Repression of the *PDCD2* gene by BCL6 and the implications for the pathogenesis of human B and T cell lymphomas

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The human *BCL6* gene on chromosome 3 band q27, which encodes a transcriptional repressor, is implicated in the pathogenesis of human lymphomas, especially the diffuse large B-cell type. We previously identified the human *PDCD2* (programmed cell death-2) gene as a target of BCL6 repression. *PDCD2* encodes a protein that is expressed in many human tissues, including lymphocytes, and is known to interact with corepressor complexes. We now show that BCL6 can bind directly to the *PDCD2* promoter, repressing its transcription. Knockdown of endogenous BCL6 in a human B cell lymphoma line by introduction of small interfering RNA duplexes increases PDCD2 protein expression. Furthermore, there is an inverse relationship between the expression levels of the BCL6 and PDCD2 proteins in the lymphoid tissues of mice overexpressing human BCL6 (high BCL6 levels, minimal PDCD2) and controls (minimal BCL6, high PDCD2) as well as in tissues examined from some human B and T cell lymphomas. These data confirm *PDCD2* as a target of BCL6 and support the concept that repression of *PDCD2* by BCL6 is likely important in the pathogenesis of certain human lymphomas.

human lymphomas | target of BCL6

BCL6, a gene on chromosome 3, band q27, encodes a nuclear zinc finger protein that is a transcriptional repressor (1–3). This protein is expressed at high levels in human lymph node germinal center B cells, most cortical thymocytes, and some human B and T cell lymphomas (4). The *BCL6* gene was identified (5–7) through its involvement in chromosomal translocations that occur in ≈40% of diffuse large-cell B cell lymphomas. In other lymphomas, mutations occur 5′ to the BCL6 coding region (8). The BCL6 protein binds DNA in a sequence-specific fashion through its C-terminal zinc finger region (9, 10). It conveys transcriptional repression through an N-terminal POZ domain and a second domain that is more centrally located (1–3, 11) and interacts with a number of corepressors (12–14). It is thought that the BCL6-repressive effects are mediated through multiprotein repression complexes with histone deacetylase activity. A peptide that specifically binds BCL6 and blocks corepressor recruitment leads to apoptosis and cell-cycle arrest of BCL6-positive lymphoma cells (15). Mice overexpressing human (16) or murine (17) BCL6 develop lymphomas.

We previously generated a dominant-negative cell system (18) with the use of a construct expressing the BCL6 zinc fingers, which compete with the binding of endogenous BCL6 in BJAB cells (an Epstein–Barr virus-negative Burkitt lymphoma cell line expressing high levels of BCL6) (19). Because BCL6 is a repressor, competition for binding of the full-length endogenous BCL6 protein by the exogenously transfected zinc fingers results in up-regulation of BCL6 target genes. We used subtractive hybridization techniques to amplify differentially expressed sequences. These studies led to the identification of the *PDCD2* (programmed cell death-2) gene as a target of BCL6. Immunohistochemistry performed on human tonsil with antibodies specifically directed against the BCL6 and PDCD2 proteins revealed a differential staining pattern of these proteins with an inverse relationship between the localization of their expression. Analysis of the sequences flanking the first exon of *PDCD2* showed an exact match to a previously identified high-affinity BCL6 binding site located ≈3.4 kb upstream of exon 1 (18). However, a direct relationship between these genes has not been demonstrated previously.

The role of BCL6 as a transcriptional repressor in lymphomagenesis requires detailed study of its effect on its target promoters. We now report that (*i*) BCL6 binds to the *PDCD2* promoter and functionally represses PDCD2 expression at the transcriptional level, (*ii*) direct silencing of BCL6 results in augmented PDCD2 expression and likely significant downstream effects thereof, and (*iii*) an inverse relationship between BCL6 and PDCD2 expression is present in the lymphoid tissues of *BCL6* transgenic mice and, more importantly, in 10 human lymphoma patient samples.

Results

The BCL6 Protein Binds to the *PDCD2* Promoter. *In vitro* binding studies with EMSAs revealed that the HA-tagged BCL6 protein binds specifically to the high-affinity consensus site we identified in the *PDCD2* promoter [GGACCTACCC*TTCTAGGAA*-AAAACCATCC; the 9- of 9-nt exact match is italicized (18)] (Fig. 1*a*). An excess of nonradioactive double-stranded oligomer containing the BCL6 consensus site competed for binding with the radioactive double-stranded oligomer, and binding was reduced by the addition of antibodies specifically directed against BCL6 and HA; in contrast, an unrelated antibody (CD45RB; Lab Vision, Fremont, CA) and PBS did not affect binding. The EMSA was performed additionally with recombinant BCL6 protein and vector not containing BCL6 (control) generated by *in vitro* transcription/translation (Fig. 1*b*). Whereas the transcription/translation BCL6 zinc-finger protein binds to the ^{32}P-labeled double-stranded oligomers containing the BCL6 consensus binding site in the *PDCD2* promoter, the vector does not. These data show that the binding between BCL6 and its consensus binding site in the *PDCD2* promoter is specific. We and others (figure 2 in ref. 3 and figure 5B in ref. 20) have previously demonstrated a reduction in binding with the use of HA antibodies. Reduction in binding without a supershift may

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The authors declare no conflict of interest.

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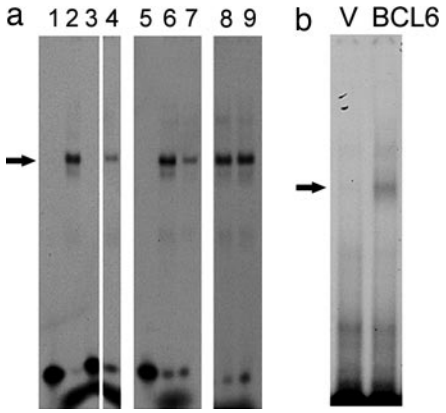

Fig. 1. EMSA. (*a*) The BCL6 protein, which was prepared from nuclear extracts of COS cells transfected with a full-length *BCL6* expression construct, binds specifically to the *PDCD2* promoter. Lane 1, nonprotein control (no shifted bands). The BCL6 protein binds specifically to the *PDCD2* promoter sequences containing the BCL6 binding site because the shifted band (lane 2, arrow) is competed with a 310-fold molar excess of the same nonradiolabeled double-stranded oligonucleotide (lane 3), and its binding is reduced by the addition of antibodies to BCL6 (lane 4). Lane 4 is from the same gel as lanes 1–3. Lane 5, nonprotein control (no shifted bands). Lane 6, ^{32}P-labeled double-stranded *PDCD2* promoter oligonucleotides. The heavy shifted band (arrow) is reduced by the addition of antibodies to the HA tag in the BCL6 expression construct (lane 7) but not by an unrelated antibody (lane 8) or by PBS (lane 9). (*b*) The *in vitro* transcribed/translated BCL6 zinc-finger protein binds to the *PDCD2* promoter sequences containing the BCL6 binding site (arrow), whereas the vector control (V) does not bind.

be caused by antibody interfering with the DNA binding of the protein.

Furthermore, ChIP assays confirmed that in a B cell lymphoma line, endogenous BCL6 was bound to the region in *PDCD2* containing the high-affinity BCL6 binding site, because DNA from this region was enriched in chromatin immunoprecipitated with anti-BCL6 (Fig. 2*a*). This genomic region was not enriched in material immunoprecipitated with anti-HA or in the absence of antibody (controls). No binding of BCL6 to the coding region of *PDCD2*, which lacks a putative binding site, was observed (Fig. 2*b*). Similarly, BCL6 does not bind to the GAPDH gene (data not shown), confirming the specificity of BCL6 binding to the *PDCD2* promoter.

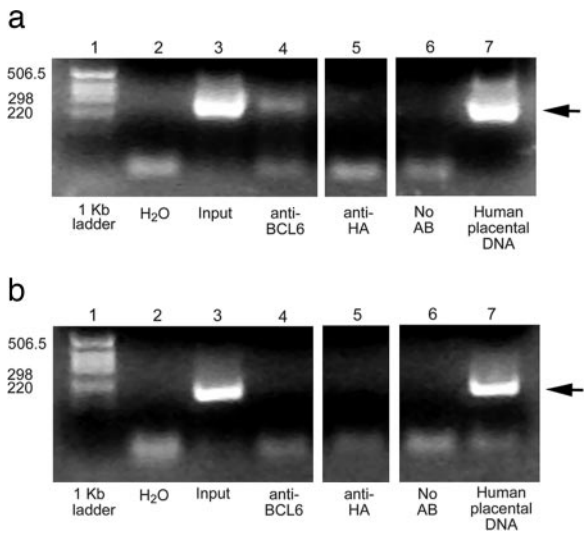

Fig. 2. The BCL6 protein binds to the *PDCD2* promoter in living cells (ChIP assay). (*a*) PDCD2 promoter region. In Daudi cells, BCL6 is bound to the region of the *PDCD2* promoter containing the putative BCL6 binding site (arrow), because DNA from this area is enriched in chromatin immunoprecipitated with anti-BCL6 (lane 4). Controls [anti-HA antibody and no antibody (no AB), lanes 5 and 6] do not enrich this genomic region. All of the lanes in *a* are from the same gel. (*b*) PDCD2 coding region. Binding of BCL6 to the coding region of *PDCD2* (lane 4, arrow) is not observed, because there is no putative binding site in this area. All of the lanes in *b* are from the same gel.

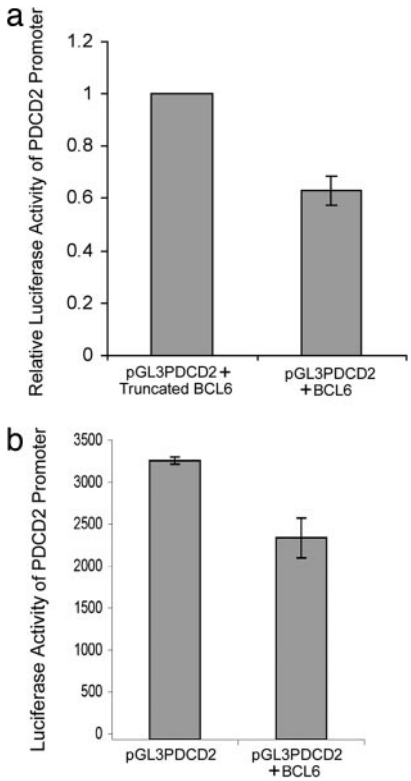

Fig. 3. The BCL6 protein represses transcription from the *PDCD2* promoter (transfection assays). (*a*) Relative luciferase levels in NIH 3T3 cells reveal significant repression ($P < 0.0005$) of *PDCD2* promoter activity by the full-length BCL6 protein (mean ± SE, 0.63 ± 0.06) as compared with a truncated control that cannot bind DNA because it lacks the BCL6 zinc fingers, which are needed to bind DNA. For purposes of comparison, the data were normalized with results for the control designated as 1. (*b*) Similarly, in HeLa cells, *PDCD2* promoter activity (mean ± SD, 3,327 ± 104 in the absence of BCL6) was repressed significantly ($P = 0.01$) by the full-length BCL6 protein (mean ± SD, 2,407 ± 584).

The BCL6 Protein Represses Transcription From the *PDCD2* Promoter. Transient transfections were performed in NIH 3T3 cells [which express BCL6 only weakly (21) and have minimal levels of PDCD2 (unpublished data)]. Relative luciferase levels, depicted in Fig. 3*a*, reveal significant repression ($P < 0.0005$) of *PDCD2* promoter activity by the full-length BCL6 protein (mean ± SE, 0.63 ± 0.06) as compared with a truncated control that cannot bind DNA (for purposes of comparison, the absolute value of the control was set at 1). Similar studies were performed in HeLa cells, which contain very little, if any, endogenous BCL6 protein (19). In this second cell line, *PDCD2* promoter activity normalized for transfection efficiency also was repressed significantly ($P = 0.01$) by the full-length BCL6 protein (mean ± SD, 2,407 ± 584) (Fig. 3*b*, second bar) as compared with the significantly higher level of luciferase activity in the absence of BCL6 (mean ± SD, 3,327 ± 104) (Fig. 3*b*, first bar). These studies demonstrate that BCL6 directly represses *PDCD2* promoter activity and thus provide evidence for transcriptional regulation of *PDCD2* by BCL6.

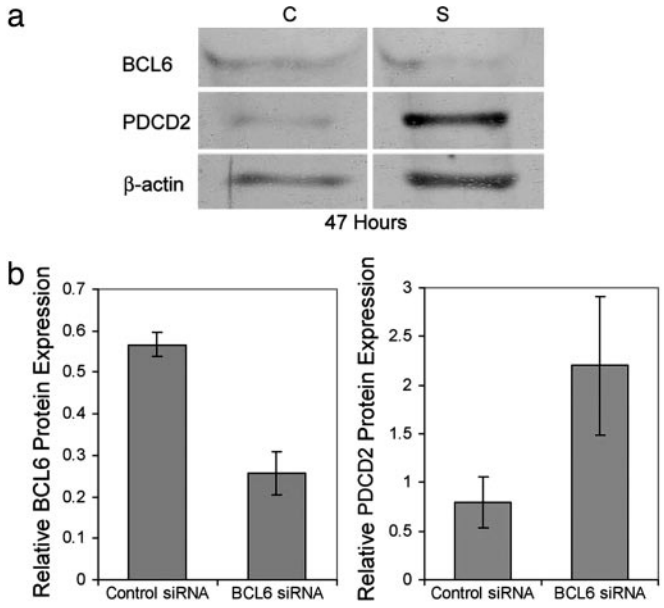

Fig. 4. Knockdown of BCL6 protein levels by siRNA increases PDCD2 protein expression. (*a*) Representative Western blot analysis of BCL6, PDCD2, and β-actin protein expression in BJAB cells 47 h after transient transfection with siRNA duplexes [C, control siRNA; S, study (BCL6) siRNA]. (*b*) To determine the relative difference in protein expression, the results were normalized based on the level of intensity of β-actin. (*Left*) BCL6 protein expression (mean ± SE: control siRNA, 0.57 ± 0.03; BCL6 siRNA, 0.26 ± 0.05; $P = 0.04$). (*Right*) PDCD2 protein expression (mean ± SE: control siRNA, 0.8 ± 0.26; BCL6 siRNA, 2.2 ± 0.71; $P = 0.005$). The graphs represent analysis of reproducible data from four independent experiments.

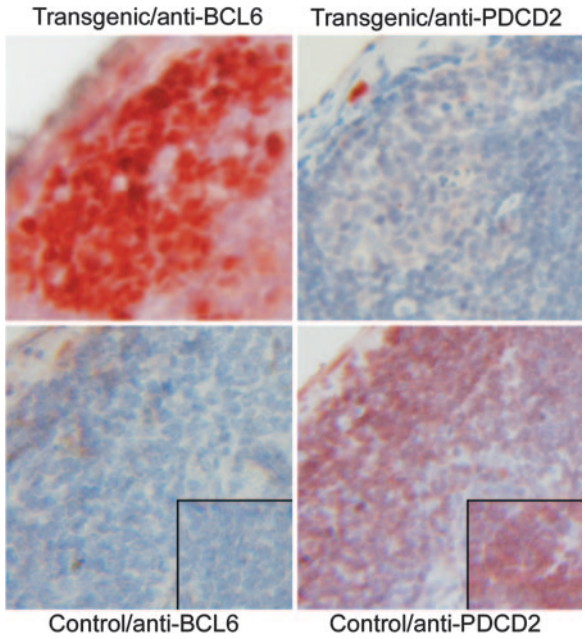

Fig. 5. Reciprocal relationship between BCL6 and PDCD2 protein expression *in vivo* (paraffin-embedded sections, Peyer's patches of mouse intestine). (*Upper*) An intestinal lymphoid nodule with a germinal center from a *BCL6* transgenic mouse. (*Upper Left*) Positive (red) staining in the nuclei of most of the lymphocytes by anti-BCL6. The lymphocytes, both within and outside the germinal center, in a sequential section of this Peyer's patch from the same transgenic mouse (top right of image) do not stain with anti-PDCD2. (*Lower*) A Peyer's patch from a control mouse that contains no germinal centers. (*Lower Left*) Because endogenous BCL6 is largely restricted to germinal centers in mice that do not carry the *BCL6* transgene, this tissue does not stain with antibodies to BCL6. (*Lower Right*) In contrast, a sequential section from this Peyer's patch in the control mouse reveals positive cytoplasmic staining with anti-PDCD2. (Magnification, ×333.)

Knockdown of the BCL6 Protein Increases PDCD2 Expression. Transient transfection of siRNA duplexes specifically targeting endogenous BCL6 in BJAB cells resulted in knockdown of BCL6 protein levels that could be detected by 24 h and persisted until at least 47 h (overall knockdown was 57% as compared with control; mean ± SE: control siRNA, 0.57 ± 0.03; BCL6 siRNA, 0.26 ± 0.05; $P = 0.04$) (Fig. 4). Higher levels of PDCD2 protein in these cells (2.8-fold increase as compared with control; mean ± SE: control siRNA, 0.8 ± 0.26; BCL6 siRNA, 2.2 ± 0.71; $P = 0.005$) were most marked at 42–47 h (Fig. 4). The calculations (Fig. 4*b*) take into account differences in the level of intensity of β-actin expression, which was used to determine the amount of protein loaded when quantitating band intensities on Western blots. These data were analyzed from four independent experiments and confirm a direct effect of BCL6 knockdown on PDCD2 expression.

Reciprocal Relationship Between BCL6 and PDCD2 Protein Expression *in Vivo*. We used immunohistochemistry to determine the relationship between BCL6 and PDCD2 protein expression in nonneoplastic murine lymphoid tissues and in murine and human B and T cell lymphomas.

Mouse Tissues. BCL6 expression is largely restricted to germinal centers in normal mice (22). Thus, as expected, in a mouse that is not transgenic for *BCL6*, the BCL6 protein was not detected in a Peyer's patch that has no germinal centers (Fig. 5 *Lower Left*). However, the lymphocytes in this tissue showed strong staining for PDCD2, a cytosolic protein (Fig. 5 *Lower Right*). In contrast, as anticipated, the BCL6 protein was expressed in the Peyer's patch lymphocytes of the *BCL6* transgenic mouse (Fig. 5 *Upper Left*), whereas the PDCD2 protein was not detected in these lymphocytes (Fig. 5 *Upper Right*). The inverse relationship between the expression patterns of BCL6 and PDCD2 also was observed in two BCL6-positive tumors (one B cell and one T cell) that we examined from our *BCL6* transgenic mice (data not shown). The histology of these neoplasms has been described previously (16). These findings demonstrate a reciprocal relationship between BCL6 and PDCD2 expression *in vivo*.

Human Lymphomas. In human lymphomas, both B and T cell, immunohistochemistry similarly confirmed the inverse relationship between BCL6 and PDCD2 protein expression (Fig. 6). We analyzed six B cell tumors (representative sections) (Fig. 6*a*). Two tumors were classified as high-grade follicular lymphomas, and the other four were diffuse large B cell neoplasms. BCL6 was expressed in four of these lesions (one follicular and three diffuse, either B5- or formalin-fixed), and PDCD2 staining was negative in the malignant lymphocytes in all of these lesions. Of note, positive PDCD2 staining was observed in some of the nonneoplastic cells in the B5-fixed tissues and in the formalin-fixed tissues, indicating that neither of these fixatives adversely affected the staining by this antibody. Two of the B cell tumors did not stain for either BCL6 or PDCD2. We are not certain whether these lymphomas are indeed BCL6-negative B cell neoplasms or whether adverse tissue preservation might be responsible for the lack of staining in these two cases.

We studied six human T cell lymphomas (representative sections) (Fig. 6*b*). Three of these lymphomas were BCL6-positive but PDCD2-negative (Fig. 6 *bI* and *bII*). The other three neoplasms were BCL6-negative but PDCD2-positive (Fig. 6 *bIII* and *bIV*).

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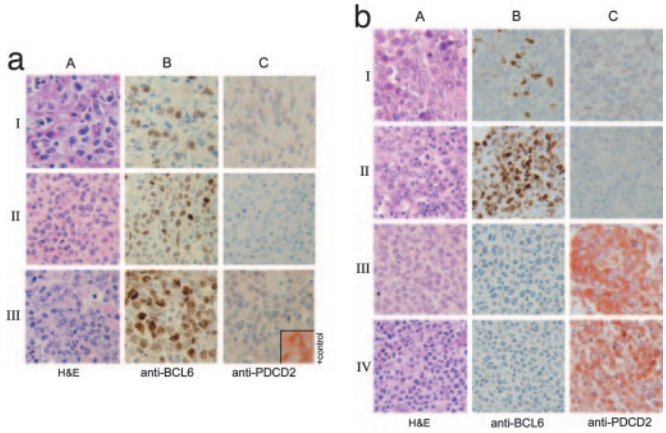

Fig. 6. Reciprocal relationship between BCL6 and PDCD2 expression in human lymphomas. (*a*) Human large B-cell lymphomas. (*aI*) Follicular lymphoma, grade III, from uterine cervix. (*aII*) Diffuse lymphoma in supraclavicular lymph node. (*aIII*) Follicular origin, predominantly diffuse architecture (atypical cleaved) lymphoma in submandibular lymph node. (*aA*) H&E stains of paraffin-embedded tissues reveal effacement of normal architecture by infiltrates of malignant lymphocytic tumor cells classified as large B cell lymphomas. (*aB* and *aC*) In each case, the malignant B cells are positive for BCL6 (*aB*, brown nuclear stain) but negative for PDCD2 (*aC*). The boxed area in a*IIIC* shows a positive control for PDCD2 from oral mucosal epithelium (red cytoplasmic staining). (*b*) Human T cell lymphomas. (*bI* and *bII*) Anaplastic large T cell lymphomas in lymph nodes. (*bIII*) Anaplastic large T cell lymphoma in skin from arm. (*bIV*) Peripheral T cell lymphoma, mixed medium and large cell type, in a breast mass. (*bA*) H&E stains of paraffin-embedded tissues show that tissue architecture is obliterated by infiltrates of malignant lymphocytic cells. (*bB* and *bC*) Two of the T cell neoplasms are BCL6-positive (*bIB* and *bIIB*, brown nuclear staining) but PDCD2-negative (*bIC* and *bIIC*). The other two lymphomas (*bIII* and *bIV*) are BCL6-negative (*bIIIB* and b*IVB*) but PDCD2-positive (*bIIIC* and *bIVC*, red cytoplasmic staining). (Magnification, ×143.)

Discussion

Our studies show that PDCD2 is a target of BCL6 through a number of observations. First, the *PDCD2* promoter region contains an exact match to a 9-nt sequence to which BCL6 has been shown to bind with high affinity (18). Second, an EMSA (Fig. 1) and a ChIP assay (Fig. 2) demonstrate that BCL6 binds specifically to the consensus region we identified in the *PDCD2* promoter. Furthermore, functional studies reveal that the BCL6 protein can repress transcription from the *PDCD2* promoter in transient transfection assays (Fig. 3). Knockdown of the endogenous BCL6 protein in a B cell line by siRNA molecules specifically targeting BCL6 leads to significantly higher levels of the PDCD2 protein (Fig. 4). Additionally, immunohistochemical studies previously performed on human tonsil (18), and now on transgenic mouse tissues (Fig. 5) and some B and T cell human lymphomas (Fig. 6), confirm the inverse relationship in the expression patterns of BCL6 and PDCD2.

Human *PDCD2* is an evolutionarily conserved gene that is expressed in many tissues. It contains an ORF encoding 344 aa (23) with a zinc-finger MYND [myeloid translocation gene 8 (*MTG8*, also called *ETO*), *Drosophila* Nervy, and DEAF-1] domain (24). Both PDCD2 and BCL6 interact with N-CoR/mSin3A corepressor complexes, which repress transcription by recruiting histone deacetylase (14, 25), and PDCD2 has been shown to negatively regulate host cell factor 1, a multidomain protein required for cell cycle progression (25). Human *PDCD2* is located on chromosome 6q27, a region where translocations have been reported in acute myelogenous leukemias and where deletions may occur in cutaneous T cell lymphomas, acute lymphoblastic leukemias (23), and non-Hodgkin lymphomas, including follicular B cell lymphoma (26), and it is believed to contain a putative tumor suppressor gene (27, 28). Fan *et al.* (29) found that *PDCD2* was among several other proapoptosis genes that were expressed in decreased levels in a multidrug-resistant human colon carcinoma cell line.

The *PDCD2* gene is highly homologous to *Rp8* (23), a rat gene that was identified in association with programmed cell death in immature thymocytes (30). Rp8 mRNA was found to be expressed 1 h after rat thymocytes were induced to die but before the occurrence of DNA fragmentation (30). Subsequently, Rp8 expression again was shown to be correlated with regions of cell death in a study of developing intracerebral transplants of ventral mesencephalic tissues in rats (31). However, others found that mouse Rp8 expression was not altered in a factor-dependent myeloid line induced to undergo apoptosis by growth factor withdrawal or when a lymphoid cell line was triggered by irradiation to undergo apoptosis (32). Similarly, D'Mello and Galli (33) reported that Rp8 mRNA was not induced in neuronal apoptosis triggered in a culture system by nerve growth factor deprivation. Recently, proteomic methodology was used to study proteins in rat kidney fibroblasts during prolonged hypoxia (34). In this study, Rp8 levels were found to increase at least 2-fold in cells subjected to 1% oxygen for a 12-h period.

These studies indicate that the function, or functions, of the PDCD2 protein remain to be clarified. Like the BCL6 protein, PDCD2 appears to be a transcriptional repressor (25). Prior published reports of other targets of BCL6 are consistent with the concept that, through repression of its target genes, BCL6 may influence apoptosis (35) and cell cycle progression (36, 37). Specifically, others have reported that knockdown of the BCL6 protein by siRNAs leads to retardation of cell cycle progression (36), an effect that we now hypothesize might be mediated through the associated increased levels of PDCD2 which, in turn, exert a negative regulatory effect on host cell factor 1 (25). In summary, our data confirm PDCD2 as a legitimate target of BCL6 repression and suggest that the interactions among BCL6, PDCD2, and other regulatory factors are likely to be very important in the development of human B and T cell lymphomas.

Materials and Methods

EMSAs. Nuclear extracts were prepared from COS-7 cells grown under 5.5–7% CO_2 in DMEM supplemented with 10% FCS and transiently transfected by electroporation/diethylaminoethyl-dextran with full-length BCL6 subcloned in the pCGN expression vector containing an HA epitope tag (3). The nuclear extracts were incubated with ^{32}P-labeled double-stranded oligomers encompassing the putative high-affinity BCL6 consensus binding site (18) identified in *PDCD2* (GGACCTACCC*T-TCTAGGAA*AAAACCATCC; the high-affinity exact base pair match is italicized) and electrophoresed on a polyacrylamide gel as described (10). The antibodies used were polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) directed against the BCL6 N terminus (*sc*-858) or HA epitope tag (*sc*-805) and an unrelated antibody, CD45RB (clone Bra-11; Lab Vision, Fremont, CA). The EMSA was repeated with *in vitro* transcribed/translated proteins. Recombinant BCL6 zinc-finger protein was generated from human BCL6 zinc-finger cDNA cloned into a pcDNA3 plasmid (18) by using the TNT T7 Quick Coupled Transcription/Translation system (Promega, Madison, WI). The pcDNA3 vector without the BCL6 zinc fingers (control) was subjected to the same transcription/translation system.

ChIP Assay. ChIP was performed with an Upstate Biotechnology (Lake Placid, NY) ChIP assay kit with some modifications of the manufacturer's protocol. Daudi cells (a B lymphoblastoid cell line derived from a Burkitt lymphoma, 5×10^6 per sample), which are rich in endogenous BCL6, were cross-linked with 1% formaldehyde for 5 min at room temperature. The reaction was terminated with an excess of glycine. Cross-linked chromatin was

sonicated to an average size of ≈400 bp and was immunoprecipitated with antibodies to BCL6 (*sc*-858), anti-HA as a negative control (*sc*-805) (Santa Cruz Biotechnology), or no antibody. Input was 3.5% chromatin, and 7% chromatin was used for each of the immunoprecipitated samples. ChIP DNA was amplified by PCR and detected by ethidium bromide staining after electrophoresis on 1% agarose gels. Primers amplified the *PDCD2* promoter region [nucleotides upstream of exon 1, −3519 to −3244, containing the putative BCL6 binding site we identified (forward primer, 5′-CCCTTCCTCCCCAAATTTTCT-3′; reverse primer, 5′-GCCCTAAGCAGTTCCCAGC-3′)] and coding region [nucleotides +872 to +1060, which did not contain any putative BCL6-binding sites (forward primer, 5′-CTCATATGAGCCACCTTCTGA-3′; reverse primer, 5′-TGCTTATGTCCCAATCTCCAG-3′)]. Human placental DNA was used as a positive genomic DNA control for PCR.

Transfection Assays/Functional Analysis. To clone a portion of the *PDCD2* promoter region, 1.43 kb of genomic DNA from the *PDCD2* gene (nucleotides −3574 to −2140), encompassing the putative BCL6-binding site, were amplified from human genomic DNA by PCR; 2 μl of the PCR was used for ligation to the pT-Adv vector (Clontech Laboratories, Mountain View, CA). The appropriate insert was isolated from the pT-Adv vector with EcoRV–BamHI restriction enzyme digests and ligated to the SmaI–BglII sites of the pGL3 basic luciferase reporter vector (Promega) (pGL3PDCD2). NIH 3T3 cells, which contain minimal levels of PDCD2 (unpublished data), were grown under standard conditions and plated at 4.5×10^5 cells per 60-mm dish. The cells were transfected the next day by calcium phosphate DNA-coprecipitation with pGL3PDCD2 (1 μg per 60-mm plate) and either full-length *BCL6* cDNA (1–2.5 μg) subcloned in the pCGN expression vector (pCGNBCL6) or an equivalent quantity of a truncated BCL6 expression construct (control) also subcloned in the pCGN vector. This truncated construct, which we have used in prior studies as a control (3), does not bind DNA because it lacks the zinc-finger DNA-binding region of BCL6. Cells were harvested at ≈72 h. A CMV-driven β-galactosidase expression construct was cotransfected, and the data were normalized for transfection efficiency by using the values of β-galactosidase assays as described previously (3).

Relative luciferase activity was defined as the luciferase levels obtained by using the BCL6 construct divided by the luciferase levels obtained with the truncated control in three independent experiments; duplicate plates were transfected in two studies, and triplicate transfections were performed in the third study. A one-sample *t* test was used to evaluate whether the mean relative luciferase was significantly different from 1.

Additionally, HeLa cells, which contain little or no endogenous BCL6 protein (19), were plated at 1×10^5 cells per well in a six-well dish and transfected by using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) per the manufacturer's protocol with 1 μg of the pGL3PDCD2 reporter as described previously (no BCL6 present) or 1 μg of pGL3PDCD2 plus 1 μg of full-length *BCL6* cDNA (pCGNBCL6). All plates received 0.1 μg of pTKRL (*Renilla* expression construct, an internal control used for normalization of luciferase activity). Cells were harvested 48 h after transfection. Luciferase assays were performed with the Dual-Luciferase Reporter Assay system (Promega). A *t* test with unequal variances was used to compare luciferase activity of the *PDCD2* promoter in the cells transfected with or without *BCL6* cDNA from three independent studies (duplicate wells in each).

Transfection of siRNAs and Western Blot Analysis. BJAB cells were grown under 5% CO_2 in RPMI medium 1640 supplemented with 10% FCS and transiently transfected by electroporation with si-CONTROL nontargeting siRNA 1 or human BCL6 siGENOME SMARTpool reagent (Dharmacon, LaFayette, CO) by using a Gene Pulser transfection apparatus (Bio-Rad Laboratories, Hercules, CA) with capacitance extender. Conditions, initially optimized with a fluorescently labeled RNA-induced silencing complex-free scrambled oligomer, were: 500 μF, 330 v, and 150 nM siRNAs for 4×10^6 cells in 0.8 ml of serum-free RPMI medium 1640. Whole-cell extracts were boiled and sheared in a reducing buffer [10% glycerol/2 gm% SDS/1% 1 M Tris·HCl (pH 6.8)/5% 2-mercaptoethanol/0.005 gm% bromophenol blue]. The extracts were electrophoresed by SDS/7% PAGE and transferred to PVDF membranes (Bio-Rad Laboratories), which were blocked in 2–3% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 and incubated with rabbit polyclonal antibodies to BCL6 (*sc*-858 or *sc*-368; Santa Cruz Biotechnology), rabbit polyclonal antiserum to PDCD2 (18), and affinity-isolated actin antibody produced in rabbit (catalog no. A2066; Sigma, St. Louis, MO). The membranes were washed and incubated with anti-rabbit IgG (Fc), alkaline phosphatase conjugate (Promega), and then washed again. Protein bands were detected with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT Color Development Substrate; Promega).

Quantitation of relative band intensity was normalized to β-actin by scanning densitometry. Relative protein expression is usually not normally distributed because it ranges from zero to infinity and is not symmetrically distributed around 1. Thus, a log transformation was conducted. The paired *t* test was used to compare the log-transformed BCL6 and PDCD2 protein levels, respectively, with the levels of the corresponding controls.

Immunohistochemistry. The relationship between BCL6 and PDCD2 expression in our *BCL6* transgenic mice (16) was tested with rabbit polyclonal antibodies specifically directed against either BCL6 (*sc*-858; Santa Cruz Biotechnology) or PDCD2 (18), which were used to stain lymphoid tissue (Peyer's patches) in the intestines of *BCL6* transgenic and control mice and two BCL6-positive (one B cell and one T cell) murine lymphomas. Mice transgenic for human *BCL6*, which were handled in accordance with approved institutional protocols, express the transgenic BCL6 protein constitutively in lymphocytes and develop BCL6-positive lymphomas (16). PDCD2 antibodies have been described (18). Tissues were fixed in 10% buffered formalin, paraffin-embedded, and sectioned. BCL6 staining was performed as described previously (16), except that antigen–antibody binding was detected with 3-amino-9-ethylcarbazole chromogen, which gives a red color. Tissues were counterstained with hematoxylin. An aqueous mounting medium was used for coverslipping. Staining for PDCD2 was performed similarly, except that pH 6 antigen retrieval buffer (DAKO, Carpinteria, CA) was used instead of the pH 10 buffer used for BCL6 staining.

Additionally, BCL6 and PDCD2 antibodies were used on B5- or formalin-fixed paraffin-embedded tissues from six human B and six T cell lymphomas obtained from the University of Chicago Surgical Pathology tissue archives in accordance with an Institutional Review Board-approved protocol. We used tumor tissues that remained from diagnostic studies in which tumors were classified by histologic criteria and multiple marker analyses. BCL6 staining was performed as previously described (18) with a mouse monoclonal antibody to human BCL6 (DAKO), except that tissues were deparaffinized, quenched, and incubated in pH 10 antigen retrieval solution (DAKO) in a pressure cooker for 20 min and then cooled before staining. Antigen–antibody binding was detected by the diaminobenzidine substrate, which gives a brown color. For PDCD2 staining, tissues were deparaffinized, quenched, and incubated in pH 6 antigen retrieval solution (DAKO) in a 97.5°C water bath before staining with rabbit polyclonal antibodies previously shown to be specifically directed against PDCD2 (18). Staining was performed overnight at 4°C. The secondary antibody was peroxidase-conjugated,

affinity-purified donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA). Antigen–antibody binding was detected with 3-amino-9-ethylcarbazole chromogen. Tissues were counterstained with hematoxylin. An aqueous mounting medium was used for coverslipping.

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