Transfer of E2F-1 to Human Glioma Cells Results in Transcriptional Up-Regulation of Bcl-2

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Abstract

Strong evidence exists to support the tenet that activation of E2F transcription factors, via alterations in the p16-cyclin D-Rb pathway, is a key event in the malignant progression of most human malignant gliomas. The oncogenic ability of E2F has been related to the E2F-mediated up-regulation of several proteins that positively regulate cell proliferation. However, E2F may indirectly enhance proliferation by activating anti-apoptotic molecules. In this work, we sought to ascertain whether E2F-1-mediated events involve the up-regulation of the antiapoptotic molecule Bcl-2. Western blot analyses showed up-regulation of Bcl-2 but not of Bcl-xL by 24 h after the transfer of E2F-1. Northern blot studies showed that transfer of E2F-1 also up-regulated Bcl-2 RNA. In support of these findings, the concept that E2F-1 has a direct effect in the induction of several proteins that positively regulate cell proliferation. We undertook this work to ascertain whether E2F-1 can activate the expression of Bcl-2. We found that E2F-1 can up-regulate the expression of Bcl-2 at the protein and mRNA levels. We also identified an E2F binding site in the promoter region of Bcl-2 and showed that E2F-1 protein binds this site. We additionally demonstrated that E2F-1, E2F-2, and E2F-4 can transactivate responsive Bcl-2 elements. This report links for first time the regulation of Bcl-2 to E2F-1 transactivation function. Our results suggest that activation of antiapoptotic genes, in addition to up-regulation of positive modulators of cell cycle progression and DNA replication, may favor the oncogenic function of E2F-1.

Materials and Methods

Cell Lines and Culture Conditions. The U-251 MG, U-87 MG, and T98 G cell lines were obtained from the American Type Culture Collection (Manassas, VA). All of the cell lines were maintained in DMEM/F12 medium (1/1, v/v) supplemented with 10% heat-inactivated FCS in a humidified atmosphere containing 5% CO2 at 37°C. Synchronization procedures are described elsewhere (14). Briefly, T98 G cells were serum-starved for 3 days by culturing in MCDB-105 serum-free medium (Sigma Chemical Co., St. Louis, MO) and then stimulated into synchronous cell cycling progression by replacing the medium with DMEM containing 10% FCS.

Adenoviral Vectors and Infection Conditions. The generation and characterization of the recombinant-deficient adenovirus vectors carrying E2F-1 and the virus control Ad5CMV-pA have been described in detail elsewhere (5). The adenoviral vectors carrying the cDNA of E2F-2 or E2F-4 (15) were the generous gift of Dr. Joseph R. Nevins (Duke University Medical Center, Durham, NC). Cell lines were cultured and infected as reported previously (16). We used a multiplicity of infection (defined as the ratio of the number of infectious virions to the number of susceptible cells) of 100.

Immunoblotting Assay. Total cell lysates were prepared by incubating cells for 1 h at 4°C in radioimmunoprecipitation assay buffer [150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM EDTA, and 50 mM Tris (pH 7.4)] at different times after infection. Then, 20 μg of protein from each sample was subjected to 7% or 15% SDS-Tris-glycine gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). The membrane was blocked with Blotto-Tween [3% nonfat

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milk, 0.05% Tween 20, 0.9% NaCl, and 50 mM Tris (pH 7.5) and incubated with the following primary antibodies: mouse anti-E2F-1 (KH95; Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-E2F-2 (C-20; Santa Cruz Biotechnology Inc.), rabbit anti-E2F-4 (C-108; Santa Cruz Biotechnology Inc.), mouse anti-Bcl-2 (C-2; Santa Cruz Biotechnology Inc.), mouse anti-Bcl-x (PharMingen, San Diego, CA), mouse antianthuman p53 (DO–7; DAKO), and mouse antianthuman actin IgG (Amersham Corp., Arlington Heights, IL). The secondary antibodies were horseradish peroxidase-conjugated antirabbit IgG, anti-rabbit IgG (both from Amersham), and antigoat IgG (Santa Cruz Biotechnology Inc.). The membranes were developed according to Amersham’s enhanced chemiluminescence protocol.

Northern Blotting. U251 MG cells (5 × 10^6) were seeded onto a 10-cm plate and allowed to adhere overnight. The next day, the cells were infected with Ad5CMV-E2F-1, AdE2F-2, AdE2F-4, or Ad5CMV-pA at a dose of 100 multiplicity of infection. The total cellular RNA was isolated 36 h after infection by the acid-phenol guanidine thiocyanate method. For the Northern blotting, 15 μg of total cellular RNA prepared from each sample were subjected to electrophoresis on a 1% agarose gel containing 2% formaldehyde, stained with ethidium bromide, photographed, transferred to a nylon membrane (Zetaprobe; Bio-Rad Laboratories, Hercules, CA), and hybridized to an [α-32P]dCTP-labeled Bcl-2 cDNA probe. Random priming was performed with the Prime It kit (Stratagene, La Jolla, CA), after which the membrane was washed in high-stringency conditions and autoradiographed for 24–48 h.

Nuclear Extracts and Electrophoretic Gel Mobility Shift Analysis. U-251 MG cells (10^6 cells in 100-mm dishes) were washed with PBS and resuspended in 400 μl of hypotonic buffer [20 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin] and incubated at 4°C for 15 min. Cells were then lysed by adding 1.0% NP40 and vortexing; nuclei were pelleted and resuspended in 200 μl of hypertonic buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 10 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin]. After thorough mixing on a rotating wheel at 4°C for 15 min, the suspension was centrifuged at 14,000 × g for 5 min. The supernatant was used as the nuclear extract for the gel mobility shift assay.

Bandshift assay were performed as follows: nuclear extracts (5 μg) were preincubated with 2 μg of the polydeoxynucleotide poly-dl-dC for 15 min at 4°C and then incubated with labeled oligomer DNA for 15 min at 4°C in the presence of 10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 20% glycerol, 1 mM DTT, and 5 mM MgCl2. An oligonucleotide (25-bp) containing the E2F(2) binding site (in capital letters; Ref. 14; sense: 5′-atttaacGAGCGCGggaaagtt5′; antisense: 3′-taatacGAGGGGCGGaaagagt5′) was synthesized (Life Technologies, Inc., Rockville, MD), annealed to each other, and forward-labeled with [γ-32P]ATP using T4 polynucleotide kinase. For competition experiments, cold wild-type E2F(2) or mutant E2F(2) (mE2F(2), similar to mE2F(2); Ref. 14) was added at a 50-fold excess to the labeled probe. After thorough mixing on a rotating wheel at 4°C for 15 min, the suspension was centrifuged at 14,000 × g for 5 min. The supernatant was used as the nuclear extract for the gel mobility shift assay.

Luciferase Reporter Assays. Luciferase reporter assays were also performed with the E2F-1 expression vector pXCIL-E2F-1 (18). This plasmid contains an E2F-1 expression cassette comprising the human cytomegalovirus promoter E2F-1 cDNA and the SV40 early polyadenylation signal. A similar construct, pXCIL-CMV-pA, lacking the E2F-1 cDNA was used as a control.

Flow Cytometric Analyses of Cell Cycle. To measure DNA content, 10^6 cells were trypsinized, fixed in 70% cold ethanol, and incubated with propidium iodide (5 mg/ml) and RNase A (1 mg/ml) for 20 min at 37°C. All of the measurements were made with an EPICS profile flow cytometer (Coulter Corp., Hialeah, FL) equipped with an air-cooled argon ion laser emitting 488 nm at 15 mW. Multicycle (Phoenix Flow System, San Diego, CA) program was used for data analysis.

Results

Cell Cycle Analyses and Expression of Endogenous E2F-1 and Bcl-2. Because E2F activity plays a major role in inducing cell progression from G1 to S phase, we assessed whether the up-regulation of E2F correlated temporally with up-regulation of the endogenous Bcl-2 protein in T98 G cells as follows. T98 G cells were made quiescent by serum starvation and then stimulated with serum and harvested at different time points from 0 to 28 h (Ref. 14; Fig. 1). After serum stimulation, we observed a more or less synchronized cell-cycle progression with a predominant G0/G1 phase at 0 h, a progressive accumulation of cells in the S phase of the cell cycle between 16 and 20 h, and an increased presence of the number of cells in the G2/M phase by 24 h. As expected, in the Western blot analyses, we detected an increase in the expression level of the endogenous E2F-1 protein 12 h after serum stimulation, immediately before cells
accumulated in S phase, and continued increase up until 24 h (exit of cells from S phase). The expression level of Bcl-2 protein began to increase in parallel with the increase in the expression level of E2F-1. These results suggested that the expression of E2F-1 and Bcl-2 molecules might be connected.

**Transfer of E2F-1 to Glioma Cells Enhances Expression of Bcl-2.** Next, we investigated whether overexpression of E2F-1 could up-regulate Bcl-2 in human glioma cells. Analysis of protein extracts from E2F-1-transduced U-251 MG cells, growing asynchronously, revealed large increases in Bcl-2 protein levels (Fig. 2). Consistent with the previous analyses of the endogenous E2F-1 and Bcl-2 expression in a synchronized population of human glioma cells, in these experiments, up-regulation of Bcl-2 was evident by 16 h after transfer of an exogenous E2F-1 and increased progressively, achieving their maximum by 24–48 h after infection (Fig. 2). Interestingly, extending the Western blot analyses to include Bcl-xL showed that the transfer of E2F-1 did not up-regulate Bcl-xL, a member of the Bcl-2 family of proteins, with antiapoptotic activity. The E2F-1-mediated up-regulation of Bcl-2 was also observed in U-87 MG cells. Moreover, transduction of U-251 MG and U-87 MG cells with adenoviral vectors carrying E2F-2 or E2F-4, two other members of the E2F family of transcription factors of which their DNA-binding domains share a high homology with that of E2F-1, also produced up-regulation of Bcl-2. These results suggest that the E2F family of transcription factors was involved in the modulation of Bcl-2 expression in these gliomas cells.

**E2F-1 Up-Regulates Bcl-2 RNA.** Because E2F-1 is a transcription factor, we hypothesized that E2F-1-mediated up-regulation of Bcl-2 should be associated with a concomitant increase in bcl-2 gene expression. Northern blot analyses of U-251 MG cells infected with adenovirus constructs containing E2F-1, E2F-2, or E2F-4 revealed increases in Bcl-2 RNA levels 36 h after infection (Fig. 3), indicating that E2F-1 either transcriptionally activated Bcl-2 or stabilized Bcl-2 RNA.

**Identification of a Putative E2F Binding Site in the Human Bcl-2 Promoter.** The presence of E2F-responsive elements in the bcl-2 promoter would be a strong indication that E2F regulates Bcl-2 at a transcriptional level. When we used the consensus E2F binding sequence (TTTCGGCGC) to seek sequence homology within the bcl-2 gene sequence, we found no homologous E2F sites in the bcl-2 promoter. However, when we examined the bcl-2 promoter sequence using another E2F binding sequence (CTTCGGCGC) [E2F(a)] present in the human p21/cip1 promoter (14), we identified a 100% homologous sequence in the −1448/−1441 P1 region of the bcl-2 promoter.

**E2F Proteins Bind to the Putative E2F Site.** After identifying the putative E2F binding site in the bcl-2 promoter, we confirmed that nuclear extract-derived proteins could form DNA-protein complexes with the E2F probe (Fig. 4). In competition experiments with nuclear extracts from U-251 MG human glioma cells, binding of the cellular proteins to the E2F probe was competed out by the unlabeled wild-type probe but not by the unlabeled mutated probe. Addition of an anti-E2F-1 antibody supershifted the complex, indicating that the E2F-1 protein was forming part of the complexes (Fig. 4).

**The Bcl-2 Promoter Is Regulated by E2F.** Although these findings provide strong evidence that E2F-1 transcriptionally regulates bcl-2, the ultimate test is to show that the exogenous E2F-1 protein can transactivate a chimeric construct encompassing the putative responsive elements of the E2F protein in the bcl-2 promoter and a reporter gene. For these experiments, we transfected U-251 MG glioma cells with one of three plasmid constructs, each containing different lengths of the bcl-2 promoter linked to a luciferase reporter gene: pGL3–2.8 bcl, pGL3–748 bcl-2, or pGL3–308 bcl-2. After transfection, the cells were infected with Ad5CMV-E2F-1 and examined for luciferase activity 24 h later. Cells that had been transfected with pGL3–2.8 bcl, which contained both the P1- and P2-responsive elements, showed 70.8 ± 22 times the activity of the bcl-2 promoter as that of the control (Fig. 5). In contrast, no significant induction of luciferase activity was noted in cells that had been transfected with the shorter constructs that contained only the P2-responsive elements (Fig. 5). To ascertain whether the up-regulation of bcl-2 was attributable to the adenoviral-mediated high level of expression of the exogenous E2F-1, we performed a similar luciferase assay transiently transfecting glioma cells with the plasmid construct pXCJL-E2F-1. These experiments showed that cells that were cotransfected with pGL3–2.8 bcl-2 displayed an increase in the luciferase activity that was 17.2, 4, and 3.4 times higher than that of the pXCJL-CMV-pA-transfected cells in three independent experiments. These results suggest that E2F-1 transactivates the bcl-2 promoter through the CTCCGGCG site located in the P1 promoter region. Transfer of
E2F-1 transactivates Bcl-2-responsive elements. A, schematic representation of Bcl-2-luciferase reporter constructs. Note that three constructs comprise the P2 region but only the pGL3–2.8 bcl2 construct encompasses the P1 region. B, U-251 MG glioma cells were cotransfected with the bcl-2 reporter constructs and the pRL-CMV vector, and 1 h later the cells were treated with adenoviral vectors carrying E2F-1, E2F-2, E2F-4, or an empty expression cassette (CMV). Luciferase activity was determined 24 h after the infection. Nontransfected cells were used as the background. All values were normalized for expression of Renilla luciferase, which served as internal control for transfection efficiency and expressed as x-fold induction relative to that of the adenovirus control-infected cells (equal to 1). Each experiment was performed at least three times in duplicate. Shown are means of normalized luciferase measurements; bars, ± SE. The E2F-mediated induction of the bcl-2 responsive elements within the pGL3–2.8 bcl2 construct was at least 30-fold higher than the control-mediated induction.
formed under similar conditions to those reported previously (5). The up-regulation of Bcl-2 (antiapoptotic molecule and negative regulator of cell cycle) by E2F-1 (positive regulator of cell and proapoptotic) seems to be counterintuitive. However, it is often the case for transcription factors involved in cancer that produce both positive and negative survival signals. In this regard, c-myc and E1A are able to induce proliferation and apoptosis signals. Importantly, the regions of E1A and c-myc that are responsible for transformation are also necessary for induction of apoptosis, indicating that the growth-promoting activities of c-myc and E1A are linked to its death-inducing properties (recently reviewed in Ref. 27). Transfer of p53 to cancer cells results in the up-regulation of pro-apoptotic molecules such as Bax, but also in the up-regulation of molecules that negatively regulate its apoptotic function, such as hdm-2 (28). The up-regulation of Bcl-2 is not the only paradoxical effect of the transfer of E2F-1 to cancer cells. E2F-1 induces the expression of several genes related to cell-cycle progression but, at the same time, up-regulates proteins that negatively influence cell cycle progression, including p18INK4c (21). Because transfer of E2F-1 results in the production of several lines of decision, the fate of an E2F-1-treated cell may rely on the status and expression of other genes. For instance, the level of E2F-3 may be important in the proliferation/apoptosis decision-making process. Thus, it has been postulated that E2F-1 and E2F-3 contribute to a pool of free E2F activity that activates inappropriate proliferation once it reaches one critical threshold level (proliferation threshold) but apoptosis once it exceeds a second, higher threshold level (29). The “free” E2F-3 (like the free E2F-1 activity) is arguably high in cancer cells with abnormally regulated retinoblastoma pathway, playing a role in the neoplastic phenotype. Increasing the level of E2F-1 in this background will surpass the proliferative threshold and trigger apoptosis. Finally, despite the fact that overexpression of Bcl-2 also follows retroviral- (21) and plasmid-mediated transfer of E2F-1, we cannot completely rule out the possibility that the induction of bcl-2 is only seen because of the use of an adenoviral system where E2F can be highly overexpressed.

The full spectrum of E2F target genes remains to be determined. The E2F-1 up-regulation of Bcl-2 is consistent with the up-regulation of other molecules with antiapoptotic activities such as p21 (16, 30). Interestingly, the p21 and bcl-2 promoters share the same E2F-1 binding site (4). Because expression of these two molecules may enhance the resistance of cells to apoptosis and, therefore, favor the ability of E2F-1 to behave as an oncogene, it is relevant to mention that Bcl-2 and p21 are overexpressed in the majority of malignant gliomas (31, 32). In this regard, it will also be interesting to see whether tumors generated by the transgenic expression of E2F-1 overexpress Bcl-2 and p21. Because E2F-1 is able to induce apoptosis, this hypothesis also predicts that the apoptosis observed in the null-Bcl-2 mice might be attributable, at least in part, to E2F-1.

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