THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 50, pp. 38351–38357, DECEMBER 15, 2006

A Novel Role for Proline- and Acid-rich Basic Region Leucine Zipper (PAR bZIP) Proteins in the Transcriptional Regulation of a BH3-only Proapoptotic Gene*

Received for publication, July 24, 2006, and in revised form, September 19, 2006 Published, JBC Papers in Press, October 20, 2006, DOI 10.1074/jbc.M607004200

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Proline- and acid-rich (PAR) basic region leucine zipper (bZIP) proteins thyrotroph embryonic factor (TEF), D-site-binding protein (DBP), and hepatic leukemia factor have been involved in neurotransmitter homeostasis and amino acid metabolism. Here we demonstrate a novel role for these proteins in the transcriptional control of a BH3-only gene. PAR bZIP proteins are able to transactivate the promoter of *bcl-gS*. This promoter is particularly responsive to TEF activation and is silenced by NFIL3, a repressor that shares the consensus binding site with PAR bZIP proteins. Consistently, transfection of TEF induces the expression of endogenous *bcl-gS* in cancer cells, and this induction is independent of p53. A naturally occurring variant of DBP (tDBP), lacking the transactivation domain, has been identified and shown to impede the formation of active TEF dimers in a competitive manner and to reduce the TEF-dependent induction of *bcl-gS*. Of note, treatment of cancer cells with etoposide induces TEF activation and promotes the expression of *bcl-gS*. Furthermore, blockade of *bcl-gS* or TEF expression by a small interfering RNA strategy or transfection with tDBP significantly reduces the etoposide-mediated apoptotic cell death. These findings represent the first described role for PAR bZIP proteins in the regulation of a gene involved in the execution of apoptosis.

Two genes, cell death specification protein 1 (*ces-1*) and *ces-2*, control the decisions of neuro-secretory motor sister cells in *Caenorhabditis elegans* to undergo apoptosis. A genetic approach showed that these factors regulate the genes required for apoptosis and that a gain of *ces-1* function or a reduction of *ces-2* function prevents these cells from dying (1). The pro-apoptotic CES-2 protein negatively regulates CES-1, which prevents the death of the neuro-secretory motor sister cells by transcriptional silencing of Egl-1, a BH3-only protein required for apoptosis in *C. elegans* (2, 3). CES-2 is similar to members of the proline- and acid-rich (PAR)[4] subfamily of basic region leucine zipper (bZIP) transcription factors, and both share the DNA binding specificity (4).

Mammalian homologs of CES-2 include thyrotroph embryonic factor (TEF), albumin D-site-binding protein (DBP), and hepatic leukemia factor (HLF) (5–7). These PAR bZIP proteins have recently been shown to be involved in amino acid and neurotransmitter metabolism through transcriptome profiling analyses in both liver and brain (8). Thus, despite the pro-apoptotic activity described for CES-2 promoting the induction of Egl-1 through an indirect pathway, none of its human homologs have been associated with the transcriptional regulation of BH3-only genes or other executors of apoptosis.

BH3-only proteins are a pro-apoptotic subgroup of the Bcl-2 family of apoptosis regulators, which share only the short BH3 region with the rest of the family (9). Genetic experiments have shown that these proteins are essential initiators of programmed cell death in species as distantly related as mice and *C. elegans*. They are regulated transcriptionally and by post-translational modifications such as phosphorylation, ubiquitination, and proteolytic cleavage (10–13). BH3-only proteins include Bid, Bad, Bim, Puma, Noxa, Hrk, Bik, Bmf, Bnip3, and Bnip3L (14, 15). Another member, Bcl-gS, the short splice variant of the *bcl-g* gene, which is uniquely expressed in testis, has recently joined this list (16). BH3-only proteins serve as upstream sentinels that selectively respond to apoptotic stimuli inducing activation of other members, mainly bax and bak, required for execution of cell death (17). Blockade of expression or activation of different BH3-only proteins has been associated with cancer cell survival. To this end, Hrk is methylated in colorectal and gastric cancer cell lines, which correlates with loss of expression of this proapoptotic gene. Restoration of Hrk promotes apoptosis and enhances adriamycin-induced cell death (18). We previously described that Hrk expression is silenced in hematopoietic progenitors by a growth factor-dependent transcriptional repressor mechanism that avoids inappropriate

* This work was supported by Fondo de Investigaciones Sanitarias grants PI040123 and RTICCC C03/10 (to J. L. F.-L.), and Grant 01/3037 (to A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[4] The abbreviations used are: PAR, proline- and acid-rich subfamily; bZIP, basic region leucine zipper transcription factors; TEF, thyrotroph embryonic factor; DBP, albumin D-site-binding protein; tDBP, truncated DBP; HLF, hepatic leukemia factor; EMSA, electrophoretic mobility shift assay; RT, reverse transcription; GFP, green fluorescent protein; siRNA, small interfering RNA.

apoptosis in these cells (19). Blockade of other BH3-only genes such as *Noxa* or *Bim* also promotes resistance to chemotherapeutic agents in different tumor cell models (20, 21). Therefore, inactivation or transcriptional repression of BH3-only proteins may represent an advantage for tumor cells to escape chemotherapy-induced apoptosis, and consequently, deciphering the regulation of these genes is crucial to understand the mechanism by which they contribute to cell fate.

We report here a novel transcriptional pathway, mediated by PAR bZIP proteins, mainly TEF, that promotes the expression of a BH3-only gene. TEF binds to a consensus site in the promoter region of *bcl-gS* and induces its expression. *bcl-gS* is upregulated by transfection of tumor cells with TEF or drastically reduced by NFIL3, a putative antagonist of PAR bZIP proteins, or by a dominant negative isoform of DBP. Additionally, the TEF-*bcl-gS* transcriptional pathway can be triggered by chemotherapeutic agents and mediates in part the apoptotic response. This is the first description of PAR bZIP transcription factors that activate the expression of a BH3-only gene.

EXPERIMENTAL PROCEDURES

Cells—Human embryonal carcinoma cell line NTERA-2 and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, CA). Colorectal carcinoma cells HCT116 and their p53 ($^{-/-}$) derivatives (22), a gift from B. Vogelstein, were grown in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal calf serum. In some experiments, cells were treated with the indicated concentrations of etoposide or cisplatin (Sigma).

Antibody Production—TEF cDNA was cloned into NdeI and XhoI sites of the pET30b expression vector (Novagen, Madison, WI), and this construct was used for transformation of BL21pLysS bacteria (Stratagene, La Jolla, CA). Expression of TEF was induced by treating cells with 1 mM isopropyl-β-D-thiogalactopyranosid, and the recombinant protein was purified by using the nickel-nitrilotriacetic acid spin kit (Qiagen, Hilden, Germany). C57BL/6 mice were immunized subcutaneously with 50 μg of the purified protein emulsified 1:1 in complete Freund's adjuvant. After 21 days, the mice were boosted with the same amount of protein, and by day 35, blood was collected, and the serum was tested for the presence of TEF-specific antibodies.

Analysis of Apoptosis—Apoptosis was assessed by an enzyme-immunoassay method that quantifies the histone-associated DNA fragments present in the cytosol (Roche Applied Science, Mannheim, Germany) as described previously (23). Values were represented as the percentage of apoptosis with respect to a positive internal control. Apoptosis was also confirmed by using a flow cytometry analysis with an active caspase 9 staining kit (MBL Int., Woburn, MA).

Electrophoretic Mobility Shift Assay (EMSA)—Cells were lysed, and nuclear fractions were resuspended in 20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 20% glycerol. Nuclear extracts (5–10 μg of total protein) were incubated with a ^{32}P-labeled double-stranded DNA probe from the promoter region of *bcl-gS* (5′-CAGATACATAAGCA-3′). Samples were run on a 5% non-denaturing polyacrylamide gel in 200 mM Tris borate, 2 mM EDTA. Gels were dried and visualized by autoradiography. Supershifts were performed using monoclonal antibodies specific for FLAG (Sigma) and Myc (BD Biosciences) tags.

Analyses of RNA—Total RNA was prepared using TRIzol reagent (Invitrogen). To assess mRNA expression, semiquantitative RT-PCR and quantitative real-time PCR were performed as described previously (24). The generated cDNA was amplified by using primers for human *bid*, *bim*, *bik*, *bnip3L*, *gapdh* (23), *bcl-gS* (5′-AAGGCCACGTGCCTGTAGC-3′ and 5′-CCCTGGATGCTGGTGTCAAC-3′), *puma* (5′-ACGACCTCAACGCACAGTACG-3′ and 5′-TGGGTAAGGGCAGGAGTCC-3′), *tef* (5′-TGGTCCTGAAGAAGCTGATGG-3′ and 5′-TCCAGGTCCATGTACTCCAGG-3′), and mouse *dbp* (5′-GCGCGGCCTGTGAGCGACAGGA-3′ and 5′-TCACAGGGCCCCGTGCTGGGC-3′).

Western Blot Analysis—Protein expression was determined by Western blotting as described previously (25). Proteins (30–60 μg) were separated on a 12% polyacrylamide gel and transferred to nitrocellulose. Blots were blocked with 3% bovine serum albumin and incubated with rabbit anti-Bcl-g antibodies, which recognize both long and short isoforms (Abgent, San Diego, CA), or mouse anti-FLAG and anti-α-tubulin (both from Sigma) antibodies and then incubated with goat anti-rabbit or anti-mouse antibodies conjugated to alkaline phosphatase (Sigma). Bound antibody was detected by a chemiluminescence system (Applied Biosystems, Foster City, CA).

Gene Silencing—Cells were transfected with a 100 nM solution of a pool of four *SMART*selection^{TM}-designed siRNA duplexes specific for *TEF*, for all splicing variants of the *bcl-g* gene, or with a similar pool of irrelevant siRNA duplexes (Dharmacon, Chicago, IL) by using Lipofectamine 2000 (Invitrogen). After 24 h of transfection, cells were incubated in the presence or in the absence of 80 μM etoposide, and 24 h later, transfected cells were analyzed for both *bcl-gS* mRNA expression and apoptosis.

Transfections and Gene Reporter Assays—A genomic PCR fragment from the promoter region of *bcl-gS* (413 bp) was cloned into KpnI and HindIII sites of the pGL2-basic luciferase reporter vector (Promega Corp., Madison, WI). The authenticity of the construct was confirmed by sequencing. HEK293T cells were cotransfected with 1 μg of the promoter-containing pGL2 construct, 1 μg of *TEF*, *DBP*, *HLF*, or *NFIL3* cDNAs cloned into FLAG-containing pcDNA3 expression vector, and 20 ng of pRSV-*β-gal* by lipofection using Superfect (Qiagen). When indicated, cells were cotransfected with pGL2-*bcl-gS* promoter and a combination of both TEF and a truncated isoform of DBP (tDBP). 24 h after transfection, cell extracts were prepared and analyzed for the relative luciferase activity by a dual-light reporter gene assay system (Applied Biosystems). Results were normalized for transfection efficiency with values obtained with pRSV-*β-gal*. PCR site-directed mutagenesis of the PAR bZIP consensus site at position −13 in the *bcl-gS* promoter was carried out by an overlap extension method (26). The following primers were used to generate two DNA fragments having overlapping ends (changed nucleotides are underlined): sense, 5′-TCTGCCCATGTGGCTGGGGAGGT-

ATGCAATG-3′, antisense, 5′-CCAGCCACATGGGCAGAG-AAGACTGAGTAACG-3′.

These fragments were then combined, allowing the 3′ overlap of each strand to serve as a primer for the 3′ extension of the complementary strand. The *bcl-gS* promoter DNA inserts were sequenced to verify the mutations. Transfection of cells with the pcDNA3 vector containing FLAG-tagged *TEF* or *tDBP* or with the vector alone (2 μg each) was performed using Lipofectamine 2000.

In some experiments, NTERA2 cells were cotransfected with 1 μg of *TEF* or *NFIL3* cDNAs and 0.2 μg of a green fluorescent protein (GFP)-containing vector (Genscript, Piscataway, NJ) by using Lipofectamine 2000. After 24 h of transfection, GFP-positive cells were selected by a cell sorter (BD Biosciences, Heidelberg, Germany) and cultured for another 24-h period. These cells were then subjected to gene expression analyses.

RESULTS

PAR bZIP Proteins Promote the Expression of a BH3-only Gene—To assess whether PAR bZIP transcription factors were able to regulate the expression of BH3-only genes, we first looked for consensus sequences within the promoter regions of these genes and found that *bcl-gS* contains a putative binding sequence located 13 bases upstream from the transcription start site (Fig. 1*A*). To study the functionality of this sequence, HEK293T cells were cotransfected with a promoter fragment from the *bcl-gS* gene cloned into a luciferase reporter vector and each of the PAR bZIP genes inserted into a FLAG-containing expression vector (pcDNA3-FLAG). As shown in Fig. 1*B*, TEF increased luciferase activity more than 35-fold. DBP also activated the *bcl-gS* promoter (about 10-fold), whereas HLF had no transcriptional activity. Interestingly, the transcriptional repressor NFIL3, which shares the DNA binding site with PAR bZIP proteins, down-regulated the basal activity of the *bcl-gS* promoter (Fig. 1*B*). Furthermore, activation of the *bcl-gS* promoter was abolished when a mutagenesis strategy changed four bases within the PAR bZIP site. After transfection with this construct, the TEF-mediated luciferase activity reached the same value as that obtained with the promoterless vector (Fig. 1*C*).

To directly prove the binding of PAR bZIP proteins to the *bcl-gS* promoter, HEK293T cells were transfected with FLAG-tagged *HLF*, *DBP*, *TEF*, and *NFIL3* (Fig. 2*A*), and then nuclear extracts were incubated with a radiolabeled probe from the *bcl-gS* promoter and subjected to EMSA. In all cases, we detected a protein-DNA binding complex that was supershifted in the presence of anti-FLAG antibodies (Fig. 2*B*).

Next, we studied the expression of *bcl-gS* in a number of tumor cell lines and found the highest mRNA and protein levels in NTERA2 and 2102Ep embryonal cancer cells (Fig. 3, *A*, and *B*). Additionally, *tef* mRNA was detected in these cells as well as in RT112 and HCT116 (data not shown). To confirm the TEF-dependent transcription of *bcl-gS*, NTERA2 cells were cotransfected with *TEF* and a *GFP*-containing vector, and then the GFP-positive cell population was sorted and analyzed for expression of BH3-only genes by real-time RT-PCR. As shown in Fig. 3*C*, TEF promoted a 5-fold induction of *bcl-gS* mRNA levels when compared with empty vector-transfected cells,

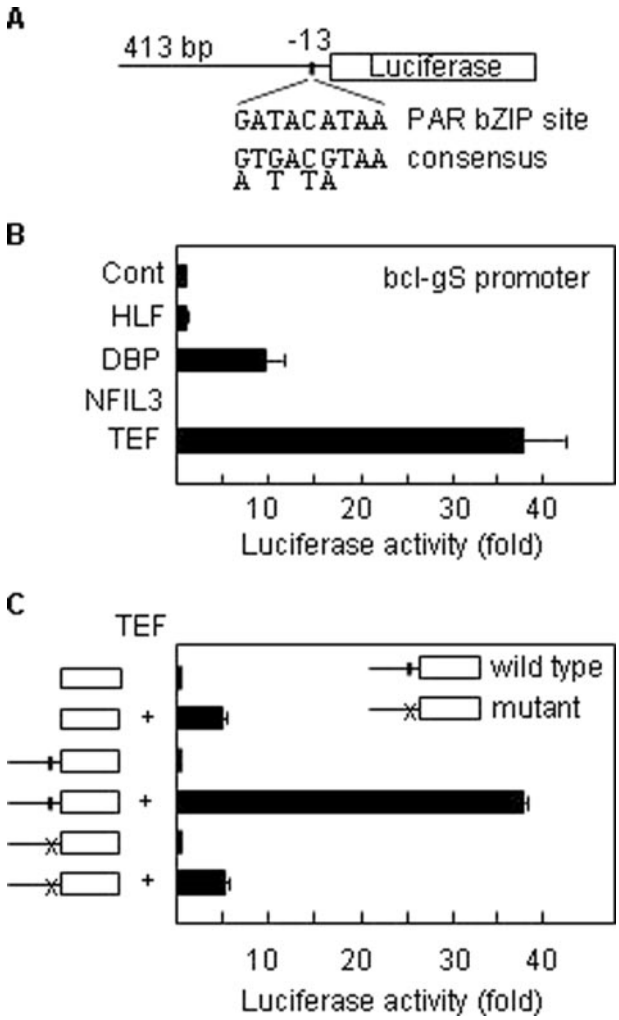

FIGURE 1. **PAR bZIP proteins transactivate the *bcl-gS* gene.** *A*, scheme of a *bcl-gS* promoter fragment cloned upstream of the luciferase gene showing the sequence of the PAR bZIP site. *B*, HEK293T cells were cotransfected with a luciferase reporter vector containing the promoter region of *bcl-gS* and with DBP, HLF, TEF, or NFIL3. Following 24 h of transfection, cell extracts were prepared and analyzed for the relative luciferase activity. *Cont.*, control. *C*, a reporter vector containing wild type or mutant PAR bZIP sites (the mutant site is marked with an *x*) was introduced into HEK293T cells, and luciferase activity in response to TEF was determined after 24 h. The *first two bars* represent the activity of the promoterless vector in the absence or in the presence of TEF. Results were normalized for transfection efficiency with values obtained with pRSV-*β-gal*. All data points represent the means ± S.D. of three independent experiments.

whereas other BH3-only genes did not show significant variations in response to TEF. Consistent with our previous data, NFIL3 down-regulated *bcl-gS*.

Recently, it has been described that p53 induces *bcl-g* (no variant specified) in Saos cells (27). To assess whether p53 was able to modify the TEF-mediated expression of *bcl-gS*, we transfected p53-deficient and wild type HCT116 cells with pcDNA3-FLAG-*TEF* and then analyzed the expression of *bcl-gS* by RT-PCR. Western blot analyses revealed the levels of the transfected protein (Fig. 3*D*). As shown in Fig. 3*D*, HCT116 express no or very little constitutive levels of bcl-gS, which are significantly increased in response to TEF regardless of the presence of p53. Thus, TEF was able to up-regulate bcl-gS in a p53-independent manner, although no significant apoptotic response was observed (data not shown).

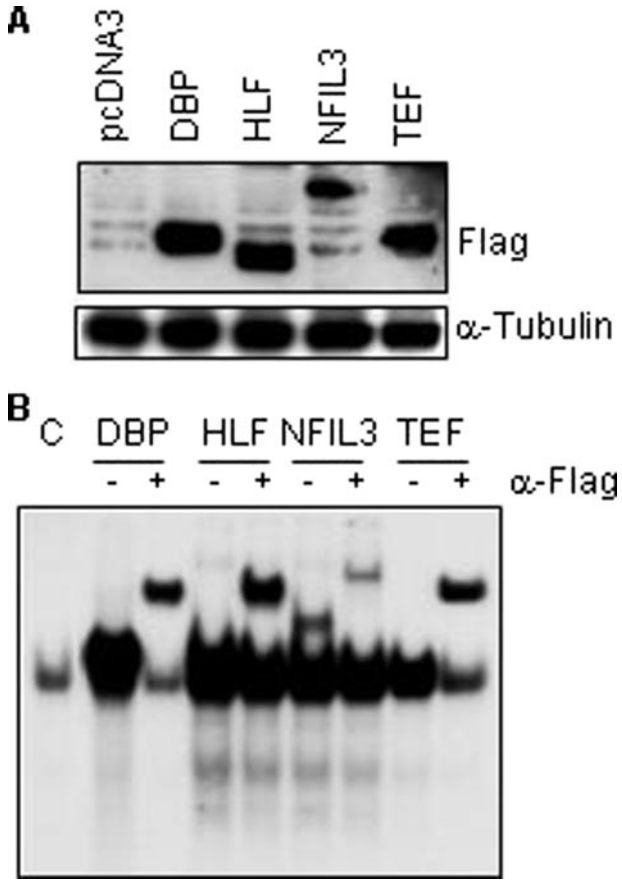

FIGURE 2. **Binding of PAR bZIP proteins to the promoter of *bcl-gS*.** *A*, HEK293T cells were transfected with FLAG-tagged PAR bZIP cDNAs, and the protein expression was analyzed by Western blot with anti-FLAG antibodies. The levels of α-tubulin were determined to assure equal loading. *B*, nuclear extracts from transfected cells were obtained and analyzed for the formation of protein-DNA binding complexes by EMSA. Anti-FLAG antibodies were used to show the specific binding of PAR bZIP and NFIL3 proteins. *C*, control.

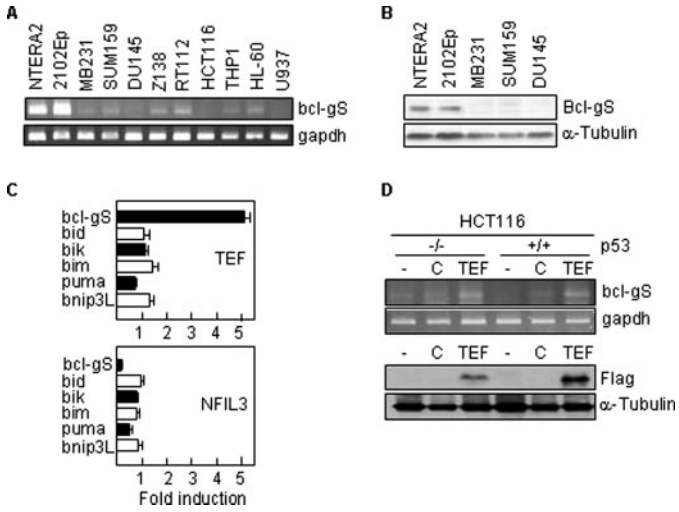

FIGURE 3. **TEF induces the expression of *bcl-gS* in tumor cells.** *A*, total RNA was obtained from a number of cancer cell lines and analyzed for *bcl-gS* expression levels by semiquantitative RT-PCR. *gapdh* mRNA was used as an amplification control. *B*, cell lysates were subjected to Western blotting to determine the expression of Bcl-gS protein. The levels of α-tubulin were determined to assure equal loading. *C*, NTERA2 cells were transfected with TEF or NFIL3 and a GFP-containing vector. Following 24 h of incubation, transfected cells were sorted, cultured for another 24-h period, and analyzed for expression of BH3-only genes by real-time quantitative PCR. Data are shown as fold induction when compared with cells transfected with the empty vector. All data points represent the means ± S.D. of three independent experiments. *D*, HCT116 cells null for the p53 alleles ($^{-/-}$) or containing wild type p53 ($^{+/+}$) were transfected with *TEF* cDNA fused to the FLAG epitope sequence and then analyzed after an overall culture time of 48 h for *bcl-gS* expression by semiquantitative RT-PCR. Transfection efficiency was determined by Western blot with anti-FLAG antibodies. *C*, control.

tDBP Functions as a Competitive Inhibitor of TEF—Gene expression analyses by RT-PCR in mouse tissues revealed the presence of a shorter (truncated) form of *DBP* (*tDBP*) in the liver (Fig. 4*B*) that retains the PAR bZIP region but lacks the transactivation domain (Fig. 4, *A* and *C*). A similar study in more than 30 human cell lines failed to detect a *tDBP*-like isoform but identified two frameshift deletions that created premature stop codons, which are predicted to result in the production of truncated proteins lacking the PAR bZIP region and all or part of the transactivation domain (Fig. 4*A*). Fig. 4*C* shows that tDBP gives rise to a shorter protein of the expected size. Consistently, EMSA experiments demonstrated that tDBP, as well as DBP and TEF, binds specifically to a probe from the *bcl-gS* promoter containing the PAR bZIP binding site (Fig. 4*D*). The bZIP sequence contains the DNA binding and protein dimerization domains. Thus, tDBP could act as a non-functional competitor of TEF that is able to bind DNA and to dimerize with itself and with other PAR bZIP proteins. To test this hypothesis, we cotransfected HEK293T cells with pcDNA3-FLAG-*TEF* and increasing amounts of pcDNA3-Myc-*tDBP*. As shown in Fig. 5*A*, tDBP dimerizes with TEF to form heterodimers that bind to a consensus sequence from the *bcl-gS* promoter. Both anti-FLAG and anti-Myc antibodies confirmed the specificity of the protein-DNA binding complexes. A similar approach was used to analyze the transcriptional activity of TEF-tDBP heterodimers. Cells were cotransfected with TEF, tDBP, and a vector containing the luciferase gene driven by the *bcl-gS* promoter. The luciferase activity was progressively down-regulated in the presence of increasing amounts of tDBP (Fig. 5*B*). To further confirm the blocking activity of tDBP, we determined the endogenous expression of *bcl-gS* in HCT116 cells cotransfected with TEF and tDBP. As shown in Fig. 5*C*, both proteins were coexpressed in these cells following transfection. Consistent with our previous results, TEF up-regulated the mRNA levels of *bcl-gS*, which were significantly reduced (about 3-fold) in the presence of tDBP (Fig. 5*C* and data not shown). These data indicate that tDBP is a convenient molecular tool to study the TEF-*bcl-gS* transcriptional pathway.

Chemotherapy Promotes Activation of the TEF-bcl-gS Transcriptional Pathway—BH3-only proteins may be up-regulated after treatment with chemotherapy, contributing to the apoptotic response of the cell. Thus, we asked whether the TEF-*bcl-gS* transcriptional pathway was activated by chemotherapeutic agents. HCT116 cells were treated with increasing concentrations of etoposide or cisplatin, and the endogenous expression of *bcl-gS* was determined by real-time quantitative PCR (Fig. 6*A*). Both treatments provoked at least a 4-fold induction of *bcl-gS* mRNA levels when compared with untreated cells. The highest inductions were obtained with 40 μM etoposide (12-fold) and with 5 μM cisplatin (about 10-fold). However, no variation in the levels of tef mRNA was observed (data not shown). To confirm that chemotherapeutic agents activated

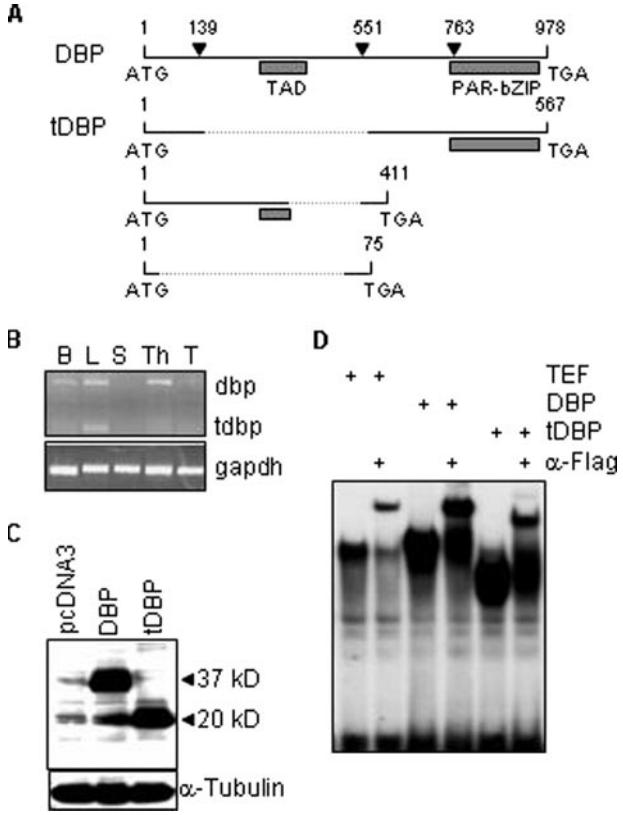

FIGURE 4. **A truncated isoform of DBP lacking the transactivation domain, binds to the *bcl-gS* promoter.** *A*, scheme of the open reading frame of DBP isoforms showing the position of functional domains. *TAD*, transactivation domain. The *arrowheads* represent splice junctions, and dotted lines the truncated sequence. *B*, RNA from mouse brain (*B*), lung (*L*), spleen (*S*), thymus (*Th*), and testis (*T*) was analyzed by RT-PCR with *dbp*-specific primers. Note the presence of a shorter band (*tDBP*) in the liver. *C*, to assure the proper expression of TAD-defective DBP (tDBP), HEK293T cells were transfected with tDBP cDNA fused to FLAG, and then cell lysates were analyzed by Western blot with anti-FLAG antibodies. FLAG-tagged wild type DBP as well as the empty vector were also included as size and specificity controls. *D*, HEK293T cells were transfected with FLAG-TEF, FLAG-DBP, or FLAG-tDBP, and the formation of protein-DNA binding complexes was determined by EMSA using a radiolabeled probe from the *bcl-gS* promoter containing the PAR bZIP site. Anti-FLAG antibodies were used to show the specific binding of transfected proteins.

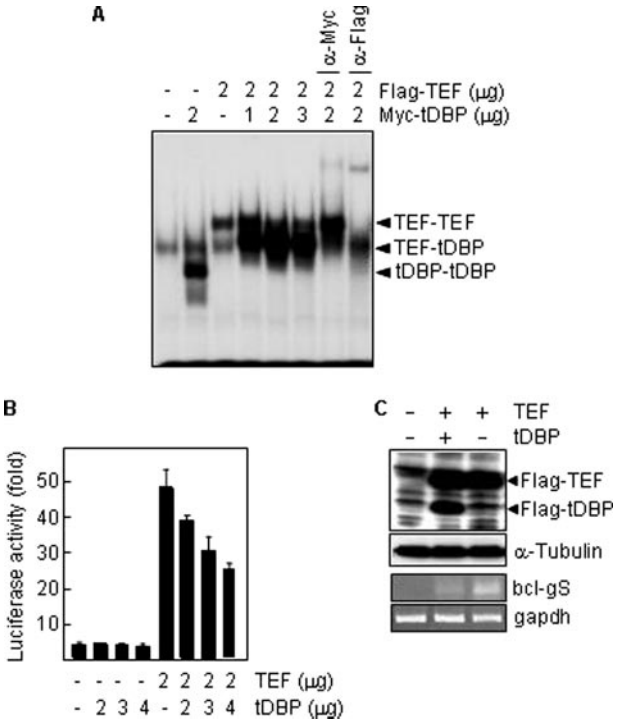

FIGURE 5. **tDBP down-regulates the expression of *bcl-gS*.** *A*, radiolabeled probe containing the PAR bZIP site of the *bcl-gS* promoter was incubated with nuclear lysates from HEK293T cells transfected with FLAG-tagged TEF, Myc-tagged tDBP, or both, and the formation of binding complexes was analyzed by EMSA. Anti-FLAG or anti-Myc antibodies were used to show the specific binding of overexpressed proteins. *B*, HEK293T cells were transfected with a luciferase reporter vector containing the *bcl-gS* promoter in the presence of TEF, tDBP, or both. Following 24 h of transfection, cell extracts were prepared and analyzed for the relative luciferase activity. Results were normalized for transfection efficiency with values obtained with pRSV-β-*gal*. *C*, HCT116 cells were transfected with TEF or TEF plus tDBP (both tagged with the FLAG epitope), and the expression of the overexpressed proteins was determined by Western blot with anti-FLAG. Transfected cells were analyzed for the expression of *bcl-gS* mRNA by semiquantitative RT-PCR. *gapdh* mRNA was included as an amplification control. All histograms represent the means ± S.D. of triplicate analyses.

TEF, we analyzed the binding of this transcription factor to the consensus site in the *bcl-gS* promoter by EMSA. As shown in Fig. 6*B*, both etoposide and cisplatin increased the formation of a protein-DNA complex in HCT116 cells. To detect the presence of endogenous TEF in this binding complex, we generated a polyclonal anti-TEF antibody that detected a single protein of the right size by Western blot analysis (Fig. 6*C*). This antibody partly supershifted the protein-DNA complex, confirming that TEF binds to the *bcl-gS* promoter in response to chemotherapy. A more direct way to demonstrate that TEF mediates the chemotherapy-induced transactivation of the *bcl-gS* gene is to block the transcriptional activity of TEF. HCT116 cells were transfected with either an empty vector or a FLAG-tagged *tDBP* (Fig. 6*D*) and then cultured in the presence or in the absence of etoposide. After 24 h of treatment, the etoposide-dependent induction of *bcl-gS* mRNA was virtually blocked in tDBP-expressing cells (Fig. 6*E*). Quantification of the histone-associated DNA fragments present in the cytosol, a feature of apoptosis, demonstrated that the level of apoptosis following treatment with etoposide decreased from 23% in control cells to 13% in tDBP-expressing cells (Fig. 6*E*). To further confirm the relevance of TEF in the chemotherapy-induced expression of *bcl-gS*, we used an RNA interference approach to deplete cells of endogenous TEF. Specific siRNA significantly reduced the *TEF* mRNA levels and impaired the up-regulation of *bcl-gS* (Fig. 6*F*). Furthermore, this interference strategy promoted a decrease in apoptotic cell death as assessed by quantification of the histone-associated DNA fragments (Fig. 6*F*) and annexin V binding (Fig. 6*G*). Blockade of *bcl-gS* expression gave similar results. A representative experiment is shown in Fig. 6*H*. Specific siRNA impaired the etoposide-induced expression of *bcl-gS* mRNA and gave rise to reduced levels of apoptotic cell death (from 28% to 14%) after treatment with etoposide. This result shows the relevance of bcl-gS in chemotherapy-induced apoptosis.

DISCUSSION

BH3-only proteins are essential for the appropriate execution of apoptotic cell death. Thus, understanding the regulation of BH3-only genes is necessary to gain insight into the mechanisms of response to apoptotic stimuli. We have identified a DNA binding site for PAR bZIP proteins in the promoter region

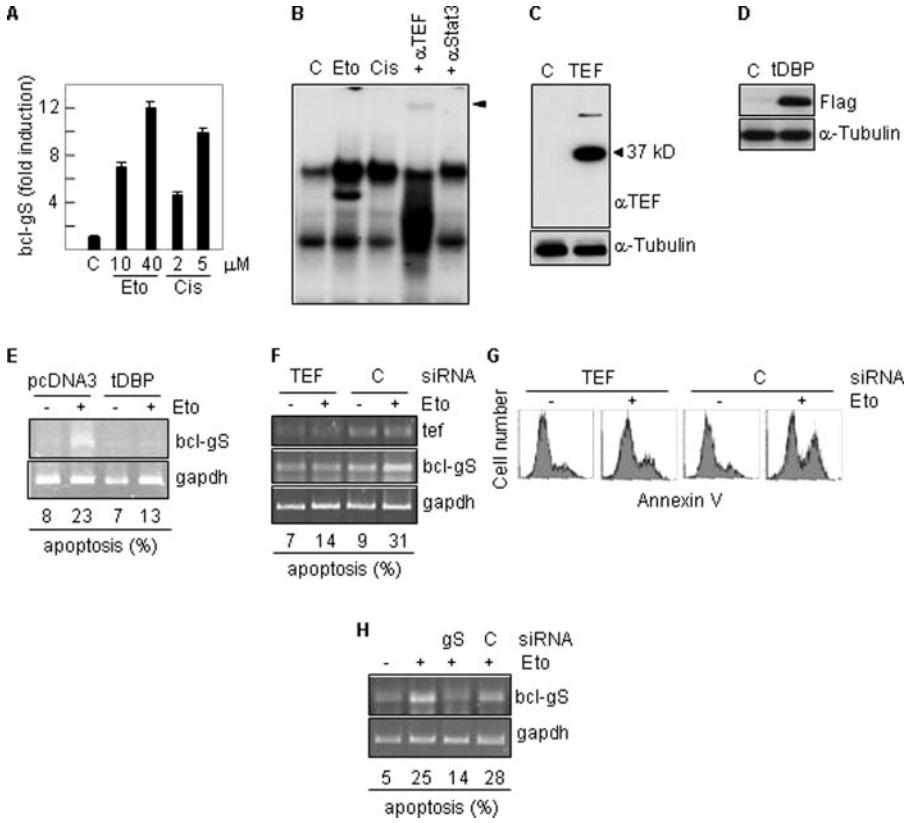

FIGURE 6. **Chemotherapy regulates the TEF-*bcl-gS* transcriptional pathway.** *A*, HCT116 cells were treated with the indicated concentrations of etoposide (*Eto*) or cisplatin (*Cis*). After 24 h, total RNA was obtained and analyzed for *bcl-gS* mRNA levels by real-time quantitative PCR. Data points represent the means ± S.D. of triplicate experiments. *B*, formation of protein-DNA binding complexes in chemotherapy-treated HCT116 cells was determined by EMSA with a PAR bZIP site-containing probe from the *bcl-gS* promoter. An autoradiograh of the EMSA revealed the TEF-DNA complex and a nonspecific band underneath this complex in *lane 2*. Polyclonal anti-TEF and irrelevant anti-Stat3 antibodies were used to show the specific binding of TEF. The *arrowhead* indicates the position of the supershifted TEF-DNA complex. *C*, the specificity of the newly generated mouse anti-TEF antibody was analyzed by Western blotting using extracts from cells transfected with the empty vector (*C*) or with TEF. *D*, HCT116 cells were transfected with empty vector or FLAG-tagged tDBP, and the overexpressed protein was detected by Western blot with anti-FLAG. *E*, tDBP-expressing cells were cultured for 24 h in the presence or in the absence of 80 μM etoposide, and the mRNA levels of *bcl-gS* were determined by semiquantitative RT-PCR. *F*, HCT116 cells transfected with irrelevant (*C*) or *tef*-specific siRNA duplexes were treated with 80 μM etoposide, and after 24 h, the expression of *tef* and *bcl-gS* mRNA was analyzed. *G*, apoptosis following treatment with etoposide was determined by flow cytometry with fluorescein isothiocyanate-labeled annexin V. *H*, cells were transfected with bcl-gS-specific siRNA duplexes and treated as in *F*. *gapdh* mRNA was included as an amplification control. *Numbers* under the figures indicate the level of apoptosis as determined by a quantitative enzyme-immunoassay method in a representative experiment.

of *bcl-gS*, a BH3-only gene. We also found that TEF promoted the strongest induction. TEF has been shown to induce the expression of thyroid-stimulating hormone β (6), pyridoxal kinase, an enzyme involved in the activation of vitamin B6 (8), telokin, a smooth muscle-restricted protein (28), and the LMO2 transcription factor (29). More recently, TEF has been associated with down-regulation of the common β chain of cytokine receptors, but no transcriptional mechanism was defined (30). Although this transcription factor is expressed in the pituitary gland during embryonic development, it appears in several tissues in the mature organism, including brain, lung, liver, spleen, and kidney (7). We have determined the expression of *TEF* mRNA in NTERA2, 2102Ep, HCT116, and RT112 cells. However, we were unable to clearly detect the endogenous protein with our polyclonal anti-TEF antibody, most likely because of the low affinity of this primary antibody, as it readily revealed the overexpressed protein in transfected cells. We determined that the highest expression levels of *bcl-gS* were found in NTERA2 and 2102Ep, which are testicular embryonal carcinoma cells. Consistently, *bcl-gS* mRNA has been found only in testis (16). Overexpression of Bcl-gS in COS-7 cells induces apoptosis, which is suppressed by coexpression of anti-apoptotic Bcl-xL protein (16). We have observed that transfection of HCT116 and NTERA2 cells with TEF promoted the expression of *bcl-gS* but had no effect on cell survival. One possible explanation for these apparently contradictory results may be in the endogenous levels of Bcl-gS, which are presumably higher in cells transfected with this gene than in cells overexpressing its transcription factor. Alternatively, the levels of Bcl-2 or Bcl-xL in the different cell lines used in both experiments may determine the cell fate in response to Bcl-gS overexpression, as both anti-apoptotic proteins counteract the action of BH3-only family members (12, 31, 32).

We also identified a splicing variant of *DBP* (*tDBP*) in mouse liver that retains the DNA binding capacity but has lost the transactivation domain. TEF and DBP bind the same DNA sequence (33) and form heterodimers (6). Consistently, we showed that tDBP competes with TEF for binding to the *bcl-gS* promoter and counteracts the TEF-mediated induction of endogenous *bcl-gS*. Although we use tDBP here as a convenient molecular tool for the study of TEF-*bcl-gS* transcriptional pathway, it may be that this or a similar truncated protein in human normal or tumor cells is acting as a regulator of TEF and other PAR bZIP proteins. Splice variants of transcription factors involved in the expression of apoptosis regulators have been previously described. To this end, a naturally occurring repressor isoform of Stat3 has been shown to be coexpressed with the activator Stat3 in a variety of cell types including leukemia cells (34). This variant inhibits the transactivation potential of Stat3 and may interfere with the anti-apoptotic activity of this transcription factor. In addition, *p73* gene generates a truncated isoform that lacks the transactivation domain, frequently overexpressed in human cancers (35). This variant acts as a potent dominant inhibitor of wild type p53 and transactivation-competent p73, blocking their apoptotic response.

Chemotherapeutic agents have been shown to promote apoptosis in numerous cell systems, including cancer cells, and

BH3-only proteins play an essential role in mediating this process. In line with this, paclitaxel has been recently described to induce apoptosis *in vitro* and in tumors *in vivo* through a Bim-dependent mechanism (20). In this model, inactivation of Bim conferred resistance of cancer cells to paclitaxel. Other BH3-only proteins such as Noxa, Puma, Bik, Bid, and Hrk have also been associated with sensitivity to chemotherapy-induced apoptosis (36–38). Consistent with this, we found that etoposide and cisplatin, two commonly used chemotherapeutic agents, activated the TEF-*bcl-gS* transcriptional pathway. Furthermore, tDBP or siRNA duplexes specific for all splicing variants of the *bcl-g* gene efficiently reduced the levels of *bcl-gS* in response to chemotherapy and partly blocked apoptosis, indicating a significant participation of this pathway in chemotherapy-induced apoptosis. Based on these data, TEF-*bcl-gS* may participate in a regulatory network that controls the apoptotic response to chemotherapeutic agents.

Together, this study shows a novel role for PAR bZIP proteins in the transcriptional regulation of BH3-only genes, mainly *bcl-gS*, and supports that this pathway can be activated by chemotherapeutic agents in cancer cells, thus contributing to the apoptotic response to chemotherapy. Further studies will need to deepen the understanding of the physiological relevance of this transcriptional pathway in normal and cancer cells. It will also be of interest to find other targets of PAR bZIP proteins that may be involved in apoptosis in different cell systems.

Acknowledgment—We are grateful to Valentina Riancho for valuable technical assistance.

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