Biological function of the vaccinia virus Z-DNA-binding protein E3L: Gene transactivation and antiapoptotic activity in HeLa cells

Jin-Ah Kwon* and Alexander Rich*

Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Room 68-233, Cambridge, MA 02139-4307

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The vaccinia virus (VV) E3L protein is essential for virulence and has anti-apoptotic activity. In mice, Z-DNA-binding activity of the N-terminal domain of E3L (Zα) is necessary for viral lethality. Here, we report that inhibition of hygromycin-B-induced apoptosis in HeLa cells depends on Z-DNA binding of the E3L Zα domain. Z-DNA-binding domains of other proteins are equally effective in blocking apoptosis. Using a transient reporter assay, we demonstrate transactivation of human IL-6, nuclear factor of activated T cells (NF-AT), and *p53* genes by E3L. This activation also requires Z-DNA binding of the N-terminal domain of E3L. Overall, this work suggests that the important role of E3L in VV pathogenesis involves modulating expression of host cellular genes at the transcriptional level and inhibiting apoptosis of host cells through Z-DNA binding.

apoptosis | interleukin-6 | p53 | poxvirus

Vaccinia virus (VV) is a large double-stranded DNA virus encoding ≈190 genes, and it causes major changes in the host cell machinery shortly after infection. *E3L* is a host range gene, necessary for efficient VV replication in several cell lines (1) and is required for VV pathogenesis (2). The *E3L* gene is expressed early during infection, and the protein is present in both the nucleus and cytoplasm of infected and transfected cells. E3L accumulates in the nucleus (3), but little is known about its activities there. E3L has two domains, an N-terminal Z-DNA-binding protein domain (Zα) and a C-terminal double-stranded RNA-binding domain (Fig. 1*A*). The N-terminal region is similar to the Zα domain of several Z-DNA-binding protein families that include the RNA editing enzyme ADAR1 (double-stranded RNA adenosine deaminase) (4), the tumor-related DLM1 (or ZBP1) protein (5), and the recently described PKR-like kinase of bony fish (6). The crystal structures of two Zα domains, $Z\alpha_{ADAR1}$ (Fig. 1*B*) (7) and $Z\alpha_{DLM1}$ (5), have shown them complexed to Z-DNA. Both N-terminal and C-terminal domains are required for infection and full pathogenesis in the mouse model (2). It has been shown that viral pathogenicity requires E3L binding to Z-DNA (8). The N-terminal half of the E3L protein is highly conserved among distantly related poxviruses, and E3L from another poxvirus has been cocrystallized with Z-DNA (9), but the functional role of this region has not been well characterized. It has been suggested that the N-terminal domain of E3L is involved in the direct inhibition of protein kinase R (PKR) activation, nuclear localization, and Z-DNA binding (8, 10, 11). A VV mutant lacking E3L induces apoptosis in HeLa cells (12). Furthermore, it has been reported that E3L inhibits dsRNA-induced apoptosis in NIH 3T3 cells and has some oncogenic properties (12). This suggests the possibility that E3L acts by modifying transcription, as do proteins from other DNA viruses (see *Supporting Text*, which is published as supporting information on the PNAS web site).

In this report, we have inquired whether E3L functions as a transcriptional regulator on several genes that are related to apoptosis, the immune response, and viral pathogenesis. We show the nuclear localization of E3L and its Zα domain alone in HeLa cells. We demonstrate the transcriptional activation by E3L of human *IL-6*, *NF-AT*, and *p53* genes, which may be a direct or indirect effect. We also demonstrate the importance of Z-DNA binding in the activity of E3L as a viral transactivator. Finally, we show the key role of Z-DNA binding in the inhibition of hygromycin-B-induced apoptosis by E3L in HeLa cells. Taken together, these experiments illustrate the biological functions of Z-DNA binding of E3L in HeLa cells.

Materials and Methods

For details, see *Supporting Materials and Methods* in *Supporting Text*.

Cell Culture, Cell Viability Assay, Plasmids, and Transfections. HeLa cells were maintained in DMEM supplemented with 10% FBS, and cell viability was determined by trypan blue exclusion. All plasmids were cloned into the pCMS-EGFP vector (Clontech). The pIL-6-Luc plasmid was constructed by inserting eight repeats of the IL-6-response-element consensus sequence into the pLuc-MCS plasmid (Stratagene). All constructs were sequenced. Cells were transfected at 70–80% confluence by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions for 24 h.

Luciferase Reporter Assays. Reporter assays used the PathDetect cis-reporting system (Stratagene) as recommended by the manufacturer's instructions. Briefly, the cells, in six-well plates, were transfected with a total of 3.5 μg of reporter (1 μg), effector (2 μg), and internal control pRL-null plasmid (0.5 μg, Promega) DNAs for 24 h. Luciferase activity was measured in a TD-20/20 luminometer with the Dual-Luciferase reporter assay system (Promega).

RT-PCR Analysis. Total RNA was isolated by using the RNeasy Mini kit (Qiagen) and then reverse transcribed by using the AccessQuick RT-PCR System (Promega) according to the manufacturer's instructions, with specific primers. The β-actin was used as an endogenous reference.

Direct Immunofluorescence Microscopy. Cells were fixed, permeabilized, and incubated with Anti-FLAG M2 monoclonal antibody-Cy3 conjugate (Sigma) according to the manufacturer's protocols. Cells were visualized by using a Nikon fluorescence microscope, and representative images were taken with a Spot 4.3 digital camera.

Apoptosis Assay. Cells were treated with hygromycin B (Invitrogen), fixed by 3% paraformaldehyde, and stained with Hoechst

Abbreviations: NF-AT, nuclear factor of activated T cells; VV, vaccinia virus; Zα, Z-DNA-binding-protein domain.

*To whom correspondence may be addressed. Fax: 617-253-8699 (A.R.) or E-mail: jakwon35@mit.edu (J.-A.K.).

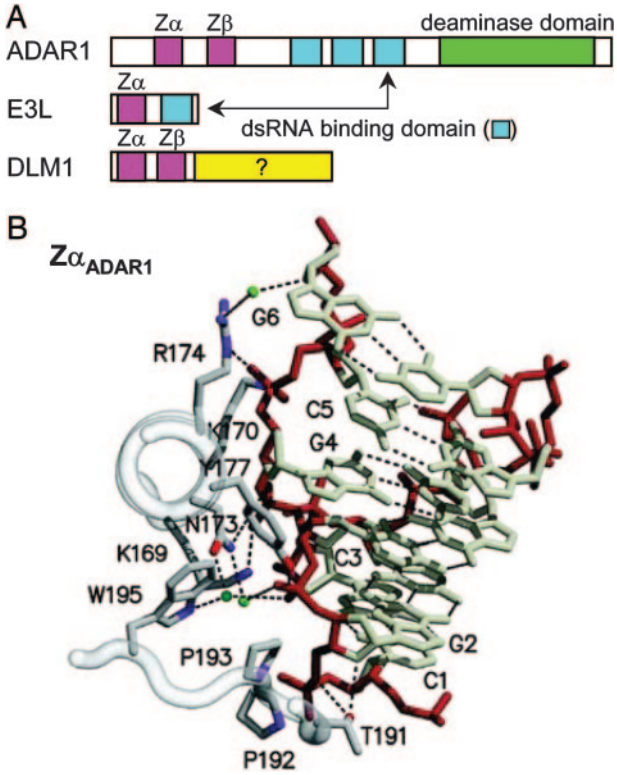

Fig. 1. Schematic representation of three known Z-DNA-binding protein families and the crystal structure of the Zα domain of the ADAR1 (Zα_{ADAR1}) bound to Z-DNA. (*A*) Three different families of proteins are represented, with domains shown as colored boxes. The Z-DNA related domains (Zα and Zβ) are represented in pink, dsRNA-binding domains in blue, and the catalytic deaminase domain of ADAR1 in green. The C-terminal domain of DLM1 (yellow) has no known function or homology. (*B*) Protein–DNA interaction in the Zα_{ADAR1}–Z-DNA complex. A portion of Zα_{ADAR1} is shown complexed to a fragment of Z-DNA, as demonstrated in the structure of the cocrystal (7). The view is down the recognition helix of the protein (on the left), and a number of amino acids are indicated that interact with left-handed Z-DNA, stabilized by electrostatic and van der Waals interactions. The Z-DNA backbone is red, and water molecules are green. Tyr 177 has an edge-to-face van der Waals interaction with the purine base guanine 4 (G4), characteristic of Z-DNA. There are van der Waals interactions between prolines 192 and 193, and the Z-DNA backbone.

33258. Samples were examined with phase-contrast or fluorescence microscopy (Nikon). The percentage of apoptotic cells was calculated as the number of apoptotic cells per number of total cells (expressing EGFP) $\times$ 100%.

DNA Fragmentation Assay. DNA fragmentation was analyzed and quantified by the diphenylamine assay. The percent fragmentation was calculated as the ratio of DNA in the supernatants to the total DNA.

Results and Discussion

Nuclear Localization of E3L and Its Z-DNA-Binding Domain in HeLa Cells. To investigate subcellular localization of E3L, we used confocal immunofluorescence microscopy with a polyclonal anti-E3L antibody (data not shown) and Western blotting with nuclear and cytoplasmic extracts from HeLa cells (see Fig. 6, which is published as supporting information on the PNAS web site, and *Supporting Text*). The results were consistent with a previous report (3). Experiments testing subcellular localization of C-terminal FLAG-tagged E3L and its deletion derivatives by direct immunofluorescence microscopy with an anti-FLAG M2 monoclonal antibody-Cy3 conjugate (Fig. 2) also confirmed E3L

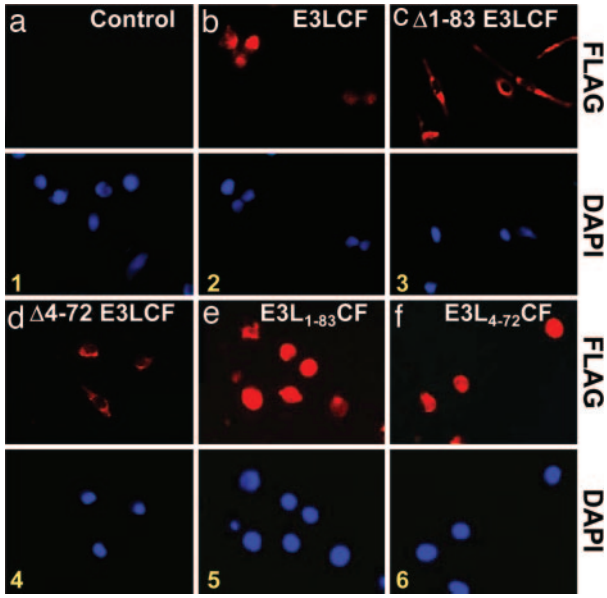

Fig. 2. Localization of E3L and its Z-DNA-binding domain in HeLa cells. The N-terminal Z-DNA binding residues (4–72) are necessary and sufficient for nuclear accumulation. Subcellular localization of C-terminal FLAG-tagged proteins (CF) was shown by direct immunofluorescence microscopy using a monoclonal Cy3-conjugated anti-FLAG antibody (*a–f*, labeled as FLAG, red). The same slides were stained with DAPI (*1–6*, blue) for nuclear counterstaining. Nuclear accumulation of E3L in HeLa cells is also shown by confocal immunofluorescence microscopy using polyclonal anti-E3L antibody as a primary antiboby and Cy3-conjugated secondary antibody (data not shown).

localization in both the cytoplasm and nucleus of transfected HeLa cells and nuclear accumulation of E3L (Fig. 2*b*). Furthermore, the Z-DNA-binding domain by itself (Fig. 2*f*) showed clear nuclear localization in transfected HeLa cells.

Transactivation Activity of E3L on Human *IL-6*, *NF-AT*, and *p53* Genes, and the Importance of the Z-DNA-Binding Domain. Although E3L accumulates in the nucleus, consistent with previous reports (3, 11), its function there is unknown. It has been demonstrated that the E3L N-terminal domain binding to Z-DNA is necessary for pathogenicity in mice (8). Z-DNA-forming sequences are found near the transcription start site of many human genes (14), and negative supercoiling generated behind a moving polymerase during transcription (15) stabilizes Z-DNA formation. Z-DNA formation has been detected in the promoter region of actively transcribed genes in mammalian cells (16). It has been demonstrated that forming Z-DNA near the promoter of the colony-stimulating factor 1 gene stimulates transcription, probably through maintaining a nucleosome-free region (17). This finding suggests the possibility that the N terminus of Z-DNA-binding proteins, such as E3L, may stabilize Z-DNA in the promoter region in some genes, possibly resulting in transactivation of the gene.

To analyze whether E3L can function as a viral transcriptional regulator and to understand its regulatory function in VV pathogenesis, a standard transient luciferase assay for transcriptional regulation was used. HeLa cells, which are highly susceptible to VV infection, were transiently cotransfected with an E3L expression plasmid or an empty vector along with a firefly luciferase reporter plasmid. Each reporter plasmid we used contains repeats of the cis-acting DNA element (usually an enhancer) of a well defined transcription factor, such as IL-6, NF-AT, p53, NF-κB, Ap-1, and the cAMP response element (CRE)-binding protein upstream of the TATA box. As shown in Fig. 3*A*, expression of E3L strongly increased the activity of

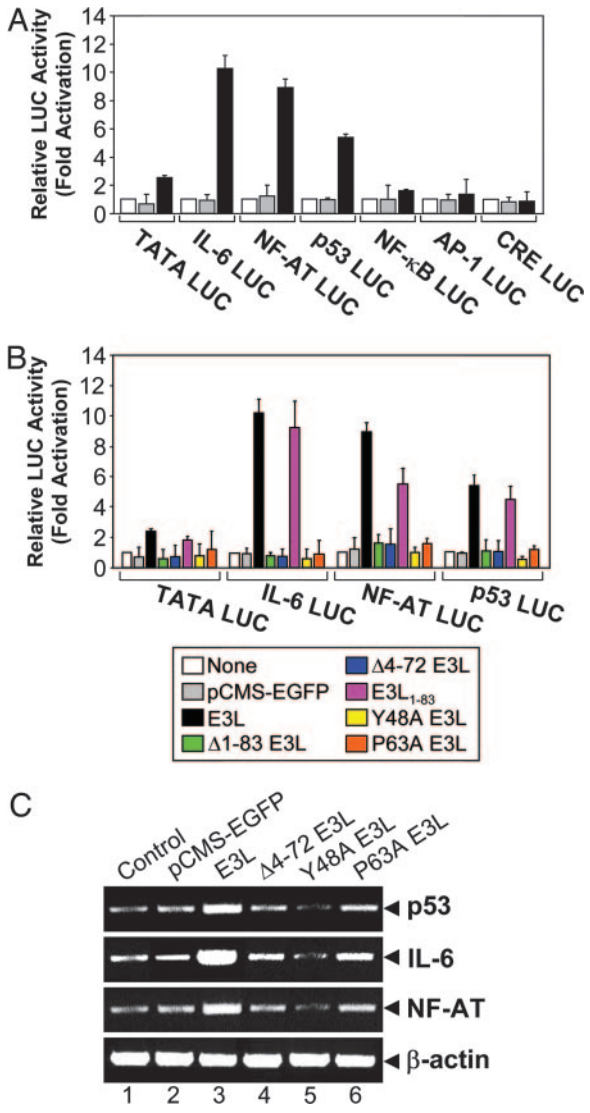

Fig. 3. Transactivation of human *IL-6*, *NF-AT*, and *p53* genes by E3L and functional involvement of E3L Z-DNA binding. A transient transfection assay was used with cis-acting DNA elements with a luciferase reporter gene. (*A*) Effect of E3L on the activity of cis-acting DNA enhancer elements. HeLa cells were cotransfected with 1 μg each of the reporter plasmids and 2 μg of either an E3L expression plasmid (pCMS-E3L) or empty plasmid (pCMS-EGFP). The reporter plasmids pLuc-MCS, pIL-6-Luc, pNF-AT-Luc, p53-Luc, pNF-κB-Luc, pAp-1-Luc, and pCRE-Luc were labeled as TATA LUC, IL-6 LUC, NF-AT LUC, p53 LUC, NF-κB LUC, AP-1 LUC, and CRE LUC, respectively. (*B*) The role of Z-DNA binding of E3L in transactivation of the human *IL-6*, *NF-AT*, and *p53* genes. Typically, 1 μg of each reporter was cotransfected into HeLa cells with each 2 μg of effectors, as indicated. To demonstrate specificity of reporter-gene expression, positive and negative control experiments were performed (data not shown). The luciferase activities are represented as fold activation over the expression of each reporter plasmid, in the absence of any effectors (None). Results are the mean ± SD of at least three independent experiments. (*C*) The role of E3L as a transactivator on the human *IL-6*, *NF-AT*, and *p53* genes in HeLa cells and the importance of its N-terminal Z-DNA binding. HeLa cells were transfected with each 1.5 μg of expression plasmids or empty vector as indicated. After 24 h, total RNA was extracted and subjected to RT-PCR to analyze the expression of human IL-6, NF-AT, and p53 mRNA. Total RNA from nontransfected HeLa cells was used as a control. β-actin was used as an internal control in the same PCR reaction. The same results were found in three independent experiments.

pIL-6-Luc, pNF-AT-Luc, and p53-Luc ≈10-fold, 9-fold, and 5.5-fold, respectively, implying that E3L transactivates human IL-6, NF-AT, or p53 transcription in HeLa cells. Dose-dependent transactivation by E3L on these reporter genes with increasing expression of E3L was also demonstrated (see Fig. 7, which is published as supporting information on the PNAS web site). Interestingly, the activity of the parental plasmid, pLuc-MCS, containing only the TATA box upstream of the luciferase gene, was increased ≈2.5-fold by E3L, suggesting some activation effect of E3L on the basic promoter element (TATA box). However, there was no significant effect in the other reporter plasmids, pNF-κB-Luc, pAp-1-Luc, and pCRE-Luc (Fig. 3*A*). Also, E3L has no effect on the negative control plasmid, pCIS-CK containing only the luciferase reporter gene without the TATA box or any cis-acting DNA elements (data not shown). These results reveal the ability of E3L to activate the human *IL-6*, *NF-AT*, and *p53* genes, implying a broad yet selective up-regulation by E3L, although the underlying mechanism remains to be established.

To identify the role of the N-terminal Z-DNA-binding region of E3L on this transactivation, the N-terminal 83-aa-deleted plasmid, pCMS-Δ1–83 E3L encompassing the Zα domain was cotransfected into HeLa cells with reporter plasmids, as indicated in Fig. 3*B*. When the 83 amino acids are deleted (Δ1–83 E3L), the activation effect of E3L on the plasmids pLuc-MCS, pIL-6-Luc, pNF-AT-Luc, and p53-Luc dramatically disappeared, suggesting the importance of the Z-DNA-binding region.

To delineate further the role of Z-DNA binding, we generated E3L expression constructs containing various truncations, deletions, and point mutations in residues important for Z-DNA binding (see Fig. 8*A*, which is published as supporting information on the PNAS web site and *Supporting Materials and Methods*). As shown in Fig. 3*B*, the activation effect of E3L on these reporter plasmids was also significantly decreased when the N-terminal 4 to 72 aa of E3L containing only the Z-DNA-binding domain were deleted (Δ4–72 E3L). It is interesting that the N-terminal part of E3L containing amino acids 1–83 ($E3L_{1–83}$) also increased the activity of reporter plasmids, although the activation effects are slightly lower than those of wild-type E3L.

The ability to recognize the Z-DNA conformation is essential for E3L function (8), and mutations in chimeric and wild-type E3L of VV that decrease Z-DNA binding were found to have decreased viral pathogenicity (8). When Pro 63 of wild-type E3L [analogous to Pro 192 of $Z\alpha_{ADAR1}$ (Fig. 1*B*), essential for Z-DNA binding] is mutated to Ala (P63A E3L), the transactivation activity was markedly reduced. Likewise, Tyr 177 of $Z\alpha_{ADAR1}$ forms an important interaction in Z-DNA binding, and mutating it to Ala strongly decreases Z-DNA binding (Fig. 1*B*) (8). When the analogous Tyr 48 of E3L is mutated to Ala (Y48A E3L) due to loss of an important van der Waals contact and a hydrogen bond, the activation activity of E3L is strongly decreased. These mutation results clearly suggest that Z-DNA binding of E3L is important in the transactivation of IL-6-, NF-AT-, and p53-mediated transcription.

Next, to clarify the effect of E3L and its Z-DNA binding region on the transcription of human *IL-6*, *NF-AT*, and *p53* genes, the levels of mRNA for those genes were measured by RT-PCR. In the RT-PCR assay, several E3L-related constructs were used that contain either a deletion of the Z-DNA-binding domain or point mutations in residues important for Z-DNA binding. The data shown in Fig. 3*C* indicate that the levels of the human *p53*, IL-6, and NF-AT mRNA expression in the E3L-transfected HeLa cells (lane 3) were markedly increased, compared with those of the control HeLa cells (lane 1) and the empty vector-transfected HeLa cells (lane 2). However, when the N-terminal residues 4–72 of E3L corresponding to the Z-DNA-binding domain were deleted, the increased expression disappeared (lane 4), as it did when the N-terminal 83 residues of E3L were deleted (data not shown). These results indicate that expressing E3L in the cell activates the transcription of human IL-6, NF-AT, and p53, and the Z-DNA-binding domain of E3L is necessary for

the transactivation. Experiments were also carried out with constructs containing point mutations in the residues important for Z-DNA binding. By using the mutants described in Fig. 3*B*, changing Pro 63 of E3L to Ala (P63A) repressed the transactivation activity of E3L on transcription of those genes (lane 6). Likewise, a mutation of Tyr 48 to Ala (Y48A) repressed the expression levels of human IL-6, NF-AT, and p53 mRNA (lane 5) even more, when compared with those of the control HeLa cells (lane 1). The results in Fig. 3*C* clearly indicate that E3L activates the transcription of the human *IL-6*, *NF-AT*, and *p53* genes, and this effect depends on N-terminal Z-DNA binding, which provides strong support for the functional involvement of E3L binding to Z-DNA in its transactivation activity, even though the underlying molecular mechanisms remain to be demonstrated.

Hygromycin B Induces Apoptosis of HeLa Cells in a Dose- and Time-Dependent Manner. Apoptosis is one of the important host defense mechanisms, and its inhibition is thought to be a major mechanism in viral persistence, allowing the virus to replicate in the cell. Such inhibition is also an important step toward malignant transformation. Many viruses are known to possess apoptosis-inhibiting proteins, such as adenovirus E1B, human papillomavirus E6, cowpox virus CrmA, Epstein–Barr virus BHRFI and LMP1, hepatisis B virus X protein, and hepatitis C virus core protein (18). A VV mutant lacking E3L induced apoptosis in HeLa cells (12), whereas E3L expression in NIH 3T3 cells conferred resistance to dsRNA-triggered apoptosis and displayed oncogenic properties (13).

Hygromycin B is an aminoglycoside antibiotic widely used to establish mammalian cell lines carrying a bacterial gene conferring resistance to the drug. It induces apoptosis in Chinese hamster ovary LR73 cells, and that apoptotic response to the drug does not require a functional p53 protein (19). Although p53-induced growth arrest and apoptosis are well known cellular responses to DNA damage, it is also known that apoptotic pathways can be induced that are independent of p53, both during normal developmental programs (20) and in response to a number of genotoxic agents (21). Hygromycin B belongs to this p53-independent category. Adenovirus E1B 19k protein has been shown to inhibit apoptosis induced by hygromycin B (19). It has also been reported that the E6 protein of oncogenic human papillomavirus types prevents apoptosis induced by both p53-dependent and p53-independent pathways (22).

To determine whether hygromycin B induces apoptosis in HeLa cells, we first examined cell viability after treatment with or without hygromycin B by trypan blue dye exclusion. As expected, hygromycin B induced cell death in a dose- and time-dependent manner in HeLa cells (see Fig. 9, which is published as supporting information on the PNAS web site). Cell viability was diminished by 50% at 12 h after treatment with 800 μg/ml hygromycin B, and most of the cells died within 24 h. To confirm whether the loss of cell viability was due to the induction of apoptosis, cell morphologies after hygromycin B treatment were analyzed by phase-contrast microscope at 12, 18, 24, and 48 h. HeLa cells displayed the hallmark characteristics of apoptosis, including membrane blebbing, nuclear condensation, and retraction of cellular processes, after the addition of hygromycin B (data not shown). In addition, the nuclear morphological changes of apoptotic cells were observed with Hoechst 33258 staining by using fluorescent microscopy. As shown in Fig. 4*A*, in HeLa cells, marked blebbing nuclei and granular apoptotic bodies were observed at 18 h after hygromycin B (800 μg/ml) incubation. The apoptotic effect of hygromycin B on HeLa cells was also confirmed by analysis of DNA fragmentation using gel electrophoresis (data not shown). Furthermore, typical DNA fragmentation was observed in hygromycin B-treated HeLa cells in a dose- and time-dependent manner (Fig. 4*B*).

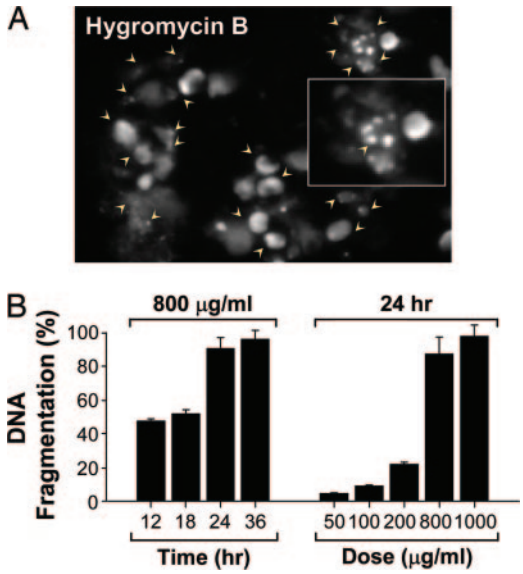

Fig. 4. Hygromycin-B-induced apoptosis of HeLa cells. (*A*) Hygromycin-B (800 μg/ml)-induced morphological changes of cell nuclei in HeLa cells shown after 18-h incubation. Staining of nuclear chromatin with Hoechst 33258 was used for identification of morphologic changes by fluorescence microscopy. (*Inset*) A representative of a nucleus scored for apoptosis (on the left), as compared with a normal nucleus (on the right). Arrowheads indicate parts of apoptotic nuclei. (*B*) Internucleosomal DNA fragmentation was investigated in a time- and dose-dependent manner with hygromycin B treatment in HeLa cells. The percent DNA fragmentation per total cellular DNA was determined by the diphenylamine assay. Results are the mean ± SD of at least three independent experiments.

E3L Inhibits Hygromycin-B-Induced Apoptosis of HeLa Cells by Z-DNA Binding. To investigate the effect of E3L on hygromycin-B-induced apoptosis of HeLa cells, we examined cell viability of transiently transfected HeLa cells with an E3L expression plasmid or an empty vector in response to hygromycin B by trypan blue staining. As shown in Fig. 5*A*, E3L-transfected HeLa cells were significantly protected from hygromycin-B-induced apoptosis, compared with empty-vector transfected or nontransfected control HeLa cells, in a dose- and time-dependent manner. The antiapoptotic effect of E3L on hygromycin-B-induced apoptosis was also confirmed by studying the morphology under phase-contrast microscopy and DNA-fragmentation analysis using gel electrophoresis (data not shown). We also observed that stable E3L-expressing HeLa cells inhibit hygromycin-B-induced apoptosis (data not shown). Furthermore, the inhibition effect of E3L on hygromycin-B-induced apoptosis disappeared when the 83 N-terminal amino acids of E3L, encompassing the Zα motif, were deleted (Fig. 5*A*, Δ1–83 E3L), suggesting the involvement of the Z-DNA-binding region of E3L in the antiapoptotic activity in HeLa cells. Similar results were obtained with stable transfected cells (data not shown).

To elucidate the importance of Z-DNA binding of E3L in the inhibition of hygromycin-B-induced apoptosis, we constructed several E3L-related plasmids, containing different deletions in the N-terminal or the C-terminal region, and with point mutations in the N-terminal Z-DNA-binding domain (see Fig. 8). After transient transfection with wild-type E3L or its deletion and mutation derivatives for 24 h, HeLa cells were incubated with hygromycin B (800 μg/ml) for 24 h, and apoptotic cells were then determined by staining with Hoechst 33258 under the fluorescence microscope. EGFP-positive cells were examined morphologically to assess apoptosis. As shown in Fig. 5*B*, the N-terminal deletion mutants Δ1–83 E3L and Δ4–72 E3L, which are lacking the entire N-terminal 83 amino acids of E3L or only

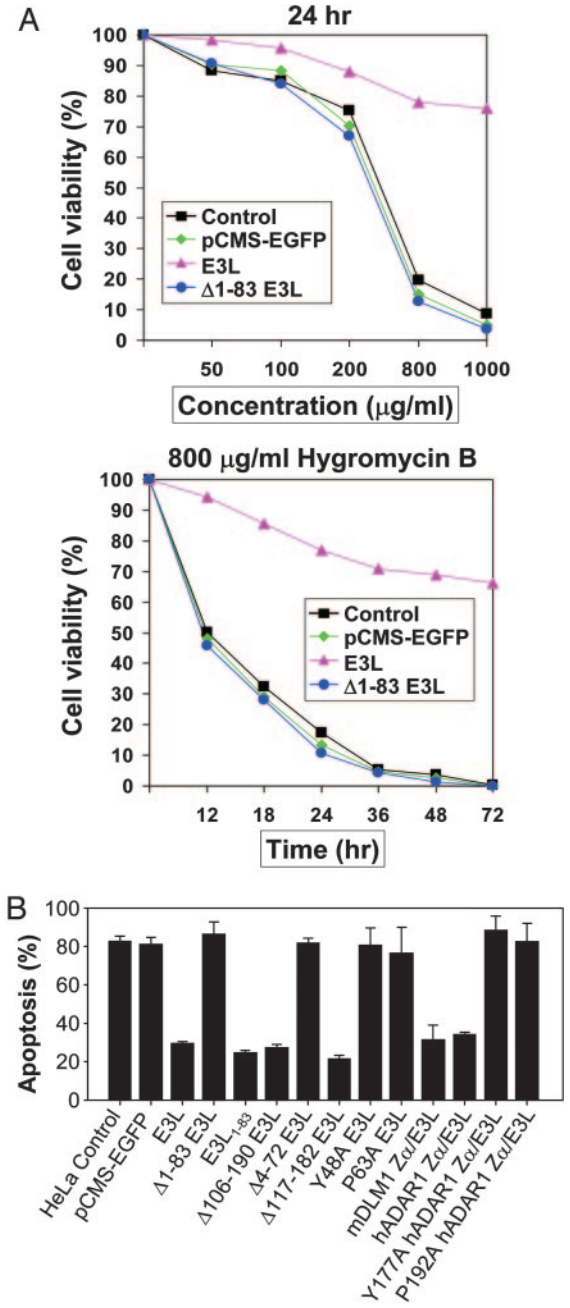

Fig. 5. Role of Z-DNA binding in the inhibition activity of E3L on HeLa cell apoptosis induced by hygromycin B. (*A*) Effect of E3L and its N-terminal Z-DNA-binding region on hygromycin-B-induced apoptosis of HeLa cells. HeLa cells were transiently transfected with each plasmid (1.5 μg), as indicated, for 24 h, and then hygromycin B was added, as indicated. Cell viability was checked by trypan blue staining. Apoptotic cells were also identified by the rounded-up morphology under microscope (data not shown). Similar results were obtained in the stably transfected HeLa cells (data not shown). (*B*) Role of Z-DNA binding in the inhibition of hygromycin-B-induced apoptosis in HeLa cells. HeLa cells grown in six-well culture plates were transiently transfected with 1.5 μg of each expression plasmid, as indicated. After 24-h transfection, hygromycin B (800 μg/ml) was added for 24 h. Apoptosis analysis was carried out by DNA staining with Hoechst 33258. Each percentage scoring for apoptotic nuclei was established by counting ≈500 cells for each condition. The data are mean ± SD from three independent experiments.

the Z-DNA-binding domain, respectively, abolished the antiapoptotic activity of E3L in hygromycin-B-induced apoptosis of HeLa cells. Furthermore, the point mutations known to block Z-DNA binding Y48A E3L and P63A E3L (8) failed to prevent hygromycin-B-induced apoptosis of HeLa cells, strongly indicating a functional role of Z-DNA binding of E3L in its apoptosis-inhibition activity. In contrast, the C-terminal deletion mutants Δ106–190 E3L or Δ117–182 E3L, which lack the entire C-terminal of E3L or only the dsRNA-binding domain, respectively, still showed as active an antiapoptotic effect as did the wild-type E3L. Also, the N-terminal half of E3L, containing the Z-DNA-binding domain ($E3L_{1–83}$) by itself, significantly suppressed the apoptosis of HeLa cells induced by hygromycin B, strongly suggesting Zα involvement in the antiapoptotic activity.

The N-terminal Z-DNA-binding domain of E3L (Z_{E3L}) has sequence similarities to $Z\alpha_{ADAR1}$ and $Z\alpha_{DLM1}$ (See Figs. 1*A* and 9*B*). Both have been cocrystallized with Z-DNA (5, 7), and both are IFN-inducible. Previously, it was reported that a chimeric VV in which the N-terminal domain of E3L is replaced with $hZ\alpha_{ADAR1}$ or $mZ\alpha_{DLM1}$ is as pathogenic as the wild-type virus (8). To further explore our finding that antiapoptotic activity is associated with Z-DNA binding, domain swap constructs were created in which either of the two known Z-DNA-binding domains ($hZ\alpha_{ADAR1}$ and $mZ\alpha_{DLM1}$) were substituted for the Zα domain of E3L (see Fig. 8*A*). Fig. 5*B* reveals that these chimeric proteins (hADAR1 Zα/E3L and mDLM1 Zα/E3L) prevented the hygromycin-B-induced apoptosis as well as wild-type E3L. Additionally, mutations in the hADAR1 Zα/E3L (8) that decrease Z-DNA binding (Y177A hADAR1 Zα/E3L and P192A hADAR1 Zα/E3L, containing a mutated Tyr 177 or Pro 192 of $hZ\alpha_{ADAR1}$ to Ala, respectively) strongly suppressed the antiapoptotic ability of the chimeric protein. These results support the correlation between Z-DNA binding and antiapoptosis. Apoptosis of cells was further confirmed by counting the rounded or cleaved cells without fixation, among the cells expressing EGFP, by using a fluorescence microscope to assay apoptosis in transfected cells. The results were similar to those shown in Fig. 5*B* (data not shown). In addition, DNA fragmentation of apoptotic cells was quantitated by the diphenylamine assay (see Table 1, which is published as supporting information on the PNAS web site), yielding results similar to those shown in Fig. 5*B*. Taken together, these results clearly demonstrate that E3L acts as a viral transactivator, inhibiting the hygromycin-B-induced apoptosis of HeLa cells. Z-DNA binding appears to be essential for these activities, although the underlying mechanism remains to be elucidated.

An important point in the apoptosis pathway, downstream of the initial signaling, is the judgement phase, which forms a second major controlling point, either ameliorating or reinforcing upstream signals before irrevocable entry into the apoptotic process (23, 24). The adenovirus E1B 19k protein inhibits apoptosis at this point in the apoptotic pathway by interacting with several proapoptotic proteins belonging to the bcl-2 family, including Bak and Bax (25). In this regard, the adenovirus E1B 19k protein has been shown to be functionally equivalent to the cellular antiapoptotic proteins Bcl-2 and Bcl-XL (26). Also, human papillomavirus E6 proteins that are frequently found in the nucleus inhibit Bak-induced apoptosis in a p53-independent manner (22).

In this study, we have shown that E3L expression of VV (Western Reserve strain) in HeLa cells transactivates the *p53* and *IL-6* genes and also inhibits apoptosis induced by hygromycin B, which does not require expression of wild-type p53, suggesting a p53-independent antiapoptotic activity in E3L. Mounting evidence indicates that the multifunctional cytokine IL-6 can act as an apoptosis inhibitor. For example, the antiapoptotic role of IL-6 in human cervical cancer cells was recently reported (27). Furthemore, it has been shown that IL-6 inhibition of p53-induced apoptosis is not mediated by a general inhibition of the transcription-regulation activity of p53 (28), and it did not degrade the p53 protein. Also, IL-6 did not affect the

p53-induced up-regulation of various proapoptotic genes implicated in the ability of p53 to induce apoptosis. Instead, IL-6 up-regulates certain antiapoptotic genes in a p53-independent manner (28). This up-regulation of antiapoptotic genes changes the intracellular balance between expression of proapoptotic and antiapoptotic genes and can explain the ability of these components to inhibit p53-mediated apoptosis. IL-6 might bypass the effect of wild-type p53 on gene expression and inhibit apoptosis, which would also facilitate tumor development. These reports may thus provide an explanation for the p53-independent antiapoptotic activity of the VV *E3L* gene.

Conclusion

In this article, we have shown that E3L accumulated in the nucleus of HeLa cells, and the Z-DNA-binding domain of E3L by itself localizes in the nucleus. We have also demonstrated that E3L activates transcription of the *IL-6*, *NF-AT*, and *p53* genes as well as increasing the mRNA levels of these genes. These results show that E3L can function as a transactivator and that Z-DNA binding of E3L is necessary for this transactivation activity. Hygromycin B, a p53-independent apoptosis inducer, is shown here to induce apoptotic programmed cell death in HeLa cells. We then found that E3L inhibits the hygromycin-B-induced apoptosis of HeLa cells and that Z-DNA binding of E3L is essential for this inhibition effect. The E3L Zα domain swaps confirmed the role of Z-DNA binding in hygromycin-B-induced apoptosis inhibition. Altogether, these results suggest that the observed transactivating effect of E3L, and others not observed, is probably involved in apoptosis inhibition. The Z-DNA-binding activity of the E3L protein may play an important role in VV pathogenesis when E3L acts as a viral transactivator and inhibits apoptosis of host cells, as seen in other oncogenic viral proteins. We have demonstrated the central role of Z-DNA-binding in these regulatory activities.

We do not know the underlying mechanisms for these activities. Indeed, the phrase "Z-DNA-binding" protein is used, but we have previously shown that the Zα domain of ADAR1 also binds Z-RNA (29). However, we can make a plausible argument for Z-DNA binding of E3L by citing the work on Z-DNA-facilitated transcriptional activation of the colony-stimulating factor 1 gene (17). There, unwrapping nucleosomes provide negative torsional strain that stabilizes a Z-DNA-forming sequence. Because Z-DNA cannot form nucleosomes (30), it leaves a nucleosome-free region near the transcription start site, allowing transcription factors to be recruited, so that transcription ensues. Because most human genes have Z-DNA-forming sequences near the transcription start site (14), it is possible that E3L binding to such sequences up-regulates transcription. Further experiments are clearly necessary.

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