Stable expression of Epstein-Barr virus BZLF-1–encoded ZEBRA protein activates p53-dependent transcription in human Jurkat T-lymphoblastoid cells

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Interaction between viral proteins and tumor suppressor p53 is a common mechanism of viral pathogenesis. The Epstein-Barr virus (EBV) BZLF-1 ORF-encoded ZEBRA protein (also denoted EB1, Z, Zta) binds to p53 in vitro and has been associated with the altered transcription of p53-regulated genes in B lymphocytes and epithelial cells. In this work, Jurkat T-lymphoblastoid cells that express ZEBRA were characterized by the use of transiently transfected p53 and p53 reporter genes. Stable expression of ZEBRA was associated with the activation of p53-dependent transcription and increased p53 dependent apoptotic cell death. In Jurkat cell lines, stably expressed ZEBRA protein was apparently localized to the cell cytoplasm, in contrast to the typical nuclear localization of this protein in other cell types. Previous studies have suggested that EBV infection of T lymphocytes may contribute to the malignant transformation of T cells and the increased replication of human immunodeficiency virus. Our observations suggest a mechanism through which ZEBRA protein expressed in human T lymphocytes could alter T-cell proliferation and apoptosis during EBV infection. (Blood. 2000;96:625-634)

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Introduction

The p53 tumor suppressor serves as a checkpoint for DNA and cellular damage in many cell types.1-3 p53 is a transcription factor4-6 that directly activates other genes such as the cyclin-dependent kinase inhibitor p21.7,8 Levels of p53 protein are regulated in part by binding between p53 and the mdm2 gene product that targets p53 for ubiquitin-dependent degradation.9 p53-dependent transcription is controlled by phosphorylation-regulated conformational changes in the protein.10 Altered p53 activation that results from hereditary conditions such as ataxia-telangiectasia11 or that exists in mice lacking p53 expression12 is associated with increased malignancy. In lymphocytes, p53 may play a particularly important role as a checkpoint for DNA recombination errors because these cells undergo site-specific recombination during generation of the T- and B-cell repertoire.

Oncogenic viruses often encode proteins that are associated with altered p53 function and altered cellular apoptosis.13,14 The p53 tumor suppressor interacts with proteins expressed by common human viruses, including adenovirus,15-16 papillomavirus,17-19 human T-cell lymphoma virus,20 human immunodeficiency virus (HIV),21-25 and herpes viruses including cytomegalovirus,26 human herpes virus 6,27 and Epstein-Barr virus (EBV).28-30 Interactions between viral and p53 proteins have been suggested to regulate viral replication.13,14 Activation or altered regulation of proapoptotic proteins, such as p53, by viral gene products may result in selection for cells lacking functional p53 or p53-regulated gene products. For example, mutated transcriptionally inactive p53 is usually present in EBV-associated Burkitt lymphoma.31

ZEBRA protein (also termed Z, Zta, and EB1) encoded by the EBV–BZLF-1 open reading frame binds physically to p53.28 Binding between ZEBRA and p53 occurs in part through sequences in the carboxyl-terminus dimerization domain of the ZEBRA protein and the carboxyl terminus of p53. ZEBRA is a site-specific transcription factor required for activation of the viral lytic cycle in lymphocytes3 that can bind and activate the transcription of AP-1 sites in the host genome and viral sequences resembling AP-1 sites.33,34 ZEBRA also binds to other endogenous transcription factors, including NF-κB p65,35 retinoic acid receptors RAR and RXR,36 the TATA binding protein TFIIID,37 and the C/EBP transcription factor.38 In EBV genome-positive B-lymphoblastoid cells, the coexpression of p53 and ZEBRA leads to a decrease in the transcription of genes by p53.28 In epithelial cell lines, the coexpression of p53 and ZEBRA leads to an increase in the transcription of genes by p53.29 Discordance between the consequences of ZEBRA expression in these 2 experimental systems has been attributed to differences in metabolism or activation of p53.29 Other gene products encoded by EBV—the LMP-1 and EBNA-2 proteins expressed during viral latency—also increase p53 expression in transformed primary B lymphocytes.30

Previously, we showed that the cell cycle-regulated expression of p53 is evident during the activation of primary peripheral human T cells.30 Detection of EBV genome and gene expression in T-cell lymphoma30-42 suggests that T cells are targets of EBV infection. In contrast to most EBV genome-positive T-cell tumors that express latency-associated gene products,41,43 lytic gene products including ZEBRA are expressed in human thymocytes.43 We suggested a model of EBV infection of T cells in which the infection of primary T cells with EBV leads to the abortive transcription of EBV lytic gene products that can rarely lead to malignant transformation and stable expression of latent gene products.41 A clinical syndrome has been described in which chronic, active EBV infection,
including expression of the ZEBRA protein in T cells, was associated with malignant T-cell lymphoma. Increased lytic replication of EBV in patients with immunodeficiency caused by HIV and congenital immunodeficiency syndromes or chronic fatigue syndrome could also lead interaction between ZEBRA and p53 in T cells. Therefore, we characterized the effects of ZEBRA expression on p53-dependent transcription in Jurkat T-lymphoblastoid cells that can be infected with EBV in vitro, through a receptor similar, but not identical, to the CD21 EBV receptor present on B lymphocytes and thymocytes.

Jurkat T-lymphoblastoid cell lines stably expressing ZEBRA protein were established. Both wild-type and mutated p53 were introduced into Jurkat cells using nontoxic lipid reagents. Transcriptional activity of p53 was monitored with reporter genes, including synthetic reporter genes regulated by the p53 consensus binding site and the more complex endogenous p21 promoter. We found that levels of p53 protein detected by Western blotting were increased in Jurkat cells stably expressing ZEBRA and that the transcription of p53 reporter genes was markedly increased. Populations of Jurkat cells expressing ZEBRA transfected with p53 also exhibited increased apoptotic cell death that was p53-dependent. Mechanisms through which interaction with the ZEBRA protein might increase p53 stability and activation are discussed, such as alterations in the ubiquitin-dependent pathways that degrade p53 and the activation of p53 through regulatory sequences in the p53 carboxy-terminus. We hypothesize that the activation of p53-dependent transcription in EBV-infected T lymphocytes may inactivate T cells required for the control of EBV infection as a mechanism of viral pathogenesis.

Materials and methods

Plasmids

pSV2-neo-WZhet ZEBRA expression plasmid and control plasmid pSV2-neo were obtained from Dr G. Miller (Yale University, New Haven, CT). Plasmids encoding wild-type p53 (pC53-SN3, denoted p53W), DNA binding mutant p53 (pC53-SCX3, denoted p53N), and reporter genes pG3PyLuc, pMG15, and p21 WWP/luc were obtained from Dr B. Vogelstein (Johns Hopkins University, Baltimore, MD). A plasmid encoding carboxy terminus deleted p53 (pCEP4-353, denoted p53C) was obtained from Dr J. Pietenpol (Tulane University, Nashville, TN). pRL-SV40 was obtained from Promega Biologicals (Madison, WI). Plasmids were prepared using the Qiagen method (Qiagen, Chatsworth, CA).

Cell culture and cell lines

Jurkat (JEG-1) cells were obtained from the ATCC (Rockville, MD). Cells were cultured in RPMI medium supplemented with 10% fetal calf serum, penicillin (100 U/mL), and L-glutamine (2 mmol/L). Stably transfected Jurkat cell lines were obtained by the transfection of cells with pSV2-neo-WZhet ZEBRA expression plasmid and control plasmid pSV2-neo by the use of Cell–Fectin (Gibco/BRL, Gaithersburg, MD). Cells were cloned in soft agar using selection for G418 resistance (100 mg/mL) and grown in 200 µg/mL G418. Two independently derived cell lines expressing ZEBRA protein/TrpE fusion protein; obtained from Dr G. Miller) at a 1/1000 dilution of human anti-serum) and precipitated with washed protein G plus/A Sephadex beads (Oncogene Science, Santa Barbara, CA). Lysates of cytoplasmic protein were generated by the lysis of cells in a hypotonic buffer containing 25 mmol/L Tris pH 7.5, 0.2% SDS, 50 mmol/L NaCl, and 0.5% SRC. Nuclear extracts were prepared as described. After separation of proteins by 12% SDS–PAGE and transfer to nitrocellulose as described previously, Western blots were bound to antibody (1/10,000 dilution of primary antibody OT20A, 1/10,000 dilution of secondary goat antimouse antisera) (Amersham, Arlington Heights, IL). Western blots were developed using the Renaissance system (NEN, Boston, MA). Anti-ZEBRA murine monoclonal antibody OT20A was obtained from Dr J. Middledorp (Organon–Teknika, The Netherlands) and reactivity of OT20A to ZEBRA protein was confirmed using Akata B-lymphoblastoid cells induced into the lytic cycle by the ligation of surface IgG (data not shown). Anti-p53 (whole p53 protein) rabbit antisera was obtained from Santa Cruz Biologicals (Santa Cruz, CA).

Cell morphology/immunofluorescence studies

For a demonstration of the altered Z+ cell morphology Z+ (Z1) and Z− cells were photographed 24 hours after plating in standard polystyrene plastic ware (Falcon; Becton Dickinson, Franklin Lakes, NJ). Cells were plated in fresh media and were not otherwise stimulated. Under these conditions, both Z+ cell lines (Z1 and ZA) had a similar adherent phenotype as shown, whereas Z− cells and parental Jurkat cells had a nonadherent phenotype typical of Jurkat cells (ZA and parental Jurkat cell data not shown). For immunofluorescence studies, Z+ and Z− cells were bound to poly-D-lysine–coated coverslips as described previously for T lymphocytes. Cells were incubated with rabbit polyclonal antisera (ZEBRA rabbit antisera generated against bacterially produced whole ZEBRA protein/TrpE fusion protein; obtained from Dr G. Miller) at a 1/1000 dilution. Cells were then washed and incubated with biotinylated donkey antirabbit secondary antibody (Jackson Research, South Park, PA), washed, and incubated with streptavidin Cy3 (Jackson Research) used at a 1/180 dilution. Cells were photographed using a Nikon Diaphot 60× oil immersion lens. Data were collected using IP Lab Spectrum software (Signal Analytics, Vienna, VA).

Transfection of cells

Reporter plasmids and p53 expression plasmids (concentration as indicated in the text) were transiently transfected into 2 × 10⁶ logarithmically growing Z+ Jurkat cells stably expressing ZEBRA and Z-control cells with Superfectin (Qiagen) following the recommended procedure for the transfection of nonadherent cells. After transfection, cells were cultured for 36 hours. In experiments shown, cells were also transfected with a plasmid expressing renilla luciferase to control for cell viability and transformation efficiency. Similar results were obtained without the cotransfection of pRL-SV40 and with luciferase activity normalized to micrograms of total protein, determined by the Bradford protein assay (Bio-Rad, Hercules, CA). Cells were irradiated using a hand-held UVB source (Ultraviolet Products, San Gabriel, CA) 3 cm above the washed cells suspended in 1 mm phosphate-buffered saline in a culture dish. For transient transfection of the Jurkat cells, a 2-stage transient transfection was used in which ZEBRA expression plasmid and p53 reporter genes were transfected 24
hours before the transfection of p53 expression plasmid to reduce the potential suppression of ZEBRA expression by the coexpression of p53.

**Luciferase assay**

Luciferase activity was determined either using the firefly luciferase or Stop-and-Glo assay systems (Promega Biologicals) and an Analytical Luminescence Laboratory (San Diego, CA) lumimeter. Results of at least 3 separate experiments were used to generate each data point in luciferase assay experiments. Mean luciferase activity and standard error were determined, as shown graphically and analyzed using the JMP Statistical Discovery Software Version 3.1 (SAS Institute, Cary, NC). Student t test was used for comparison of experiments, and significance (P < .05) was determined by the JMP program.

**Detection of apoptosis in Jurkat cells**

Exponentially growing Z+ and Z− cells 2 × 10⁶ were transiently transfected with 1 µg wild-type p53 as described above. Cells were stained with propidium iodide and annexin–fluorescein isothiocyanate (FITC) of apoptotic cell death using Apo-alert (Clontech, Palo Alto, CA) as instructed by the manufacturer. Apoptotic cells were quantitated in 3 independent experiments for each data point shown by fluorescence-activated cell sorting (FACS) of 2000 cells using a Coulter (Hialeah, FL) Epics XL. In some experiments 0.5 µg of a plasmid expressing green fluorescent protein (Green Lantern; New England Biolabs, Boston, MA) was transfected into Z+ and Z− cells to determine transfection efficiency, and approximately 10% of transfected cells expressed glial fibrillary protein under conditions used in these studies (data not shown).

**Results**

**Characterization of Jurkat cells stably expressing ZEBRA protein**

Jurkat T-lymphoblastoid cell lines stably transfected with the ZEBRA expression plasmid pSV2-neo-WZhet were established by selection for growth in G418 selection medium. pSV2-neo-WZhet has previously been shown to express a functional 43-kd variant of ZEBRA protein that is sufficient to activate the EBV lytic cycle. Jurkat cells transfected with pSV2-neo-WZhet (denoted Z+ cells) grown in G418 expressed a 43-kd protein detected by ZEBRA-specific monoclonal antibody OT20A (Figure 1). This 43-kd putative ZEBRA protein was not detected in Jurkat cells stably transfected with control plasmid pSV2-neo (control cells denoted Z− cells; Figure 1A).

Levels of putative ZEBRA protein in several independent Jurkat cell lines were uniformly lower than in nonlymphoid cell lines, such as COS cells transfected with pSV2-neo-WZhet (Figure 1A). To confirm that the 43-kd protein was in fact ZEBRA rather than a cross-reactive cellular protein, we also demonstrated that the putative ZEBRA protein was highly enriched among the proteins precipitated from Z+ cells by human serum reactive against EBV lytic gene products (Figure 1B). Precipitated 43-kd protein appeared as a closely spaced dimer, consistent with the appearance of WZhet ZEBRA expressed in B lymphocytes and in EBV-infected primary human lymphocytes. In these coprecipitation experiments, components of NF-κB, including p65, were also selectively enriched by coprecipitation with anti-ZEBRA antisera, consistent with the previous observation that ZEBRA and p65 form a complex when coexpressed in lymphoid cells. ZEBRA protein was also detected in Z+, but not in Z−, cells using rabbit polyclonal antisera generated against purified bacterially expressed ZEBRA protein in immunofluorescence studies (Figure 1C).

In immunofluorescence studies ZEBRA protein was detected largely in the cytoplasm of Z+ but not Z− cells (Figure 1C). A cytoplasmic location for the protein was also suggested by fractionation experiments in which ZEBRA protein was detected in lysates from Z+ cells but not Z− cells generated in a hypotonic lysis buffer (Figure 1D). ZEBRA protein was not detected in the nucleus of Jurkat cells by Western blotting of nuclear extracts (data
not shown). These observations suggested that when it was expressed stably in Jurkat cells, ZEBRA protein was localized to the cell cytoplasm, where it maintained its ability to associate with other cytoplasmic proteins. The cytoplasmic localization of ZEBRA protein was in contrast to the nuclear localization of the protein in EBV-positive Akata B-lymphoblastoid cells determined using similar fractionation and staining techniques (data not shown). Jurkat cells stably transfected with pSV2-neo-Wzhet also exhibited a distinct cytoplasmic cellular phenotype of increased adherence to plastic ware and an ameboid morphology not present in control cells transfected with pSV2-neo grown under identical conditions (Figure 1E).

Expression of transiently transfected p53 protein in Jurkat cells stably expressing ZEBRA protein

Because of the previously described effects of ZEBRA protein on p53-dependent gene transcription, we determined the effects of ZEBRA expression on p53 stability and p53-dependent gene transcription in Z+ cells in comparison to control Z− Jurkat cells. Endogenous p53 protein was not detected in the parental Jurkat cells used to generate Z+ and Z− cell lines (data not shown). To introduce p53 expression, Z+ and Z− cells were transiently transfected with 1 µg of expression vectors encoding wild-type p53 (pC53-SN3, denoted p53W), p53 amino acids 1 to 353 (pCEP4-353, deleted from p53 C-terminal regulatory sequences; denoted p53C), and a DNA binding null mutant form of p53 (pC53-SCX3, denoted p53N). Expression of cytoplasmic ZEBRA protein did not vary significantly with transient transfection of p53 expression vectors (Figure 1D). With the transient transfection of p53 expression vectors, p53 protein encoded by wild-type p53 expression plasmid and the pCEP4-353 C-terminal deleted protein was detected in proteins extracted from the nucleus of Z+ cells in hypertonic buffer (Figure 2A). Expression of p53C was markedly less than the expression of p53W and was barely detectable by Western blotting. Remarkably, transiently expressed p53W or other p53 proteins was not detected in the nuclear proteins of Z− cells under these conditions (Figure 2A). Transiently expressed p53 proteins were not detected in cytoplasmic proteins extracted from Z+ or Z− cells in hypotonic lysis buffer, with the possible exception of p53N, which was evident as a very faint band in the cytoplasm of both Z+ and Z− cells (Figure 2B).

The ability of pC53-SCX3 to express a stable transcriptionally inactive p53 protein was confirmed in a human neuroblastoma cell line (data not shown). Mutant, transcriptionally inactive p53 alleles, such as the protein encoded by pC53-SCX3, were stabilized, and protein levels protein were increased in many cell types, possibly accounting for the small amount of p53N protein transiently expressed in the cytoplasm of Z+ and Z− cells (Figure 2B).

Activation of a synthetic p53 reporter gene in Jurkat cells stably expressing ZEBRA protein

In the absence of activation, the p53 protein is normally rapidly degraded by a ubiquitin-dependent pathway. Binding between p53 and viral proteins in human cells can either stabilize or destabilize p53 through interference with p53 metabolism. In many instances, p53, stabilized by viral proteins, is not transcriptionally active. The transcriptional activity of transiently transfect p53 was further characterized in Jurkat cells stably expressing ZEBRA using a reporter gene pG13PYluc. pG13PYluc is a synthetic reporter gene in which 13 copies of an active p53 response element are fused to a polyclonal virus promoter. Production of luciferase by this reporter gene has previously been shown to be a sensitive and specific measure of p53-dependent transcriptional activity. Two hundred nanograms of pG13PYluc was introduced transiently with 500 ng of p53 expression vectors into Z+ and Z− Jurkat cells (Figure 3). As in Western blotting studies (Figure 2), a nontoxic transfection reagent Superfectin; Qiagen) was used to introduce DNA into cells. Cells were not activated in these experiments by radiation or other stimuli, and viability of cells was similar to that of control cells cultured without transfection.

As shown in Figure 3A, markedly increased levels of p53-dependent transcription were evident in Jurkat cells stably expressing ZEBRA transfected with a plasmid expressing wild-type p53 (pC53-SN3, denoted p53W). Transfection of a plasmid expressing p53 lacking regulatory sites in the carboxyl terminus of the p53 protein (pCEP4-353, denoted p53C) was also associated with the activation of p53-dependent transcription in cells expressing ZEBRA, though the activation of p53-dependent transcription was less than that with transfection of a plasmid expressing wild-type p53. Levels of pG13PYluc transcription, when quantitated by luciferase assay (Figure 3A), corresponded to levels of p53 protein...
detected by Western blotting (Figure 2A) in Jurkat cells. Levels of pG13PYluc transcription, with transcription of a transcriptionally inactive p53 (p53N) or with a control plasmid not encoding p53, were similar to basal levels of pG13PYluc transcription in the absence of any p53 expression plasmid transcription (data not shown). Transcription of pG13PYluc by wild-type and carboxyl terminus-deleted p53 was eliminated by a subtle mutation\(^7\) in the p53 consensus site (plasmid MG15PYLuc) and thus was specific for the transcriptionally active p53 protein for the p53 consensus binding site in pG13PYluc (Figure 3A). Transfection of more p53 plasmid into Z\(+\) cells resulted in nonlinear increases in p53-dependent transcription (data not shown), suggesting the quantity of expressed p53 was a limiting factor in p53 expression.

In these experiments, 5 ng pRL/SV40 expressing Renilla luciferase (pRL/SV40), under the control of an SV40 promoter enhancer, was introduced into cells as a control for transfection efficiency and cell viability. Renilla luciferase transcription was similar in the presence of wild-type p53 in Z\(+\) and Z\(-\) cells, and thus differences in transfection efficiency or viability between Z\(+\) and Z\(-\) cells could not account for the activation of p53-dependent transcription associated with ZEBRA protein expression. Similar results were found in 2 independently established Z\(+\) Jurkat cell lines and did not differ in the presence or absence of pRL/SV40.

To determine whether transient expression of ZEBRA could reproduce the activation of p53-dependent transcription evident in Jurkat cells stably expressing ZEBRA, the parental Jurkat cell line used to generate cells stably expressing ZEBRA was transiently transfected with ZEBRA expression plasmid pSV2-neo-WZhet, p53W expression plasmid, and p53 reporter genes (Figure 3B). Expression of pG13PYluc was compared between cells transfected with pSV2-neo-WZhet or control plasmid pSV2-neo. In the parental Jurkat cell line, levels of p53-dependent transcription of pG13PYluc were low. Transient coexpression of wild-type ZEBRA and wild-type p53 in Jurkat cells resulted in a trend toward increased transcription of p53 reporter gene pG13PYluc, but this trend was not significant because of greater interexperimental variability with cotransfected ZEBRA expression plasmid and low levels of luciferase activity. These results suggested that either prolonged or high levels of ZEBRA protein were required to activate p53-dependent transcription in Jurkat cells or that the expression of ZEBRA selected for a specific phenotype in Jurkat cells was not present in the parental cells.

**Activation of a p21 promoter reporter gene in Jurkat cells stably expressing ZEBRA protein**

A luciferase reporter gene regulated by the p21 promoter (plasmid WWP-Luc) was previously used\(^2\) to characterize the activation of a physiologic target of the p53 protein in epithelial cells. The p21 promoter is a physiologic target of p53 activation, and it was used in addition to the synthetic reporter, pG13PYluc, to determine the effects of stable ZEBRA expression in Jurkat cells. Transcription of the p21 promoter was activated by cotransfected wild-type p53 (pC53-SN3, denoted p53W) in Jurkat cells stably expressing ZEBRA but not in Z\(-\) cells (Figure 4A). In these experiments, synergy was evident between activation of the p53-responsive p21 promoter in Z\(+\) cells and other stimuli, such as ultraviolet irradiation (Figure 4B). A small but significant increase in expression of the p21 promoter was also evident in Z\(+\) cells in the absence of cotransfected p53 (Figure 4B) and also in Z\(-\) cells, but p53-dependent activation of WWP/Luc was not evident in Z\(-\) cells (data not shown). These results demonstrated that stable expression of ZEBRA in Jurkat T-lymphoblastoid cells was associated with p53-dependent activation of the physiologic p21 promoter, and this activation could synergize with other activators of p21 transcription.

**Increased apoptosis of cells coexpressing ZEBRA protein and p53**

Increased expression of p53 reporter genes including the p21 promoter was demonstrated in cells stably expressing ZEBRA protein transfected with p53W expression plasmid (Figures 3, 4). Although p21 promoter expression was activated by cotransfected p53 (Figure 4A), Western blotting was not sufficiently sensitive to detect a convincing increase in p21 expression in Z\(+\) Jurkat cells transfected with p53W (data not shown), possibly because a small percentage (approximately 10%) of transfected cells expressed transiently transfected genes. Further experiments were designed to determine whether populations of Z\(+\) cells demonstrated evidence of increased apoptosis (programmed cell death) in the presence of p53 expression because p53 induces apoptosis in many cell types through the transcription of gene products, including p21.\(^{13}\)

Z\(+\) and Z\(-\) cells were transfected with 1 µg of either wild-type...
p53 (p53W) or DNA binding mutant p53 (p53N) expression plasmids. Cells were incubated for 48 hours and stained with both propidium iodide and annexin–FITC, independent markers for early and late stages of apoptotic cell death, respectively. Increased staining of approximately 10% of cells with both apoptotic markers was evident in populations of Z+ cells transfected with p53W expression plasmid or to transfection of Z− cells with either p53W or p53M expression plasmids, as shown in Figure 5A. The number of cells staining with either marker relative to total cells was quantitated in 3 independent experiments (Figure 5B). To obtain normalized data shown in Figure 5B, populations of Z+ and Z− cells were transfected with either p53N or p53M expression plasmids, as described above, or with a plasmid driven by a similar cytomegalovirus promoter lacking p53 protein coding sequences (pCMV5).

The number of cells exhibiting apoptotic cell death in each population of cells transfected with either p53N or p53W was then determined and expressed as the ratio of apoptotic cells to apoptotic cells detected in a control population of cells transfected with control plasmid. As shown in Figure 5B, only the expression of p53W in Z+ cells resulted in a detectable increase in apoptotic cells compared with control cells (ratio to control cells greater than 1.0 by more than the standard error of measurement) for either annexin or propidium iodide markers. Cell death in either Z+ cells transfected with mutant p53 or Z− cells transfected with wild-type p53 was not different than that in control cells (ratio to pCMV = 1.0). Decreased cell death relative to control cells (ratio to pCMV less than 1.0) was evident in Z− cells transfected with mutant p53.

Similar numbers of dying cells were present in populations of Z− and Z+ cells transfected with p53W expression plasmid after 24 hours of incubation (Figure 5C), as determined by staining with propidium iodide. Significantly more dying cells were evident 48 hours after transfection with p53 W in Z+ cells than in Z− cells. Thus increased cell death in Z+ cells transfected with p53W expression plasmid correlated with the time course of p53-dependent transcription in Z+ cells, which increased several times between 18 and 36 hours after transfection of p53 (Figure 5C, inset graph). These experiments demonstrated increased apoptotic cell death in populations of Jurkat cells stably expressing ZEBRA protein transfected with transcriptionally active p53 expression plasmid.

Discussion

RNA transcripts encoding ZEBRA are expressed in EBV-infected T cells,42,43 but ZEBRA protein expression levels are low. A heterogeneous population of EBV-infected thymocytes contains infected and uninfected cells.43 Thymocytes from normal patients obtained during surgery45 also have endogenous p53 that may be in variable states of activation, depending on variable conditions such as the health of the tissue donor. In contrast, Jurkat cells stably expressing ZEBRA protein are a homogeneous population of cells lacking significant endogenous p53-dependent transcriptional activity. Therefore, we characterized p53-dependent transcription in Jurkat cells stably expressing ZEBRA protein (Figure 1) to test the hypothesis that ZEBRA expression modulates p53-dependent transcription in T cells. Remarkably, ZEBRA protein detected in these cells using several ZEBRA-specific antibodies (mouse monoclonal antibody, human and rabbit polyclonal antisera) was localized to the cell cytoplasm, as determined by both immunofluorescence (Figure 1C) and protein fractionation (Figure 1D). These small amounts of cytoplasmic ZEBRA protein were associated with an altered cellular morphology (Figure 1E).

Expression of ZEBRA in Jurkat T-lymphoblastoid cells was associated with increased nuclear levels of p53 protein detected by Western blot analysis (Figure 2) and increased p53-dependent transcriptional activity detected by luciferase reporter genes (Figures 3, 4). In T cells, nuclear p53 protein levels detected by Western blotting (Figure 2) correlated with transcriptional activation of p53 in Jurkat cells (Figures 3, 4). Activation of p53-dependent transcription associated with ZEBRA expression required functional p53 DNA binding sites in the reporter gene. Deletion of the p53 carboxyl-terminus that regulates the physiologic activation of p53 was associated with decreased p53-dependent transcriptional
activation (Figure 3). Increased transcription of p53 reporter genes was not evident in Jurkat cells transiently transfected with ZEBRA expression plasmid (Figure 3C). Activation of the p21 promoter, a physiologic target of p53, was also associated with the expression of ZEBRA protein in T cells (Figure 4). Transcriptional activation of the p21 promoter associated with ZEBRA expression did not require activation of p53 by physiologic signals, such as DNA damage or other cellular injury, but exhibited synergy with p53 activation by ultraviolet irradiation.

Collectively, these observations suggest that prolonged or high levels of ZEBRA protein expression, but not transient ZEBRA expression, activate p53-dependent transcription in Jurkat cells. A similar activation of p53-dependent transcription was noted previously in epithelial cell lines overexpressing ZEBRA protein from an inducible promoter in the absence of other viral gene products. The transient expression of p53 in Z+ Jurkat cells was associated with the increased apoptosis of Jurkat cells (Figure 5), consistent with the increased transcription of p53 response elements in reporter genes. Although increased p21 promoter expression (Figure 4) may contribute to this phenotype, delineation of the mechanism through which p53 mediates increased apoptosis in Z+ cells was not determined in this work. Because ZEBRA expression activates p53-dependent transcription in epithelial cells and, as described in this work, in Jurkat T lymphocytes because ZEBRA expression inactivates p53-dependent transcription in B-lymphoblastoid cells such as the Akata cell line, these discordant results require further investigation.

Because ZEBRA is a nuclear transcription factor in B lymphocytes, the cytoplasmic localization of ZEBRA and its corresponding transcriptional inactivity in Jurkat cells is novel. Possibly, mutant non-nuclear alleles of ZEBRA could be selected during the generation of stable cell lines because of the toxic nature of the protein. However, the remarkable similarity between our results and previously reported results in epithelial cells in which the inducible transient expression of ZEBRA also activated p53-dependent transcription suggests that the activation of p53-dependent transcription by ZEBRA is cell-type specific rather than related to stable or transient expression of the protein. In addition, using a variety of antibodies (Figure 1), we only detected a single species of ZEBRA protein in Jurkat cells, eliminating the possibility that a truncated or deleted protein was expressed.

Therefore, we propose that the cytoplasmic localization of the ZEBRA protein in some cell lineages, for example T lymphocytes, results from a fundamental difference in post-translational processing of the ZEBRA protein in T cells that could, in turn, account for the effects of the expressed protein on, for example, p53-dependent transcription (Figure 6). As an example, a difference in post-translational phosphorylation could result in the altered cellular localization of ZEBRA protein. Thus, the cellular lineage and localization of ZEBRA protein, rather than the expression of ZEBRA itself, may be critical in determining the effects of ZEBRA on p53-dependent transcription. These results were also consistent with our recent studies of the effects of ZEBRA protein on both transient and stable expression of the NF-κB transcription system in which ZEBRA expression blocked the cytoplasmic-to-nuclear translocation of components of NF-κB, apparently through a cytoplasmic interaction between ZEBRA and components of NF-κB.

We found in the present work that transiently transfected ZEBRA and p53 resulted in a trend toward increased p53-dependent transcription in most experiments in Jurkat cells (Figure 3C), though levels of ZEBRA expression in transiently transfected
cells were below the level of detection by Western blot analysis and in situ studies. Because the ZEBRA expression plasmid pSV2-neo-Wzhet used in this work uses a native EBV promoter32 driven by the BamHI W promoter that we have demonstrated is poorly expressed in T cells, 43,44 it is likely that low levels of expression of the protein from this construct in Jurkat cells contributed to our ability to establish stable cell lines expressing an otherwise toxic protein product.54 In contrast, a significant decrease in p53-dependent transcription was confirmed with transient cotransfection of ZEBRA and p53 in B-lymphoblastoid cells, as reported previously.28

Activation of p53-dependent transcription associated with cytoplasmic ZEBRA expression in Jurkat cells could be dependent or independent of binding between the 2 proteins.29 ZEBRA protein bound to p53 could displace factors such as mdm-2, which stimulates the degradation of p53,9 or could mask a p53 site targeting the protein for degradation by the ubiquitin pathway.48,49 Alternatively, cytoplasmic ZEBRA could stabilize p53 indirectly through activation of DNA-PK, ATM, or other kinases that phosphorylate the p53 amino terminus, thus interfering with the binding of mdm-2,9,11 or directly by displacing mdm-2 through a shared binding site. It would, therefore, be important to determine whether ZEBRA in part shares a p53-binding site with mdm-2 or interferes with mdm-2 binding and to what extent ZEBRA interacts directly or indirectly with other components of p53 activation, such as DNA-PK.

Because the ZEBRA protein binds at least in part to the carboxyl terminus of p53, as determined by in vitro binding studies,28 ZEBRA could, under some conditions, activate p53, which would be analogous to certain antibodies and small peptides binding to the p53 carboxyl terminus.10 This hypothesis is difficult to reconcile with the observations that deletion of the carboxyl terminus of p53 reduced, but did not eliminate, the activation of p53 detected by luciferase assay (Figure 3). If ZEBRA activates p53 directly by binding to the carboxyl terminus of p53, then deletion of the carboxyl regulatory terminus of p53 might be expected to eliminate p53 activation. We also did not find any evidence that purified ZEBRA protein could activate p53 DNA binding activity by a direct interaction with the carboxyl terminus of p53 using a gel-shift assay for p53-specific DNA binding55 and purified bacterially produced p53 56 (data not shown).

As a result, we favor the hypothesis that ZEBRA expression in Jurkat cells indirectly activates cellular kinases or other factors that in turn stabilize and activate p53 partly or completely, independently of binding between the 2 proteins (Figure 6). For example, nonspecific toxic effects of ZEBRA on Jurkat cells could activate cytoplasmic kinases such as the ATM kinase11 or related components in DNA-PK leading to the stabilization and activation of p53. Toxic effects of cytoplasmic ZEBRA protein are also suggested by the altered appearance of Jurkat cells stably expressing ZEBRA (Figure 1E). Activated p53 would then be translocated to the nucleus, where it could interact with components of the p53-transcriptional pathway, including CBP/p300, a p53-binding regulatory protein57-59 possibly altered by ZEBRA expression.

EBV-encoded gene products expressed in T cells could contribute to altered T-cell signaling and apoptosis.44,54 Expression of EBV-encoded proteins such as ZEBRA could also alter the replication of HIV-1 in EBV-infected primary human T cells,45 in part through the altered activation of p53.21-25 Because p53 is expressed in primary T lymphocytes during normal cellular activation and proliferation but not in resting T cells,39 activated cells in particular could be targeted by ZEBRA expression because the ZEBRA promoter is activated through T-cell activation.60 These observations may also be relevant to the pathogenesis of X-linked lymphoproliferative disease (XLP), an often fatal disorder of T-cell
proliferation and malignancy in response to EBV infection.41 Because the defect in XLP resides in what is apparently a signaling protein related to the cytoplasmic SH2 adaptor family and this protein (denoted SH2D1A) is expressed primarily in human thymocytes, it is an interesting coincidence that these thymocytes are also the T-cell subset most readily infected by EBV with subsequent expression of ZEBRA and other EBV-encoded proteins.53,54 Possibly, interactions between cytoplasmic ZEBRA or other EBV-encoded proteins and SH2D1A could be altered in XLP, leading to the often fatal lymphoproliferation typical of this disease. Further studies in EBV-infected Jurkat cells should provide a useful model system for understanding the effects of EBV infection of T cells, particularly with regard to the role of this infection in altered p53-dependent transcription in the progression to T-cell lymphoma.40

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