). As indicated in Fig. 6D, luciferase activity was dramatically increased up to 18-fold when FN promoter constructs were cotransfected with the expression vector pCMVEgr-1 into U251 cells, which compared well with the results for the pGL control vector. Mutation of either GCE-A or GCE-B greatly reduced the induction of Egr-1-induced promoter activity (3- or 4-fold compared with 18-fold, respectively; p < 0.001). These data confirm the functional importance of the sequences containing the EGR-1-binding sites for the induction of expression of the FN gene in response to EGR-1 expression. These results also suggest that the regulatory effect of EGR-1 on increased FN secretion is mediated by direct activation of the FN promoter region.

EGR-1 Binds to EGR-1-Binding Sites in the Human FN Promoter Region—The luciferase reporter studies showing functional regulation of transcription of the FN promoter element by EGR-1 predict that EGR-1 directly and specifically binds to the GC-rich region of the FN promoter. To define potential EGR-1-binding sites in the human FN promoter region, we used Transcription Element Search software to screen 742 base pairs of the 5'-flanking region (29) of the human FN promoter (exon 1) and identified two potential EGR-1 consensus sites termed GCE-A and GCE-B (Fig. 6A). Our previous studies have confirmed that the recombinant EGR-1 protein and the nuclear EGR-1 protein from the EGR-1-expressing HT-1080 cell line specifically bound to these two sites (42). To test whether EGR-1 that is expressed by U251 transfected clones specifically binds to these two sites, we performed gel shift analyses of the two putative binding sequences. Incubation of nuclear protein extracts from UE-1 with labeled oligonucleotide probes containing EGR-1-binding sites of FN (probes A and B) resulted in the formation of a complex (Fig. 7, lanes 4 and 14, arrow). This complex was not detected in nuclear extracts from control U251 and UN cells (Fig. 7, lanes 2 and 3 and lanes 12 and 13). This complex could be disrupted by addition of an excess of unlabeled probes A and B in a dose-dependent manner (Fig. 7, lanes 8–10 and lanes 18–20). Preincubation of the UE-1 nuclear protein extracts with antibodies against EGR-1 prior to addition of the probe dissociated this complex (Fig. 7, lanes 5 and 15). Also, this complex was dissociated by addition of an unlabeled EGR-1 consensus sequence (GCE), but not by a mutated EGR-1 consensus sequence (Fig. 7, lane 6 and 7 and lanes 16 and 17). Probe A also formed a slow migrating complex with all cell extracts (Fig. 7A, indicated by the line). This complex was not dissociated by anti-EGR-1 antibodies, GCEs, or unlabeled probe A itself (Fig. 7B), indicating that this complex is not related to EGR-1, but is possibly a nonspecific band. The results demonstrate that EGR-1 can specifically bind to sequences of the human FN promoter and activate transcription of the FN gene.

Enhanced Expression of FN in EGR-1-Expressing Cells Increases Cell Adhesion and Cell Migration—We asked whether
the EGR-1-dependent stimulation of the FN gene produced functional FN in human glioblastoma U251 cells. Because the interaction with extracellular matrix such as FN and PAI-1 is considered to be important for cell adhesion and migration, we tested whether the enhanced expression of FN increased cell adhesion. The EGR-1-expressing cells (UE-1) and control cells (U251 and UN) were used in attachment efficiency assays. As shown in Fig. 8, ~28% of EGR-1-expressing UE-1 cells attached to uncoated plastic plates 3 h after plating compared with ~11% for the control U251 and UN cells (p < 0.0003). Moreover, addition of the GRGDSP peptide, which is known to specifically and competitively disrupt FN binding to its receptors (44, 45), caused complete reversal of enhanced adhesion of EGR-1-expressing UE-1 cells. However, addition of equal amounts of the control GRGESP peptide, which differs by a single residue, had negligible effects (Fig. 8). This result demonstrates that enhanced FN secretion in EGR-1-expressing cell clones has a functional role in cell adhesion.

During ordinary tissue culture processing and passage, we consistently observed increased migratory activities of the U251 clones that stably expressed large amounts of FN. Thus, as an additional test, the cell migratory activity was assessed by in vitro "wound healing" assays. In this assay, the effects of EGR-1 expression on the ability of cells at ~70% confluence to "fill in" an acutely cleared zone of a standard cell monolayer are assessed after a period sufficient for migration, but considerably less than the doubling time (~30 h) of the cells. Fig. 9 shows that UE-1 cells that express maximum amounts of EGR-1 and FN had the greatest migratory ability. These cells almost filled the "wound area" at 21 h. UE-21 cells, which express less EGR-1 compared with UE-1 cells, migrated more slowly than UE-1 cells. In contrast, no significant migration was observed in control cells (U251 and UX-13) at 21 h, suggesting that enhanced expression of EGR-1 significantly increases cell migration.

**DISCUSSION**

The transcription factor EGR-1 was first cloned in nerve-like cells such as NGFI-A by differential hybridization from nerve growth factor-treated rat PC12 cells (31). Since then, the expression of EGR-1 has been extensively studied in the mammalian central nervous system. Most previous studies have shown that the basal mRNA and protein levels of EGR-1 are especially abundant in all brain mouse and rat regions and that EGR-1 plays important roles in brain injury (16) and plasticity-related phenomena such as learning and memory (17) and resting neuronal activity (18). However, the expression and function of EGR-1 in human brain tumors are not yet defined. We have observed that EGR-1 protein expression is undetectable in five human glioblastoma cell lines. Decreased or absent basal expression of EGR-1 also has been found in other tumor cell lines such as human breast carcinoma and tissues such a lung carcinoma (for a review, see Ref. 5), suggesting that the down-regulation of EGR-1 may be related to the production and development of some types of cancer. We also examined the induction of Egr-1 by various stimuli in four human glioblastoma cell lines (U251, T98G, U-87MG, and U-373MG), which leads to the induction of a full-length protein identified by specific antibodies, suggesting that the coding sequence and certain regulatory properties remain intact. These observations raise the possibility that basal expression is commonly and specifically suppressed in human tumors, leading to our
interest in determining the function of EGR-1 in human glioblastoma cells.

EGR-1 usually functions as an activator or, less often, a repressor of target gene transcription. Our previous studies on the human fibrosarcoma cell line HT-1080 indicated that expression of EGR-1 suppresses the proliferation and transformation of HT-1080 by coordinated induction of TGF-β1, PAI-1, and FN (42). EGR-1 directly binds and activates the TGF-β1 promoter, and this suppresses growth by an autocrine mechanism. EGR-1 also causes the accumulation of ECM proteins such as PAI-1 (regulation by TGF-β1 is a secondary effect of EGR-1) and FN (42). However, the mechanism of regulation of FN by EGR-1 was unclear, and it was not known whether this pathway observed in one cell line, HT-1080, is relevant to other cell types such as human glioblastoma cell lines. In this report, we further demonstrated that EGR-1 up-regulates not only TGF-β1, but also PAI-1 and FN in glioblastoma cells. The increased secretion of TGF-β1 occurs in proportion to the expression level of EGR-1 in U251 cells. The induction of PAI-1 by EGR-1 is dependent on the expression of TGF-β1. In contrast, the induction of FN is dependent on the expression of both TGF-β1 and EGR-1. Our gel mobility shift and luciferase assays provide evidence that EGR-1 specifically binds to the FN promoter and directly activates the FN gene, thereby increasing the secretion of FN. Thus, as summarized in Fig. 10, two EGR-1-dependent mechanisms regulate TGF-β1 and FN in U251 glioblastoma cells, one through direct binding of EGR-1 to the TGF-β1 and FN promoters and the other indirect through an EGR-1-stimulated TGF-β1 autocrine loop, leading to increased PAI-1 and FN.

The human FN promoter contains four GC boxes that have been shown to be bound by Sp1 in embryonic carcinoma cells (43). Sp1 is a ubiquitous transcription factor that has been detected in many mammalian cells and that regulates the transcription of a variety of mammalian and viral genes involved in many different cellular processes (19). Sp1 also contains three zinc finger motifs and binds to a GC-rich region termed the GC box (20). Since the consensus sequences of EGR-1 (GGGCGGGCG)- and Sp1 (GGGCGG)-binding sites resemble each other, overlapping binding motifs such as that found in the −75/−52 region of the FN promoter are often observed (Fig. 6). In this study, luciferase activity that did not express EGR-1 was increased ~5-fold by transient transfection of pGLFN105 into parental U251 cells compared with the pGL control vector without the FN promoter insertion (Fig. 6B, U251, black bar versus white bar), suggesting that endogenous factors in U251 cells are able to modestly activate the FN promoter. One candidate for this role is Sp1. Sp1 commonly influences transcription through sequences that contain overlapping and/or adjacent Sp1/EGR-1-binding sites (for a review, see Ref. 5). There is little direct evidence that Sp1 functions in the regulation of basal expression of FN in U251 cells. However, we showed here that re-expression of EGR-1 has the ability to directly up-regulate the expression of the FN gene. Indeed, this is the first demonstration that EGR-1 is a directly acting transcription factor of the FN gene.

The results observed here have implications for the nature of human glioblastoma tumors. Human glioblastoma U251 cells express readily detectable levels of TGF-β receptors I–III. The growth of cells is significantly inhibited by TGF-β1 (54). EGR-1 overexpression preferentially inhibits the growth of human glioblastoma U251 cells and suppresses its tumorigenicity in athymic mice (39). Thus, our observations provide one rationale for the suppression of basal expression of EGR-1 in glioblastoma cells. In addition, EGR-1 is a direct regulator of the FN gene in U251 cells. This effect is likely general since we observed that the secretion of functional FN was also greatly enhanced in human fibrosarcoma HT-1080 cells upon re-expression of EGR-1. Moreover, FN, a major component of ECM, plays an important role in promoting cell adhesion, migration, and cytoskeletal organization, thereby influencing cellular proliferation and differentiation (21). Overproduction of FN suppresses the transformed phenotype in human fibrosarcoma cells (22). Indeed, decreased FN production is often observed following oncogenic transformation, leading to decreased adhesion and increased metastatic potential (21, 23, 30). We observed that the expression of EGR-1 in U251 cells increased cell adhesion to the substratum in an RGD-specific manner (Fig. 8). Thus, based on the adhesion assays observed here, EGR-1-induced FN appears to play a main role in promoting cell adhesion. This observation provides an additional rationale for the suppression of basal expression of EGR-1 in human glioblastoma cells.

FN has also been shown to have both growth inhibitory and growth stimulatory effects on different cell types (22, 24, 46–
49. As for glioblastoma cells, FN has shown to induce migration and invasion of the tumor cells in vitro and in vivo (50–52). Re-expression of EGR-1 is also associated with increased migration. This effect may be due to the FN-inducing role of EGR-1. Moreover, it was previously observed that HT-1080 cells that exhibit increased expression of EGR-1 and FN also demonstrate increased migration, very similar to the "wound-filling" properties observed with glioblastoma cells (data not shown). However, it appears likely that any contribution that EGR-1 could make to migration is reduced or absent in many glioblastoma cell lines owing to the absence of expression of EGR-1. Moreover, EGR-1 expression appears to be substantially reduced in proportion to stage when measured in a large series of fresh surgical specimens of human glioblastoma cells. Thus, the infiltrating properties characteristic of human glioblastoma cells are unlikely to relate to EGR-1-regulated events.

It has been noted previously that EGR-1, although growth-promoting in certain specific settings, also shares many properties with so-called "tumor suppressor" factors (5). The observations presented here support this view. EGR-1 acts directly on the TGF-β1 and FN promoters, leading to coordinated expression of at least three proteins (TGF-β1, PAI-1, and FN) that work together to suppress aspects of transformation, including decreased proliferation and enhanced cell attachment. This model, summarized in Fig. 10, provides one rationale for the growing numbers of observation (41) that the normal expression of EGR-1 is commonly suppressed in tumor cell lines and tissues. Thus, like c-Myc and other central factors, EGR-1 may best be regarded as a bifunctional regulator that may potentially participate in transformation or suppression of transformation. The circumstances that determine which mechanism prevails are of considerable interest.

Acknowledgment—We thank Dr. Kinichiro Oda for kindly providing fibronectin promoter plasmids.

REFERENCES

1. Changelian, P. S., Feng, P., King, T. C., and Milbrandt J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 377–381
2. Chavrier, P., Vesque, C., Galliot, B., Vigneron, M., Dolle, P., Duboule, D., and Charnay, P. (1990) EMBO J. 9, 1209–1218
3. Crosby, S. D., Puetz, J. J., Simburger, K. S., Fahrner, T. J., and Milbrandt, J. (1991) Mol. Cell. Biol. 11, 3835–3841
4. Cao, X., Mahendran, R., Guy, G. R., and Tan, Y. H. (1993) Mol. Brain Res. 215–223
5. Christy, B. A., Lau, L. P., and Bourgeois, S. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7857–7861
6. Ciba, V., Cao, X., Chang, L. C., Tsai-Morris, C. H., Stamenkovich, D., Perre`ra, C. P. C., Cohen, D. R., and Adamson, E. D. (1988) Cell 53, 37–43
7. Schlingensiepen, K. H., Loo, K., and Brysich, W. (1991) Neurosci. Lett. 122, 67–70
8. McCormack, M. A., Rosen, K. M., Villa-Komaroff, L., and Mower, G. D. (1993) Mol. Brain Res. 12, 215–223
9. Kaplan, I. V., Guo, Y., and Mower, G. D. (1993) Dev. Brain Res. 90, 174–179
10. Cao, X., Koski, R. A., Gaskel, A., McKiernan, M., Morris, C. F., Gaffney, R., Hay, R. V., and Sukhatme, V. P. (1996) Mol. Cell. Biol. 16, 1367–1377
11. Huang, R.-P., Liu, C., Fan, Y., Mercola, D., and Adamson, E. (1995) Cancer Res. 55, 5054–5062
12. Liu, C., Adamson, E., and Mercola, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11831–11836
13. de Belle, I., Huang, R.-P., Adamson, E., and Mercola, D. (1999) J. Biol. Chem. 274, 4400–4411
14. Suzuki, M., Oda, K., Sekiya, S., and Oda, K. (1998) Mol. Cell. Biol. 18, 3010–3020
15. Kornblihtt, A. R., Pesce, C. G., Alonso, C. R., Cramer, P., Srebrow, A., Werbajh, S., and Muro, A. F. (1996) FASEB J. 10, 244–257
16. Akamatsu, H., Iihara-Tanaka, K., Ozono, K., Kamiike, W., Matsuoka, H., and Sekiguchi, K. (1996) Cancer Res. 56, 4541–4546
17. Der, C. J., and Stanbridge, E. J. (1986) Int. J. Cancer 36, 451–459
18. Ignotz, R. A., and Massague J. (1986) J. Biol. Chem. 261, 4337–4345
19. Deane, D. C., Bowlus, C. L., and Bourgeois, S. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1876–1880
20. Steel, D. M., and Harris, R. (1989) J. Cell Sci. 93, 515–524
21. Milbrandt, J. (1987) Science 235, 797–799
22. Christy, B. A., Lau, L. P., and Nathans, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1019–1101
23. Saulnier, R., Bhardwaj, B., Klassen, J., Leopold, D., Rahimi, N., Tremblay, E., Mosher, D., and Elliott, B. (1986) Exp. Cell Res. 225, 360–369
24. Sato, T., Hocking, D. C., and Sivak, P. J. (1987) J. Cell Sci. 191, 2393–2434
25. Giese, A. Rief, H. D., Loo, A., and Berens, M. E. (1994) Cancer Res. 54, 3897–3904
26. Dey, A. S., Chen, W., Loo, M., and Berens, M. E. (1995) Clin. Exp. Metastasis 12, 729–741
27. Church, G. M., and Gilbert, W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1991–1995
28. Scott, J. N., Reewestol, N. B., Brasher, P. M., Fulton, D., Hagen, N., Mackinson, J. S., Guber, S. C., and Forryth, P. A. (1998) Can. J. Neurol. Sci. 25, 197–201
29. Potapova, O., Fakhrai, H., Bbbaird, S., and Mercola, D. (1995) Cancer Res. 56, 2380–2386
30. Kim, S.-J., Denhez, F., Kim, K. Y., Holt, J. T., Sporn, M. B., and Roberts, A. B. (1987) J. Biol. Chem. 262, 19373–19378
31. Miyazono, K., Oloffson, A., Calosetti, P., and Heldin, C.-H. (1991) EMBO J. 10, 1091–1101
32. Birchenall-Roberts, M., Rusetti, F. W., Kasper, J., Lee, H.-D., Friedman, R., Geiser, A., Sporn, M. B., Roberts, A. B., and Kim, S.-J. (1990) Mol. Cell. Biol. 10, 4975–4983

2 A. Calogero, personal communication.