

The promyelocytic leukemia zinc finger protein down-regulates apoptosis and expression of the proapoptotic BID protein in lymphocytes

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The promyelocytic leukemia zinc finger (*PLZF*) gene, involved in rare cases of acute promyelocytic leukemia, encodes a Krüppel-type zinc finger transcription factor. It has been reported that *PLZF* affects myeloid cell growth, differentiation, and apoptosis. However, the function of *PLZF* in the lymphoid compartment, where *PLZF* is also expressed, remains largely unknown. To investigate a potential relationship between *PLZF* expression in lymphocytes and programmed cell death, an inducible model of stable clones of the lymphoid Jurkat cell line was created by using the tet-off system. Although induction of *PLZF* expression by itself did not produce changes in the basal levels of apoptosis, *PLZF* had a significant anti-apoptotic effect in Jurkat cells cultured in conditions of serum starvation, as measured by annexin V staining and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling. In addition, retarded loss of mitochondrial transmembrane potential was observed in the *PLZF*-expressing clones, suggesting that *PLZF* protects from cell death through a mitochondrial-dependent mechanism. To identify apoptosis-related targets of *PLZF*, a screen for differential expression identified *BID*, a proapoptotic member of the *Bcl2* family, as significantly down-regulated by *PLZF*. Furthermore, a high-affinity *PLZF*-binding site element was identified upstream of the *BID* transcriptional start site, as assessed by electrophoretic mobility-shift assays. These results suggest that *BID* is a target of *PLZF* repression and a candidate gene to mediate the *PLZF*-induced resistance to apoptosis.

The promyelocytic leukemia zinc finger (*PLZF*) gene was identified in a variant of acute promyelocytic leukemia bearing reciprocal chromosomal translocations t(11;17)(q23;q21) whose breakpoints map within the *PLZF* locus at chromosome 11 and the retinoic acid receptor α locus at chromosome 17 (1). As a result of these translocations, chimeric proteins *PLZF*-retinoic acid receptor α and retinoic acid receptor α -*PLZF* are expressed in the malignant cells. Transgenic animal experiments support the hypothesis that both chimeric proteins contribute to the leukemogenic phenotype of acute promyelocytic leukemia (reviewed in refs. 2 and 3).

PLZF encodes a transcription factor containing nine C-terminal Cys₂-His₂ zinc finger motifs and an N-terminal BTB/POZ domain. Although the zinc finger domain binds DNA in a sequence-specific manner, it has not been defined which sequences are recognized by *PLZF* (4, 5). The BTB/POZ domain is an evolutionarily conserved domain that appears to convey transcriptional repressor activity (6). In addition, a central domain of *PLZF* interacts with the nuclear corepressors (N-CoR, SMRT, and mSIN3A), which in turn recruit histone deacetylases (7–10). This interaction provides a link between *PLZF* and an enzymatic activity considered one of the major mechanisms of chromatin remodeling and transcriptional silencing. Thus, both the BTB/POZ domain and the N-CoR interac-

tion domain could contribute to the transcriptional repressor function attributed to *PLZF*.

The *PLZF* protein is localized to distinct nuclear speckles and appears to interact with the promyelocytic leukemia and *BCL6* proteins in large nuclear bodies of unknown function (11, 12). In the human hematopoietic system, *PLZF* is expressed in pluripotent progenitor cells and in peripheral blood T, B, and NK lymphocytes (13, 14). Intriguingly, a significant proportion of B cell chronic lymphocytic leukemia (B-CLL) patients exhibit down-regulation of *PLZF* expression compared with that in normal lymphocytes, and this pattern of expression correlates with a better prognosis (14). Apoptotic functions appear to be suppressed in B-CLL, and progression could be related to increased resistance to apoptosis. A hypothetical role of *PLZF* in apoptosis could link *PLZF* expression with B-CLL prognosis.

In this article, an inducible model of stable clones of the lymphocytic Jurkat cell line, created by using the tet-off transfection plasmids, was exploited to test a potential role of the *PLZF* protein in apoptosis. It was shown that in Jurkat lymphocytes *PLZF* expression increased resistance to apoptosis when cells were stressed by serum starvation. In addition, screening differentially expressed apoptosis-related genes based on the presence or absence of *PLZF* expression identified *BID*, a proapoptotic member of the *Bcl2* family, as a target of *PLZF* transcriptional repression.

Materials and Methods

Generation of Stable Clones with Inducible Expression of *PLZF* by Using the Tet-Off System. The T lymphocytic leukemia Jurkat cell line was grown in RPMI medium 1640 (BioWhittaker) supplemented with 10% heat-inactivated FCS (BioWhittaker), 50 units of penicillin per ml, 50 units of streptomycin per ml, and 2 mM L-glutamine ("complete medium"). Stable Jurkat clones with regulatable *PLZF* expression were generated by using the tet-off system, following a procedure previously described (14–16), and maintained in complete medium with 250 μ g of hygromycin B per ml (Roche) and 1 mg of G418 per ml (Amersham Pharmacia Biosciences) ["complete selection medium" (CSM)], and 10 ng of doxycycline (dox) per ml.

Induction of Apoptosis by Serum Deprivation and Quantitation by Annexin V Labeling. *PLZF*-expressing and nonexpressing clones were washed free of dox, and replicate aliquots were adjusted at

Abbreviations: *PLZF*, promyelocytic leukemia zinc finger; DiOC₆(3), 3,3'-dihexyloxocarbocyanine iodide; EMSA, electrophoretic mobility-shift assay; CSM, complete selection medium; LSM, low-serum medium; $\Delta\Psi_m$, mitochondrial transmembrane potential; dox, doxycycline; TSS, transcriptional start site; B-CLL, B cell chronic lymphocytic leukemia; RARE, retinoic acid response element.

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0.3×10^6 cells per ml in CSM with 10 ng of dox per ml or without dox. After 72 h of culture, cells were washed twice and adjusted at 0.1×10^6 cells per ml in RPMI medium 1640 with the same supplements as the CSM except that FCS was added at 0.1% ["low-serum medium" (LSM)]. dox was added again to the replicates, which had been cultured with dox, and 0.5×10^6 cells of each replicate were taken every 24 h to determine the percentage of apoptotic cells. Cells were dual-labeled with annexin V and propidium iodide (Annexin V-FITC Apoptosis Detection kit I, Pharmingen). Labeled cells were acquired in a FACSCalibur flow cytometer (Becton Dickinson), and analysis of cell populations was performed by using the CELLQUEST package. In certain control experiments, apoptosis was induced with 1 μ g of anti-CD95 Ab per ml (Immunotech, Luminy, France).

Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling. DNA fragmentation that occurs in the cell nucleus as a consequence of the apoptosis process was measured by using the *in situ* cell death detection kit, fluorescein (Roche). Cell populations were acquired and analyzed in the flow cytometer.

Loss of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$). Uptake of the fluorochrome 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] by the mitochondrial matrix decreases as a consequence of the loss of $\Delta\Psi_m$ that occurs as an early event of apoptosis (17). Cells were labeled with DiOC₆(3) (Sigma) and analyzed in the flow cytometer.

Identification of Target Gene Modulation by Semiquantitative RT-PCR. mRNA levels of 41 genes related to apoptosis were examined by a semiquantitative RT-PCR technique. Oligonucleotide primers were designed from the mRNA sequences deposited in the UniGene database (www.ncbi.nlm.nih.gov). These included members of the Bcl2 family (*BCL2*, *BCL2L1*, *BCL2L2*, *MCL1*, *BCL2A1*, *BAG1*, *BAX*, *BAK1*, *BID*, *BAD*, *BIK*, *BCL2L11*, *BNIP3*, and *BNIP3L*), caspases (*CASP2*, *CASP3*, *CASP6*, *CASP7*, *CASP8*, and *CASP9*), programmed cell death (*PDCD1*, *PDCD2*, *PDCD5*, *PDCD6*, and *PDCD8*), transcription factors (*TP53*, *MYC*, *PML*, *BCL6*, *STAT6*, and *TZFP*), and other factors involved in diverse apoptotic pathways (such as *TNFRSF6*, *TNFSF6*, *IL3RA*, *CYCS*, *APAF1*, *ADPRT*, *HSPA1A*, *DIABLO*, *BIRC1*, and *CARD4*). To avoid coamplification of contaminant DNA, primers were mapped to different exons. As a control for equal loading of cDNA, a fragment of β -actin mRNA was coamplified in each reaction. Sequences of the oligonucleotides are available on request. Briefly, 1 μ g of total RNA, obtained by using the SV Total RNA Isolation kit (Promega), was reverse transcribed by using random hexamers and 1 unit of Moloney murine leukemia virus retrotranscriptase (Roche) at 42°C for 50 min. One-tenth of this reaction was subjected to five cycles of PCR using the primers of an apoptosis-related gene and 2.5 units of *Taq* DNA polymerase (Biolone, London). Each PCR cycle consisted of 30 s at 95°C, 1 min at 55°C, and 3 min at 72°C. Then, β -actin primers were added, and 23 additional cycles were performed. Amplified products were fractionated and examined on a UV transilluminator. When necessary, gels were blotted, hybridized with [γ -³²P]ATP-labeled oligonucleotide probes, and autoradiographed.

Northern Blot Analysis. Total RNA (40 μ g) was electrophoresed, blotted, hybridized with an [α -³²P]dCTP-labeled probe, and autoradiographed as described (14). Probes included a 750-bp cDNA *BID* fragment, obtained from the RT-PCR product by using the TOPO TA cloning kit (Invitrogen), a 2.1-kb cDNA *PLZF* fragment (1), and, as a control of equivalent loading, a human glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) probe (Clontech). An estimation of the amounts of *BID* mRNA

was performed by optical densitometry after normalization with *GAPD* mRNA.

Western Blot Analysis. Immunoreactive proteins contained in 10 μ g of whole-cell protein extracts were detected as described (14). A mouse anti-human *PLZF* mAb (Oncogene Research Products), a rabbit anti-human *BID* polyclonal Ab (Pharmingen), and a mouse anti-actin mAb (Santa Cruz Biotechnology) were used as primary Abs. Horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (DAKO) was used as secondary Ab. The amounts of *BID* protein were estimated by optical densitometry after normalization with actin.

Quantitation of Target Gene Modulation by Flow Cytometry. Cells were labeled by using the Perm/Fix kit (Pharmingen) by using anti-*PLZF* or -*BID* primary Abs, and phycoerythrin-conjugated anti-mouse or rabbit IgG (Caltag, South San Francisco, CA) secondary Abs, respectively. Labeled cells were acquired and analyzed in the flow cytometer. Other apoptosis regulators were also analyzed by Western blot and/or flow cytometry and served as controls (anti-human *BCL2*, *BCLX*, *BCLW*, *MCL1*, *BAX*, *BAK*, *BAD*, *BIK*, *BIM*, *BAG1*, *P53*, *CD95*, or *FASL*, purchased from DAKO, Pharmingen, R&D Systems, or Immunotech).

Electrophoretic Mobility-Shift Assays (EMSA). Sequences flanking the transcriptional start site (TSS) of human *BID* (<http://elmo.ims.u-tokyo.ac.jp/dbtss/>) were scanned for homologies with high-affinity *PLZF*-binding sites as described (4, 5). A sequence with significant homology to the *PLZF*-binding site contained within the yeast *LEXA* operator was found \approx 2 kb upstream of the TSS. Nuclear extracts of the Jurkat clones containing 15 μ g of protein were incubated for 20 min with a double-stranded [γ -³²P]ATP-labeled probe made by using the oligonucleotide 5'-ATTTTACATACAGTAAAACCTCACCAT-TCTTCGTGCAGAGT-3', which maps at positions 1984–1945

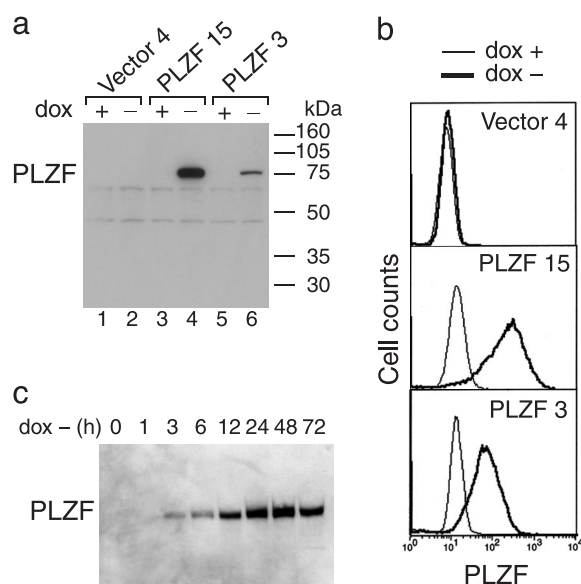


Fig. 1. Analysis of *PLZF* protein expression in the inducible clones. (a) Western blot prepared from whole-cell extracts of human Jurkat cells stably transfected with pJEF4 (clone vector 4) or pJEF4-*PLZF* (clones *PLZF* 3 and 15), obtained 72 h after washing the cells free of dox and reculturing in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of dox, incubated with a *PLZF* mAb. A band at the expected molecular mass of 80 kDa was detected in clones 3 and 15 after dox withdrawal. (b) Flow cytometry analysis of the clones by using the *PLZF* mAb and secondary labeling with anti-mouse IgG coupled to phycoerythrin. (c) Western blot of whole-cell extracts of clone *PLZF* 15 showing the kinetics of induction of *PLZF* expression after dox withdrawal.

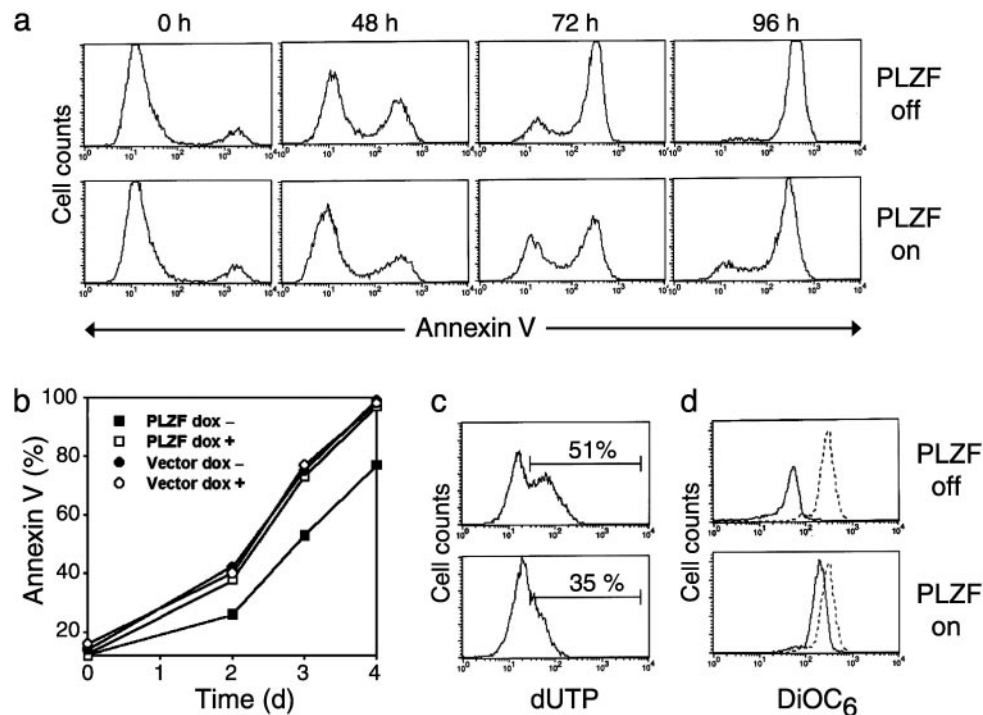


Fig. 2. Flow cytometry analysis of apoptosis of the Jurkat clones induced by serum starvation in the presence (PLZF off) or absence (PLZF on) of dox. (a) Kinetics of annexin V staining of Jurkat clone PLZF 3. (b) Graph comparing the kinetics of annexin V staining of Jurkat clone PLZF 3 shown in a with clone vector 4. (c) Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay of clone PLZF 3 after 72 h of culture in LSM. (d) Loss of $\Delta\Psi_m$ of clone PLZF 3 after 6 h of culture in LSM. The dashed line represents a control culture of clone PLZF 3 grown in CSM.

nt upstream of *BID* TSS, and its complement. A DR5-retinoic acid response element (RARE), 5'-GATCAGGGTTCACCGAAAGTTCACATGCATATATTAG-3', and a p53-binding site element, 5'-CTAGGACATGCCCGGGCATGTCCTAG-3', were used as irrelevant competitors. For supershifts, 0.1 μ g of Abs was added 10 min before the nuclear extracts. Binding reaction products were electrophoresed in 10% polyacrylamide gels for 2 h. Gels were dried and autoradiographed.

Results

Generation of Stable Clones with Inducible Expression of PLZF. To investigate the potential role of PLZF in apoptosis, stable clones of the lymphocytic leukemia Jurkat cell line with inducible expression of PLZF were generated by using the tet-off system. Two of 21 clones selected, PLZF 3 and PLZF 15, expressed PLZF upon dox withdrawal at the expected molecular mass of 80 kDa (Fig. 1a, lanes 4 and 6). dox exerted tight repression of PLZF (Fig. 1a, lanes 3 and 5). WT Jurkat cells do not express PLZF, as measured by Western blotting or a more sensitive RT-PCR assay (data not shown). Consequently, Jurkat clones obtained by transfection with pJEF4 vector did not express PLZF in the presence or absence of dox (Fig. 1a, lanes 1 and 2). After flow cytometry analysis, comparison of the mean levels of fluorescence revealed that clone PLZF 15 exhibited \approx 4-fold higher levels of PLZF expression than clone 3 (Fig. 1b). Maximum levels of PLZF expression were achieved 24 h after washing the cells free of dox and remained stable afterward (Fig. 1c).

Expression of PLZF Delays the Kinetics of Apoptosis Induced by Serum Starvation. Induction of PLZF expression upon dox withdrawal did not produce changes in the basal levels of apoptosis of Jurkat cells cultured in CSM. To study whether PLZF expression could influence the response of the cells to apoptotic stimuli, replicates of the Jurkat clones grown for 72 h in the presence and absence of dox were recultured in LSM. Analysis of cell death induced by

serum deprivation in Jurkat cells showed that annexin V-positive/propidium iodide-negative cells predominate in the earlier stages (24–48 h), and double-positive cells predominate in the later stages (72–96 h), suggesting a transition from single- to double-positive cells (data not shown). Therefore, to establish the kinetics of apoptosis, all annexin V-positive cells were counted as apoptotic cells. Clones PLZF 3 (Fig. 2a) and 15 (not shown) cultured in the absence of dox showed a significant delay in the kinetics of annexin V staining compared with the replicates cultured in the presence of dox. Maximal differences in the values of annexin V-positive cells were observed between 48 and 72 h of culture in LSM. These ranged between 20% and 25% less annexin V-positive cells in the replicates grown in the absence of dox. Control clones transfected with the empty vector did not show differences in the kinetics of apoptosis induced by serum starvation in the presence or absence of dox (Fig. 2b), ruling out that the differences observed in the positive clones might be due to a potential toxic effect of dox. Therefore, PLZF has an antiapoptotic effect in Jurkat cells cultured in conditions of serum starvation.

Protection Promoted by PLZF Involves Decreased Nuclear Fragmentation and Retarded Loss of $\Delta\Psi_m$. As a confirmation of the protective effect of PLZF, the Jurkat clones cultured in LSM were assayed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling. PLZF-expressing clones stressed by serum deprivation in the absence of dox exhibited decreased labeling compared with replicates stressed in the presence of dox (Fig. 2c). These differences were not observed in control clones transfected with empty vector (data not shown). To test the involvement of mitochondria in PLZF down-regulation of apoptosis, loss of $\Delta\Psi_m$ was analyzed by DiOC₆(3) labeling. Replicates of the positive clones cultured with dox showed decreased $\Delta\Psi_m$ after 6 h of serum starvation compared with those cultured without dox (Fig. 2d). Such differences were not observed in the

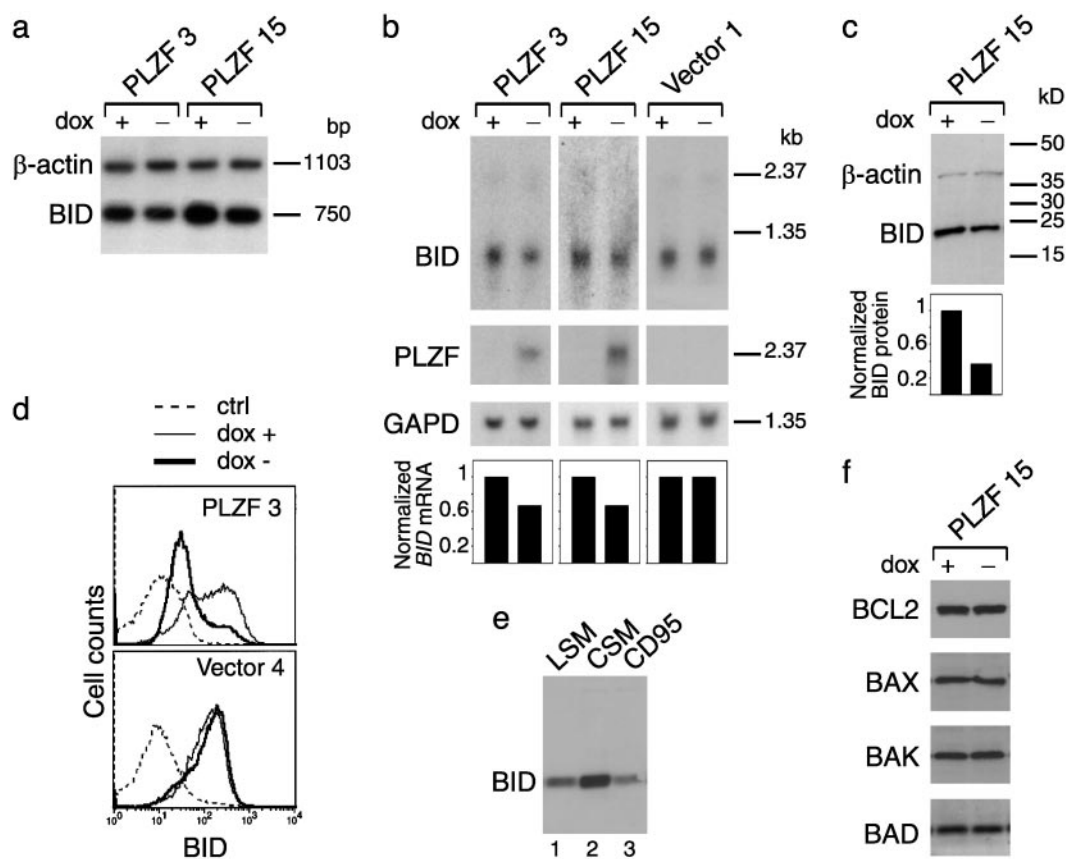


Fig. 3. Analysis of BID mRNA and protein expression in the Jurkat clones after 72 h of culture in the presence (+) or absence (–) of dox. (a) RT-PCR showing *BID* mRNA down-regulation in clones PLZF 3 and 15 after culture in the absence of dox. (b) Northern blot showing *BID* mRNA down-regulation in clones PLZF 3 and 15 but not in control clone vector 1 after culture in the absence of dox. Bar graphs depict estimations of *BID* mRNA amount after normalization with *GAPD*, expressed in relative units, 1 being the value given to the culture in the presence of dox of each clone. (c) Western blot showing BID protein down-regulation in whole-cell extracts of clone PLZF 15 after culture in the absence of dox. The bar graph depicts an estimation of BID protein amount after normalization with actin, expressed in relative units as in b. (d) Flow cytometry analysis showing BID protein down-regulation in clone PLZF 3 after culture in the absence of dox. (e) Western blot showing BID protein cleavage in whole-cell extracts of clone PLZF 15 in the presence of after culture in LSM for 48 h (lane 1) or in CSM with anti-CD95 for 4 h (lane 3) compared with control grown in CSM (lane 2). (f) Western blot showing no changes in BCL2, BAX, BAK, and BAD protein expression in whole-cell extracts of clone PLZF 15 after 72 h of culture in the presence (+) or absence (–) of dox.

control clones transfected with empty vector (data not shown). These data suggest that PLZF expression negatively interferes with serum deprivation-induced apoptosis upstream of the mitochondrial changes associated with this death pathway.

Expression of PLZF Induces Repression of BID, a Proapoptotic Member of the Bcl2 Family of Apoptosis Regulators. To identify apoptosis-related targets of the transcriptional activity of PLZF, a screen for differential expression of apoptosis-related genes was performed by semiquantitative RT-PCR. Among these genes, only *BID*, a proapoptotic member of the Bcl2 family of apoptosis regulators, was identified as significantly down-regulated in Jurkat clones PLZF 3 and 15 when PLZF expression was induced by culture in dox-free medium (Fig. 3a). Northern blotting confirmed *BID* mRNA repression in the PLZF-expressing clones upon culture in the absence of dox, whereas control clones transfected with empty vector in the presence and absence of dox showed equivalent expression (Fig. 3b). Western blotting using a polyclonal anti-human BID Ab detected a band at the expected molecular mass of BID (22 kDa), which was significantly down-regulated (2.7-fold) when PLZF expression was induced (Fig. 3c). Comparison of the mean levels of fluorescence by fluorescence-activated cell sorter analysis also revealed that expression of BID protein was ≈ 2.8 -fold down-regulated during 96 h of culture of clone PLZF 3 in the absence of dox (Fig. 3d).

BID plays an important role in apoptosis induced by diverse stimuli in different cell types, where it is cleaved to produce an active truncated form that translocates from the cytosol to the mitochondria (18–21). Among these stimuli, CD95 (FASR/TNFRSF6) stimulation induces a rapid apoptotic process in Jurkat cells, and BID cleavage contributes to this pathway (22). To establish whether BID may be involved in serum deprivation-induced apoptosis of Jurkat cells, whole-cell extracts of serum-deprived Jurkat cells were compared with nondeprived or anti-CD95 Ab-stimulated Jurkat cells by Western blot analysis. Dramatic decreases of full-length BID were observed after culture in LSM for 48 h or with anti-CD95 for 4 h (Fig. 3e, lanes 1 and 3, respectively) compared with culture in CSM (Fig. 3e, lane 2). These results suggest that, as in CD95-induced apoptosis, BID is cleaved in serum deprivation-induced apoptosis. Furthermore, in concordance with RT-PCR data, no regulation of other apoptosis regulators was observed in the PLZF-expressing or nonexpressing Jurkat clones cultured in the presence or absence of dox by Western blotting (Fig. 3f) and/or flow cytometry (data not shown). These results indicate that BID is a specific target of PLZF transcriptional repression and a candidate protein to mediate the increased resistance to serum deprivation-induced apoptosis conferred to Jurkat cells by the expression of PLZF.

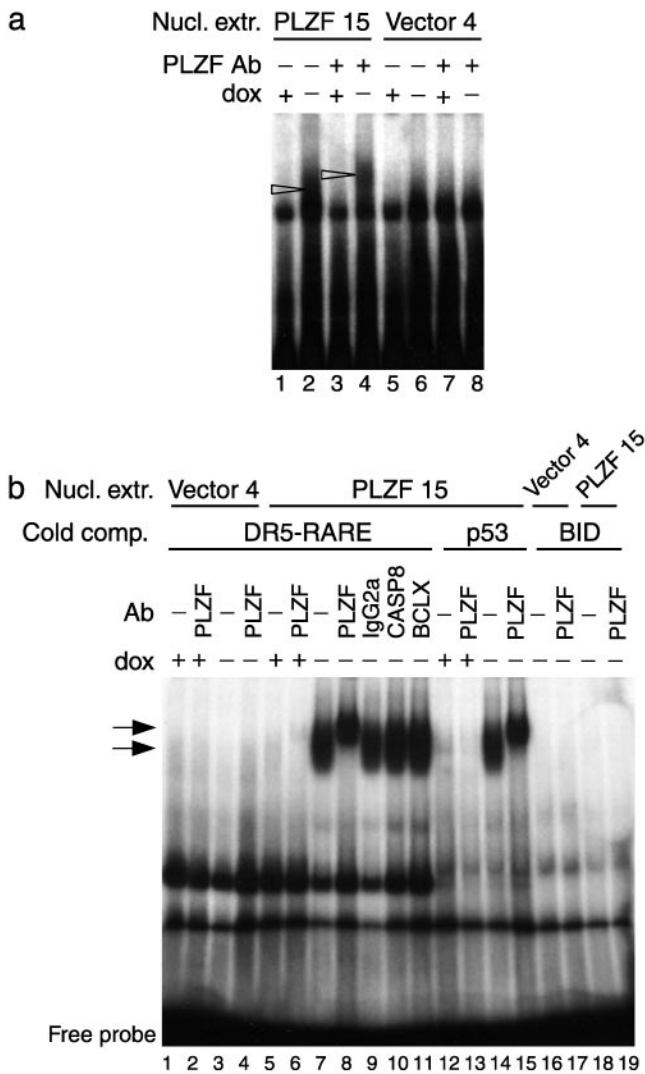


Fig. 4. EMSA analysis using a sequence contained upstream of the *BID* TSS and nuclear extracts (Nucl. extr.) of the Jurkat clones PLZF 15 and vector 4. (a) EMSA using the BID radiolabeled probe without competitors. Arrowheads denote the presence of a retarded complex observed when nuclear extracts of clone PLZF 15 cultured in the absence of dox were used (lane 2) and the shifted complex when a PLZF mAb was used (lane 4). (b) EMSA using BID-radiolabeled probe with a 1,000-fold molar excess of unlabeled competitors (a DR5-RARE, lanes 1–11; a p53-binding site probe, lanes 12–15; or the specific unlabeled probe, lanes 16–19). Arrows denote the presence of a retarded complex observed when nuclear extracts of clone PLZF 15 cultured in the absence of dox were used (lanes 7, 9–11, and 14), and the shifted complex when a PLZF mAb was used (lanes 8 and 15). Irrelevant Abs failed to produce a shift (lanes 9–11). The complex was detected in the presence of a 1,000-fold molar excess of the irrelevant unlabeled competitors (lanes 7–11, 14, and 15), whereas it disappeared in the presence of the specific unlabeled probe (lanes 18 and 19).

PLZF Is Found in a Complex That Binds a Specific Sequence Within the *BID* Promoter. To investigate the mechanism of repression, the *BID* promoter was examined. A sequence that maps ≈ 2 kb upstream of the human *BID* TSS, significantly homologous to the PLZF-binding site contained in the yeast *LEXA* operator, was tested by EMSA. A retarded complex was detected when nuclear extracts of clone PLZF 15 cultured in the absence of dox were incubated with the labeled probe (Fig. 4a, lane 2). The complex was shifted with a PLZF mAb (Fig. 4a, lane 4) and was not observed in the presence of dox (Fig. 4a, lanes 1 and 3) or when extracts of clone vector 4 were used (Fig. 4a, lanes 5–8). The

specificity of PLZF binding to this sequence was shown in competition EMSA (Fig. 4b). The retarded complex was detected in the presence of a 1,000-fold molar excess of unlabeled competitors: a DR5-RARE (Fig. 4b, lanes 7 and 9–11) or a p53-binding site probe (Fig. 4b, lane 14), whereas it disappeared in the presence of the specific competitor (Fig. 4b, lanes 18 and 19). This complex was not observed when the clone was cultured in the presence of dox (Fig. 4b, lanes 5, 6, 12, and 13) or when nuclear extracts of clone vector 4 were used (Fig. 4b, lanes 1–4). The complex was shifted with a PLZF mAb (Fig. 4b, lanes 8 and 15) but not with irrelevant isotype Abs (Fig. 4b, lanes 9–11). These findings indicate that the PLZF protein could target *BID* for transcriptional repression through binding to specific sequences of its promoter.

Discussion

PLZF, like other members of a protein family characterized by a structure that consists of an N-terminal BTB/POZ domain and C-terminal Cys₂-His₂ zinc finger domains, e.g., BCL6 and TZFP (23, 24), acts as a transcriptional repressor (4, 25). It is likely that PLZF binds specific DNA sequences within the regulatory regions of its target genes through its zinc finger domain and recruits histone deacetylase through its interaction with nuclear corepressors, e.g., N-CoR, SMRT, and mSIN3A (7–10, 26). Histone deacetylase enzymatic activity would render the regulatory regions of PLZF target genes transcriptionally inactive. Other nuclear factors that interact with PLZF, such as ETO (25), might participate in these multiprotein complexes and cooperate with PLZF to repress transcription.

Shaknovich *et al.* (27), by means of retrovirally induced overexpression of PLZF in the murine IL-3-dependent hematopoietic progenitor 32D cell line, concluded that PLZF may be an important regulator of myeloid cell growth, differentiation, and apoptosis. However, the role of PLZF in apoptosis was reported to be paradoxical. Thus, when 32D cells were grown in the presence of IL-3, a moderate proapoptotic effect of PLZF was exhibited. In striking contrast, a strong antiapoptotic effect of PLZF was observed when 32D cells were grown in the absence of IL-3. In B-CLL, the observation that decreased levels of PLZF expression are associated with a better prognosis suggested the possibility that PLZF may affect the apoptotic phenotype of B-CLL cells (14). Although the mechanism of PLZF dysregulation in B-CLL is not known, a potential antiapoptotic function of PLZF would be concordant with a lower survival of PLZF low-expressor malignant cells and a better prognosis for these patients.

The Jurkat lymphoid cell line, which does not express PLZF, has been widely used in apoptosis studies because the cells are sensitive to diverse stress stimuli, such as serum deprivation, genotoxic agents, or death receptor ligation. In addition, the frequency of apoptotic Jurkat cells can be readily quantified by flow cytometry. The tet-off inducible system developed here allows the examination of the functional consequences of PLZF expression within the same clone, thus eliminating clonality biases. By means of such a cell system, it was observed that Jurkat clones induced to express PLZF produced an antiapoptotic response of the cells stressed by serum deprivation, measured by preservation of plasma membrane integrity, nuclear DNA fragmentation, or mitochondrial membrane depolarization.

We sought to identify potential apoptosis-related PLZF targets for transcriptional regulation in Jurkat cells. A semiquantitative RT-PCR method that uses β -actin as an internal control was developed to detect modulation in the levels of mRNA expression of a variety of apoptosis regulators or effectors, including Bcl2 family members, caspases, and transcription factors, among others. Only *BID* was shown to be significantly repressed in the PLZF-expressing clones upon dox removal. The repression of *BID* was confirmed at the mRNA level by Northern

blotting and at the protein level by Western blotting and flow cytometry. Furthermore, ≈ 2 kb upstream of the *BID* TSS we detected the sequence TTTTACATACAGTA, which is significantly homologous to the sequence TTATATGTACAGTA contained within the PLZF-binding site of the yeast *LEXA* operator (4). EMSA using Jurkat nuclear protein extracts showed that PLZF participates in a complex that binds with high affinity to a double-stranded oligonucleotide synthesized from this region of the *BID* promoter. These findings suggest that PLZF targets *BID*, an apoptosis agonist, for transcriptional repression. *BID* activation during serum deprivation-induced apoptosis suggests that this mechanism may contribute to this form of apoptosis in Jurkat cells.

BID is a cytosolic protein that induces cytochrome *c* release from mitochondria in response to caspase 8, the caspase activated by cell surface death receptors such as FAS receptor and tumor necrosis factor receptor. Activated caspase 8 cleaves *BID*, and the C-terminal fragment translocates to mitochondria, where it triggers cytochrome *c* release, loss of $\Delta\Psi_m$, cell shrinkage, and nuclear condensation (18–20). The caspase-activated form of *BID* activates proapoptotic BAX or BAK to produce large membrane openings that allow the translocation of large mitochondrial proteins during apoptosis (21, 28). Krajewska *et al.* (29) reported that *BID* expression in normal tissues varies widely, being prominent in several types of short-lived cells and in apoptosis-sensitive cells. Evidence of *BID* dysregulation in B-CLL has also been reported (30). Little is known about regulation of *BID* expression. Sax *et al.* (31) reported that p53 up-regulates *BID* expression and increases

cellular chemosensitivity. Taken together, these data suggest that *BID* regulation may be relevant in human cancer.

Our findings support the notion that PLZF can repress programmed cell death in lymphoid cells stressed by nutrient deprivation and thus are consistent with previously reported data obtained by using a different cell type stressed by growth factor deprivation (27). Whether PLZF may control the response of lymphoid cells to other apoptotic stimuli remains to be studied. It is curious that both Jurkat clones expressed different PLZF levels but none of the effects observed were significantly different. One possible explanation is that PLZF expression in both clones reaches levels higher than those required for its maximal activity in Jurkat cells under these physiological conditions. Thus, whether PLZF involvement in apoptosis may depend on its level of expression or the cellular context also remains to be elucidated.

We conclude that *BID* repression could be a mechanism to mediate the antiapoptotic effect of PLZF, and we hypothesize that *BID* repression by PLZF plays a role in lymphoid malignancies where PLZF is dysregulated, such as B-CLL. Therefore, analysis of the potential role of PLZF and *BID* in lymphoid malignancies may be promising fields for future research.

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