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*J Immunol* 2009; 183:5778-5787; Prepublished online 14 October 2009;
doi: 10.4049/jimmunol.0901120
http://www.jimmunol.org/content/183/9/5778
PU.1 Regulates Positive Regulatory Domain I-Binding Factor 1/Blimp-1 Transcription in Lymphoma Cells

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The human positive regulatory domain I-binding factor 1 (PRDI-BF1) and its murine homolog Blimp-1 promote differentiation of mature B cells into Ab-secreting plasma cells. In contrast, ectopic expression of PRDI-BF1 in lymphoma cells can lead to inhibition of proliferation or apoptosis. However, little is currently known about the regulation of PRDM1, the gene encoding PRDI-BF1. This report establishes that in lymphoma cells stimulation through the BCR rapidly induces endogenous PRDM1 at the level of transcription with minor changes in mRNA stability. The induced PRDM1-encoded protein localizes to its target genes in vivo and suppresses their expression. In vivo genomic footprinting of the PRDM1 promoter in unstimulated lymphoma and myeloma cells reveals multiple common in vivo occupied elements throughout the promoter. Further functional and structural analysis of the promoter reveals that the promoter is preloaded and poised for activation in the B cell lines. The transcription factor PU.1 is shown to be required for the BCR-induced expression of PRDM1 in lymphoma cells and in PU.1-positive myeloma cells. Activation of PRDM1 is associated with loss of the corepressor transducin-like enhancer of split 4 from the PU.1 complex. These findings indicate that PRDM1 is poised for activation in lymphoma cells and therefore may be a potential therapeutic target to inhibit lymphoma cell proliferation and survival.

Received for publication April 7, 2009. Accepted for publication September 3, 2009.

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Anti-IgM cross-linking of the BCR has been reported in multiple studies to induce apoptosis in lymphoma cells (10–14). This response has been correlated with decreased levels of c-myc (6). Inducing PRDI-BF1/Blimp-1 expression in lymphoma cells with histone deacetylase inhibitors also decreased expression of the downstream targets c-myc and BSAP (15). More specifically, introduction of PRDI-BF1/Blimp-1 into lymphoma cells can induce apoptosis, suggesting PRDI-BF1 may be an important mediator of the anti-IgM-mediated apoptotic response (16, 17). However, no direct link between expression of PRDI-BF1/Blimp-1 and anti-IgM-mediated BCR activation has been described.

Recently, PRDM1 expression has been detected in a subset of diffuse large B cell lymphomas (18–20). However, inactivating mutations in the PRDM1 coding sequence were described, indicating a potential tumor suppressor role for this gene (19, 20). Similarly, proliferating myeloma cells and myeloma cell lines abundantly express the truncated PRDI-BF1 isofrom, PRDI-BF1β, which has impaired function (21). Additionally, Borson et al. (22) demonstrated PRDM1 expression in B cells isolated from myeloma patients, whereas normal donors lack expression. The mutation status of PRDM1 in these myeloma-derived B cells is as yet unknown. Together, these findings indicate that PRDI-BF1/ Blimp-1 may be important to the pathology of various hematopoietic malignancies, including lymphoma.

Very little is known as to the regulation of PRDM1 expression. Our data now demonstrate that PRDM1 is regulated primarily at the level of transcription both in myeloma cells and in lymphoma cells stimulated by cross-linking of the BCR. BCR stimulation leads to rapid increases in newly transcribed PRDM1 RNA levels, whereas mRNA stability is unchanged. Using promoter deletion constructs, we demonstrate several regions of activation in the PRDM1 promoter in both lymphoma and myeloma cells. In vivo genomic footprinting demonstrates multiple protein-DNA interactions in both lymphoma and myeloma cells. Further analysis of these interactions reveals PU.1 binding is functionally important for promoter activity in stimulated lymphoma cells. These findings demonstrate the PRDM1 promoter is poised for rapid activation in lymphoma cells, which suggests that inducing PRDM1 expression

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in lymphoma cells may be a viable target to inhibit lymphoma progression.

Materials and Methods

Cell lines and reagents

The CA46 EBV-negative Burkitt’s lymphoma and RPMI8226 multiple myeloma cell lines were maintained in RPMI (Invitrogen) supplemented with 10% FBS (HyClone), and 1% penicillin/streptomycin (Invitrogen). Goat anti-human IgM Ab (Southern Biotechnology) was used at 10 μg/ml. Actinomycin D (Sigma-Aldrich) was used at 10 μg/ml. 

Transfections and luciferase assays

Cells were transfected by electroporation using the Gene Pulser II (Bio-Rad). Cells (1 × 10^6) were pulsed with 250 V at a capacitance of 10.7 μF. Transfections for luciferase assays were done with 10 μg of luciferase reporter construct and 50 ng of the internal control plasmid pRL-TK. Firefly luciferase activity was normalized to Renilla luciferase activity in all experiments. For small interfering RNA (siRNA) knockdown experiments in CA46, cells were transfected with 3 μg of control nontargeting siRNA or PU.1 siRNA (Dharmacon) for 24 h. Live cells were separated using a Ficoll gradient and treated with 10 μg of anti-IgM for 24 h. For siRNA knockdown experiments in RPMI8226, cells were transfected with 2 μg of control nontargeting siRNA or PU.1 siRNA (Dharmacon) for 48 h.

B cell isolation

B cells were isolated from healthy human donors. Briefly, PBMCs were isolated by Ficol separation and incubated with anti-CD19 microbeads (Miltenyi Biotec) followed by magnetic separation using MS columns. The purified cells were routinely >90% B cells as confirmed by flow cytometry analysis for CD20. Isolated B cells were activated by coculture with irradiated CD40L-expressing L cells (23) in the presence of cytokines IL-2 (20 U/ml), IL-4 (50 ng/ml), IL-10 (50 ng/ml), and IL-2 (12 ng/ml) for 4 days. The cells were then divided into two flasks, contents of one stimulated with anti-IgM and those of the other unstimulated for 24 h.

Apoptosis assay

Cells were treated with anti-IgM continuously for 24 h, followed by annexin V-PE and 7-aminoactinomycin D staining per the manufacturer’s protocol (BD Pharmingen). ToPro3 staining was used to detect dead cells. Flow cytometry acquisition was done on a FACScalibur and analyzed with CellQuest software (BD Biosciences).

Quantitative PCR

Nascent RNA was isolated as previously described (24, 25). mRNA was isolated from cells using Trizol reagent (Invitrogen). One microgram of RNA was treated with DNase using RQ1 DNase (Promega), followed by first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). One-twentieth of the final cDNA reaction volume was used in each PCR reaction. PRDM1 mRNA levels were confirmed using two distinct primer sets: PRDM1-set 1 forward (FWD), 5′-TACATCAACAGGAAGCTAC-3′; PRDM1-set 1 reverse (REV), 5′-TACCTCCTCTCTGGAA TA-3′; PRDM1-set 2 was described previously (20). Additional PRDM1-specific primer pairs used to detect mRNA decay rates were: PRDM1-set 1 B FWD, 5′-ATCCAGGGCTAAGAACGG-3′; PRDM1-set 1 B REV, 5′-ATGAGGAGATCTGATAC-3′; PRDM1-set 2 C FWD, 5′-GGACCC TCTGACTCTGTA-3′; PRDM1-set 2 C REV, 5′-CTCCCTCCCTGTT TGGTT-3′. Control primer sets used were: GAPDH FWD, 5′-GAAGGT GAAGAAGGATTACAC AC-3′; and GAPDH REV, 5′-GAAGATGGTGATGGGA TA-3′; PRDM1-set C REV, 5′-TAATGGGGTACGCTTTGA-3′; and β-actin (Realltimeprimers.com). Quantitative PCR reactions were performed with iQ SYBR Green Supermix (Bio-Rad) using an iCycler (Bio-Rad). 

Chromatin immunoprecipitation (ChIP)

After 24 h of treatment with anti-IgM or control (no treatment), cells were cross-linked with 1% formaldehyde for 10 min at room temperature, and the reaction was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were washed twice with ice cold PBS and resuspended in 1 mL of 10× TX-100, Nonidet P-40 buffer (10 mM Tris, pH 8.1, 1% Triton X-100, 0.5 M EDTA, 0.5 M EGTA, 0.25% TX-100, 0.5% Nonidet P-40, 1 mg/mL PMSF, 0.5% protease inhibitors) at a density of 4 × 10^6 cells/ml. Cells were resuspended in 10 mL of ice cold salt-wash buffer (10 mM Tris (pH 8.1), 1 mM EDTA, 0.5 M EDTA, 200 mM NaCl, 1 mM PMSF, 0.5% protease inhibitors) and incubated for 10 min at 4°C. Cells were lysed by adding sonication buffer (10 mM Tris (pH 8.1), 1 mM EDTA, 0.5 M EDTA, 0.1% SDS, 1 mM PMSF, 1× protease inhibitors) at a cell density of 1 × 10^8 cells/30 μl. Lysate was sonicated using a water bath sonicator (Diagenode). ChIP was performed using 2 × 10^6 cells and 5 μg of Ab: IgG (Upstate); PU.1 (Santa Cruz Biotechnology); transducin-like enhancer of split 4 (TLE4; Santa Cruz Biotechnology). Immunoprecipitated chromatin was washed sequentially with low salt wash (20 mM Tris (pH 8.1), 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100), high salt wash (20 mM Tris (pH 8.1), 2 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton X-100) and LiCl wash (10 mM Tris (pH 8.1), 250 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholic acid, 1 mM EDTA). DNA was eluted with elution buffer (10 mM Tris (pH 8.1), 1% SDS, 1 mM EDTA), and cross-links were reversed by incubating with 312 mM NaCl at 65°C for 4 h. The immunoprecipitated DNA was treated with RNase (Ambion) at 37°C and proteinase K (Roche) for 1 h at 45°C. The DNA was purified with QuiaQuick PCR spin columns. Purified DNA was analyzed by quantitative PCR using the following primers: PRD_PU.1-binding site FWD, 5′-GCTCAAATCCCCAGGTACAA-3′; and proximal PRDM1 FWD, 5′-AGGACCAAGACAGCTCCACTG-3′; proximal PRDM1 REV, 5′-GCTCAAATCCCCAGGTACAA-3′. Primers to the HLA-DRA promoter or an exon myoglobin locus were used as negative controls for specificity. ChIP data are calculated as relative occupancy, i.e., 2^− ΔCt − Ct-specific Ab. All the primer sets were run using an annealing temperature of 60°C.
Results

Induction of PRDM1 expression and activity in lymphoma cells

Burkitt’s lymphoma cell lines respond to signaling through the BCR via anti-IgM treatment by undergoing either growth arrest or apoptosis. Similarly, although most Burkitt’s lymphoma cell lines lack detectable levels of PRDM1, ectopic expression of PRDM1 leads to growth arrest or apoptosis (16, 17). To dissect the regulation of PRDM1 in B cell lymphoma, we have initially investigated the effects of BCR cross-linking of the EBV-negative lymphoma line, CA46. A 24-h exposure to anti-IgM results in a significant increase in annexin V staining, indicative of the early stages of apoptosis (Fig. 1A) but does not significantly increase the presence of late apoptotic or dead cells (control 3% vs anti-IgM 5%) as detected by ToPro3 staining. This finding is similar to that reported by Kaptein et al. (13) in which BCR cross-linking of CA46 cells induced growth arrest and limited apoptosis along with a decrease in c-myc expression. This treatment also significantly increases PRDM1 mRNA levels ~8-fold above untreated controls (p < 0.05; Fig. 1B). The induction of PRDM1 is also detectable at the protein expression level as revealed by immunoblot analysis (Fig. 1C). We have previously reported that a PRDM1-β isoform can also be expressed from a distinct promoter within intron 3 (21). Neither mRNA nor protein for the PRDM1-β isoform was detected in the anti-IgM-treated cells (data not shown). Whether or not the PRDM1 protein is functional after induction was determined by examining its ability to silence target gene promoters. The steady-state levels of BSAP and c-myc mRNA were examined by real-time quantitative RT-PCR. Expression of both genes decreased after BCR cross-linking consistent with suppression by PRDM1 (Fig. 1D). The observed 2-fold level of suppression is consistent with previous reports using overexpression of murine PRDM1 (Blimp-1; Refs. 7 and 30). Given the rapid turnover rate of c-myc mRNA (31), this may suggest that PRDM1 can attenuate expression of some target genes but does not necessarily silence them.

PRDM1 regulation occurs at the transcriptional level

The mechanism by which PRDM1 expression is induced in lymphoma cells is unknown and could occur at multiple levels. We first examined basal levels of nascent RNA production in both lymphoma and myeloma cells. Nascent RNAs, defined as those RNAs still in the process of being transcribed, are an accurate measure of endogenous transcriptional activity (25). The nascent RNA were purified from nuclei after extensive washing to remove the released transcripts and quantified by real-time RT-PCR with specific primers directed to the 5’ end of the RNA transcript. Levels of nascent RNA production in CA46 lymphoma cells are significantly lower than that measured in myeloma cells (Fig. 2A). This finding is consistent with the high level of PRDM1 protein expression in myeloma cells (Fig. 1C). Stimulation of the lymphoma cells with anti-IgM increased production of PRDM1 nascent RNA 3-fold after 1 and 4 h (Fig. 2B), indicating a rapid transcriptional activation. Changes in mRNA stability could also contribute to the increase in PRDM1 levels. mRNA stability changes were directly measured by inducing PRDM1 mRNA for 1 h and then blocking subsequent transcription initiation with actinomycin D. The mRNA half-life was indistinguishable before and after anti-IgM treatment of the lymphoma cells (Fig. 2C). The mRNA half-life was very short (~1 h) in the lymphoma cells and <2-fold longer in the myeloma cells. This is consistent with the recent genome-wide analysis of mRNA decay rates in mouse embryonic stem cells in which PRDM1 was one of the rare transcripts with a <1-h half-life (32). The PRDM1 mRNA is present in three predominant molecular weights which vary only in the length of the 3’-untranslated region (UTR; Refs. 21 and 33). Using real-time PCR probes spanning the 3’-UTR, we determined that the largest mRNA species had a slightly faster decay rate but that this was not affected by anti-IgM treatment (supplemental Fig. 2).6 Similarly, a proximal 3’-UTR probe that detects each of the mRNA species showed a similar decay rate and no change upon anti-IgM exposure. Although this does not exclude changes in a minor mRNA species, it indicates that anti-IgM does not have a major role in altering PRDM1 mRNA stability. Together, these data indicate regulation of PRDM1 occurs primarily at the level of transcription.

6 The online version of this article contains supplemental material.
whereas the mRNA has a relatively short half-life in both stimulated lymphoma cells and myeloma cells.

Characterization of PRDM1 promoter activity

Because PRDM1 expression is primarily regulated at the level of transcription, we cloned the human promoter to assess the regions necessary for activity. Using a series of promoter deletion constructs spanning 2618 bp upstream of the transcription start site, potential regulatory regions were identified in lymphoma and myeloma cells (for schematic, see supplemental Fig. 1). PRDM1 promoter constructs containing 521, 863, or 1528 bp display robust and similar promoter activity in the myeloma cell line, RPMI8226 (Fig. 3A). Addition of promoter sequences up to 1921 bp results in a significantly higher level of activity. Similar results were obtained in a second myeloma cell line, U266 (data not shown). PRDM1 promoter activity was also analyzed in the lymphoma cell line CA46 (Fig. 3B). The pattern of promoter activity in the unstimulated lymphoma cells is similar to that observed in the myeloma cell lines. The 521-bp promoter was sufficient for activity, and the activity increased significantly with the addition of the region between −1528 and −1921 bp, but larger constructs show a partial but consistent inhibition of activity. The overall level of promoter activity in the lymphoma cell line was lower than that in the myeloma cell lines, consistent with the levels of nascent RNAs detected at the endogenous promoter in Fig. 2A. However, comparing activity in two different cell lines requires the assumption that a cotransfected minimal thymidine kinase promoter-renilla luciferase construct. Constructs containing 2618 bp of the promoter were analyzed 24 h after stimulation. Stimulation resulted in a small but not statistically significant increase in promoter luciferase activity (Fig. 3C). This finding may indicate that a region required for induction lies outside of the 2618-bp promoter. One likely candidate is the intronic regions previously shown to bind the repressor B cell lymphoma 6 (BCL6) (34, 35). It is also possible that the transiently transfected promoter

![Figure 2](http://www.jimmunol.org/)  
**FIGURE 2.** PRDI-BF1 regulation occurs at the level of transcription. *A.* Relative levels of active transcription as measured by nascent RNA levels were determined by quantitative RT-PCR. Levels in U266 myeloma cells are significantly higher than those of unstimulated CA46 lymphoma cells. Data represent three independent experiments with SEM (p < 0.005). *B,* Anti-IgM (αIgM) induces nascent PRDM1 RNA synthesis as early as 1 h. CA46 cells were stimulated with 10 μg/ml anti-IgM for 1 or 4 h before harvest of nascent RNA and analysis by quantitative RT-PCR. Data represent three independent experiments with SEM.

![Figure 3](http://www.jimmunol.org/)  
**FIGURE 3.** Characterization of PRDM1 promoter activity. RPMI8226 myeloma cells (*A*) and CA46 lymphoma cells (*B*) were transiently transfected with the indicated PRDM1 promoter deletion constructs fused to a luciferase reporter gene. CA46 lymphoma cells were transiently transfected with p2618 construct and stimulated with anti-IgM for 24 h. Luciferase activity was measured 42 h after transfection. Data are normalized to expression of a cotransfected minimal thymidine kinase promoter-renilla luciferase construct. Construct names along the x-axis represent the number of PRDM1 promoter base pairs upstream of the transcription start site included in the construct. The region between −1528 and −1921 relative to the transcription start site was required for maximal transcription activity in both cell types. Data are the means of three independent experiments with SEM. A schematic of the promoter is shown in supplemental Fig. 1.
constructs do not fully recapitulate the chromatin structure of the endogenous gene. This may prevent further activation of these promoter constructs by anti-IgM. However, these results reveal that the lymphoma cells have the necessary components to transcribe the \textit{PRDM1} gene.

In vivo protein-DNA interactions occur across the \textit{PRDM1} promoter

To define the important \textit{cis}-acting DNA elements within the \textit{PRDM1} promoter, high resolution mapping of the protein-DNA contact sites was done by in vivo genomic footprinting. Unstimulated CA46 lymphoma cells and RPMI8226 myeloma cells were treated briefly with dimethyl sulfate to induce limited methylation of guanine residues. Close protein-DNA interactions have been demonstrated to inhibit or enhance the methylation activity, which can be visualized after chemical cleavage and resolution by sequencing gel electrophoresis. These footprints of altered methylation represent the contact points of transcription factors bound to the promoter. Examination of the first 237-bp region proximal to the transcription start site demonstrated factor binding in both the lymphoma and myeloma cells (Fig. 4A). Four clusters of interaction are detected and labeled with brackets on the left side of the sequence. These contacts are indistinguishable between the two cell types. Closest to the transcription start site, nine strongly protected guanine residues map across a sequence with homology to an Sp1 consensus binding element. Sp1 binding to this element was confirmed by in vitro EMSA and specific Ab reactivity (Fig. 5). This extends the recent findings by Mora-Lopez et al. (36) and establishes Sp1 as a regulator of \textit{PRDM1} transcription in vivo. Three additional occupied sites have been designated P.A, P.B, and P.C.

\textbf{FIGURE 4.} In vivo genomic footprinting of the \textit{PRDM1} promoter reveals multiple protein-DNA interactions. The RPMI8226 myeloma and CA46 lymphoma cell lines were analyzed with eight different primer sets to reveal interactions across the proximal 2618 bp of the \textit{PRDM1} promoter. Three regions that revealed contacts are shown: A, +15 to −170 bp; B, −1717 to −1951 bp; and C, −1899 to −2072 bp. In each pane, the control lanes (cont.) show the guanine residue sequence from deproteinized in vitro methylated DNA. The DMS lanes show the in vivo methylated residues. Protections (○) and enhancements (●) are shown on the right side of each footprint panel and are indicated in the sequence below. Clusters of contacts have been assigned arbitrary names as indicated along the left side and are boxed in the sequence. Bent arrow in A, position of the transcription start site. A schematic of the footprint primers is shown in supplemental Fig. 1.

\textbf{FIGURE 5.} Sp1 interacts at the \textit{PRDM1} proximal promoter. EMSA using an oligonucleotide spanning the Sp1 consensus sequence identified at position −52 to −43 in the \textit{PRDM1} promoter. \textit{Lanes} 1 and 2 contain 0 and 2 μl of nuclear extract, respectively. The binding reactions in \textit{lanes} 3 and 4 were incubated with the specific Ab indicated at the top of each lane. Unlabeled competitor oligonucleotides as indicated at the top of \textit{lanes} 5–10 were added to the binding reaction at 150- or 300-fold molar excess. \textit{Bottom}, Sequence of the Sp1 oligonucleotide and the mutant (mt) probe. wt, Wild type; labeled arrowhead, Sp1-containing complex; smaller arrowhead, a related specific GC-box binding protein antigenically unrelated to Sp1; α, anti.
and P.C. These contact sites do not have obvious homology with known elements.

The distal promoter region from −1497 to −2641 bp was next examined by genomic footprinting using eight overlapping primer sets. Five additional clusters of contact were detected in the distal promoter (Fig. 4, B and C, and data not shown). The region that conferred transcriptional activation (−1528 to −1921) contained three contacts designated P.J, P.H, and P.G. Site-P.J, associated with contacts at −1805 and −1802 and site-P.G associated with contacts at −1648, −1645, −1643, and −1641 overlap with AP1 consensus sequences. Both of these sites were previously predicted due to sequence homology by Vasanwala et al. (37), but neither direct assessment of AP1 binding nor functional activity in B cells or unstimulated myeloma cell lines were done. These sites show conservation across species (supplemental Fig. 3). Our data demonstrate that both sites are occupied in vivo. Site P.H associated with strong contacts at −1733, −1732, −1731, and −1728 has sequence homology to consensus binding sites for Ets family members known to regulate multiple genes in the B cell lineage. These sites also show conservation across species (supplemental Fig. 3). The region associated with a partial repression of transcription in the lymphoma cell line (−1921 to −2618) contained only two consistent clusters of in vivo contacts, designated sites P.D and P.F. Site P.F was strongly protected in the CA46 lymphoma cell line and weakly protected in the myeloma cell line. Mutation of the P.F site did not effect promoter activity of the 2618 PRDM1-luciferase construct in either lymphoma or myeloma cell lines (data not shown). Site P.D associated with contacts at −2038 and −2032 only in the lymphoma cell line.

**PU.1 binds to the P.H site and regulates PRDM1 promoter activity**

To identify the transacting factor bound at site P.H, both in vivo and in vitro assays were used. First EMSA binding assays were performed with a 30-bp probe spanning the P.H site in conjunction with nuclear extracts from CA46 lymphoma cells (Fig. 6A). A fast-migrating protein-DNA complex was detected which was specifically competed by unlabeled P.H probe but not a mutated P.H probe. The P.H site has homology to consensus Ets family binding sequences. Ab to PU.1 induced the formation of a supershifted probe. The P.H site has homology to consensus Ets family binding sequences. Ab to PU.1 induced the formation of a supershifted protein-DNA complex, indicating that PU.1 is contained within the complex bound to P.H in vitro (Fig. 6A). Similar results were obtained with nuclear extracts from both the myeloma cell line RPMI8226 (data not shown).

PU.1 association at the P.H site was also measured at the endogenous PRDM1 promoter. ChIP was performed with Abs specific to PU.1, and association with the P.H site was assessed by quantitative PCR (Fig. 6B). In lymphoma cells, PU.1 factor binding at the PRDM1 promoter was ~9-fold greater than the negative control promoter HLA-DRA. Similarly, PU.1 binding was also observed in the myeloma cell line. We next examined normal primary B cells for PU.1 binding in vivo. ChIP analysis

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**FIGURE 6.** Site P.H is a PU.1 factor-binding site. A, In vitro binding assays were done using an oligonucleotide containing the P.H site sequence identified at position −1745 to −1737 of the PRDM1 promoter and CA46 nuclear extracts. Lanes 1 and 4 contain 2 μl of nuclear extract. Unlabeled competitor oligonucleotides as indicated at the top of lanes 5–10 were added to the binding reaction at 150- or 300-fold molar excess. A single complex indicated by an arrowhead is specifically competed by the wild-type (wt) but not a mutant (mt) or unrelated oligonucleotide. In lanes 2 and 3, Abs as indicated at the top were added to the binding reaction. The formation of the specific P.H complex is inhibited by addition of PU.1 Ab. Bottom, Wild type and mutant P.H site sequences. B, ChIP was performed in CA46 lymphoma cells (left) and RPMI8226 myeloma cells (right) using PU.1 Ab and quantitative PCR primers spanning the P.H site. PU.1 binding at the P.H site was significantly higher than on the negative control promoter HLA-DRA (DRA). Data are means of three independent experiments with SEM (p < 0.01). C, ChIP in activated primary human B cells. The experiment is as described in B except a myoglobin B locus is shown as the negative control. Lanes labeled αIgM (for anti-IgM) were treated for 24 h with anti-IgM, whereas lanes labeled control (cont.) were mock treated for 24 h. PU.1 binding is specifically detected at the P.H site and is not altered by anti-IgM treatment. Similar data were obtained in two independent donor samples. D, Immunoblot analysis of PU.1 expression.
clearly identified PU.1 binding at the region of the P.H site, but essentially no binding was observed at the negative control locus (Fig. 6C). PU.1 binding was unchanged by anti-IgM treatment. Consistent with this finding, the level of PU.1 protein expression in the primary B cells did not change with anti-IgM treatment (Fig. 6D).

Functional assessment of site P.H was performed initially by mutating the site in the context of either the p1921 and p2618 PRDM1 promoter-luciferase constructs. Transfection of these mutated constructs into the lymphoma cell line revealed a reduction in transcriptional activity, indicating that this site is required for maximal activity of the PRDM1 promoter (Fig. 7A). PU.1 function in the activation of the endogenous PRDM1 gene was also examined by inhibiting PU.1 protein expression using siRNA. CA46 lymphoma cells were transiently transfected with either wild-type or mutant site P.H p2618 PRDM1 promoter luciferase constructs. In addition, the cells received siRNA against PU.1 or the nontargeting control as indicated below each graph. siRNA against PU.1 diminished wild-type promoter activity. Mutation of the P.H site also significantly lowered transcription but addition of siRNA to PU.1 did not further diminish activity in the context of a mutated P.H site. Data are normalized as in Fig. 3 and represent four independent experiments with SEM.

FIGURE 7. Site P.H and transcription factor PU.1 are involved in anti-IgM-mediated transcriptional activation of PRDM1. A, The p1921 and p2618 PRDM1 promoter-luciferase constructs containing a wild-type (wt) or mutated (mut) sequence at the P.H site were transfected into CA46 cells. Mutation of the P.H site in either construct decreased promoter activity. The mutation is the same as shown in Fig. 6A. The lane marker denoted as basic represents the activity from the promoterless vector, pGL3-Basic. Promoter activity was normalized as in Fig. 3 and represents six independent experiments with SEM. B, Endogenous PRDM1 expression is inhibited by loss of PU.1. CA46 lymphoma cells transfected with either nontargeting control siRNA or an siRNA specific to PU.1 for 24 h and then either stimulated with anti-IgM (αIgM) or untreated (control) for an additional 24 h. PRDM1 mRNA was assessed by quantitative RT-PCR. Data was normalized to GAPDH and shown as fold induction relative to untreated (control) sample. Data are the means of three independent experiments with SEM. C, Immunoblot analysis of PU.1 and PRDI-BF1 expression. PU.1 siRNA decreased PU.1 protein levels and diminished induction of PRDI-BF1 in response to anti-IgM. Experimental conditions are as in B.

Similarly, to demonstrate the involvement of PU.1 in the expression of PRDM1 in myeloma cells, RPMI8226 were cotransfected with PRDM1 full-length luciferase-promoter construct and siRNA specific for PU.1. PU.1 knockdown in these cells decreases the PRDM1 promoter activity by ~60% (Fig. 8A).
P.H site results in a >80% loss of PRDM1 promoter activity. Knockdown of PU.1 does not further alter PRDM1 transcription in the context of a mutated P.H site. This confirms that the P.H site is functional in myeloma cells and that PU.1 exerts its effects through the P.H site. Loss of PU.1 also decreases endogenous PRDM1 mRNA levels in the RPMI8226 cell line (Fig. 8B), further confirming a role for PU.1 in regulating PRDM1 transcription.

Loss of TLE4 at the P.H site in response to transcriptional stimulation

PU.1 has been described to function both as an activator of transcription and as a repressor (38, 39). These divergent activities have been linked to differential PU.1-mediated recruitment of the corepressor TLE4 and the coactivator CBP. The observed PU.1-dependent activation in response to anti-IgM might be due to changes in PU.1 binding to the PRDM1 promoter or to changes in the coactivator or corepressors recruited by PU.1. To address this question, we used chromatin immunoprecipitation to profile binding of the factors in response to anti-IgM. PU.1 binding at the P.H site on PRDM1 distal promoter is unaffected by anti-IgM treatment. Data represent the means of six (P.H site) or four (prox. promoter) independent experiments with SEM. B, ChIP assay using TLE4 Ab to detect binding at P.H site. The same samples assessed in A were reassessed for TLE4 binding. TLE4 binding at the P.H site significantly decreases upon treatment with anti-IgM. Data are the means of four independent experiments with SEM.

Discussion

The transcription factor PRDI-BF1/Blimp-1 is required for the differentiation of a mature B cell to a plasma cell (3). It does this by directly repressing downstream targets, which in turn has a widespread effect on further downstream targets (40). These downstream effector cascades have been well studied; however, very little is known as to how PRDI-BF1/Blimp-1 expression is regulated.

This study demonstrates a direct link between BCR cross-linking by anti-IgM and transcriptional activation of PRDI-BF1/Blimp-1. Treatment of CA46 lymphoma cells with anti-IgM significantly up-regulated PRDM1 mRNA and protein levels and induced apoptosis. This is consistent with observations in other B cell lymphoma cell lines (10–14). One previous study using the EBV-negative CA46 cell line reported that this line was unique in responding to anti-IgM with only growth arrest, raising the possibility that EBV-negative B cell lymphomas were heterogeneous in the apoptosis response (13). However, this report used very late markers of apoptosis (DNA fragmentation), whereas we measured early markers, suggesting that only the kinetics of apoptosis induction may vary. The anti-IgM-induced growth arrest and apoptosis has been linked to down-regulation of c-myc (13). This is consistent with the induction of PRDI-BF1/Blimp-1 and its known role in directly repressing c-myc transcription (6). Suppression of PRDI-BF1 target genes was ~2-fold, which is consistent with previous reports using overexpression of murine PRDM1 (Blimp-1; Refs. 7 and 30). The lower levels of PRDI-BF1 induced by anti-IgM treatment could also be responsible for the attenuation of the suppressive activity of PRDI-BF1/Blimp-1. Alternatively, post-translational modifications of PRDI-BF1 after anti-IgM treatment could alter the functional activity of PRDI-BF1, although no such modifications have been described to date. Future investigations of PRDI-BF1/Blimp-1 posttranslational modifications may reveal additional levels PRDI-BF1/Blimp-1 regulation. The increase in PRDM1 expression occurs primarily at the level of transcription, given that we did not detect any change in mRNA stability but actively transcribing nascent RNA levels were induced within 1 h. Unexpectedly, in vivo genomic footprinting revealed that the PRDM1 promoter was extensively occupied by transcription factors even in the absence of stimulation or promoter activity. Together these findings indicate that the PRDM1 promoter is in an open and poised state in the lymphoma cells. This provides support that therapeutic approaches to trigger endogenous PRDM1 expression are feasible and could be a viable approach to induce apoptosis in lymphoma cells. Furthermore, PRDI-BF1 has been shown to be an important target in immunotherapy of myeloma by induction of PRDI-BF1-specific CTLs, an approach that could also be exploited to kill lymphomas after PRDI-BF1 induction (41).

The transcription factors and cis-acting elements controlling PRDM1 promoter activity have only begun to be investigated. A region of the murine PRDM1 promoter spanning −918 to +207 bp was previously shown to have minimal promoter activity but did not confer any cell type-specific activity (33). This is consistent with our finding that the sequences between −1528 and −1921 bp of the human promoter are required for activation of the promoter in lymphoma and myeloma cells. In vivo genomic footprinting of the proximal promoter region revealed four occupied elements within the first 170 bp. These include a bound Sp1 site proximal to the transcription initiation point, consistent with the absence of a
canonical TATA box element and the recent findings by Mora-Lopez et al. (36). In vivo genomic footprinting of the upstream activation domain revealed three occupied elements in both lymphoma and myeloma cells. Sites P.J and P.G both have homology to an AP1 consensus binding site sequence. These sites were previously predicted by sequence homology (37). Investigation of the murine PRDM1 promoter has also provided evidence that c-fos can regulate the gene (42). The authors identified an AP1-binding site at a region homologous to the site we designated P.G and demonstrated that c-fos can bind to this site. c-fos was required for maximal activity of the murine PRDM1 promoter. Together, these findings strongly support that the PRDM1 gene expression is directly regulated by AP1 through sites P.J and P.G.

The third in vivo occupied element within the PRDM1 promoter required for transcriptional activation is site P.H. In vivo ChIP assays and in vitro DNA binding assays established that Ets family member PU.1 specifically binds to site P.H. Basal and anti-IgM stimulated PRDM1 promoter activity was significantly inhibited by mutating the P.H site in lymphoma cells. Additionally, knockdown of PU.1 expression by siRNA decreased PRDM1 transcription after BCR cross-linking by anti-IgM. This indicates that PU.1 and the Ets site is a critical and required component of the PRDM1 promoter, which must be present for the promoter to fully respond to anti-IgM stimulation. However, this site is not sufficient for the anti-IgM response. These data do not exclude the possibility that other Ets family members may also function in regulating PRDM1 expression such as Elf-1 which has a DNA recognition sequence similar to that of PU.1. In the myeloma cell line RPMB226, the P.H site and PU.1 expression were also required for maximal promoter activity. PU.1 has an important role in regulating early B cell development and continues to be expressed throughout B cell maturation (43). A recent report has shown that PU.1 is also expressed in primary human plasma cells but that expression in myeloma cells and cell lines is variable (44). Our results suggest that PU.1 may contribute to the initial activation of PRDM1 expression in B cells. Furthermore, PRDM1 expression in myeloma cells is significantly enhanced by PU.1. PU.1 is bifunctional and can either increase or repress transcription of its target promoters (39, 45).

This opposing activity is mediated by differential recruitment of coregulators by PU.1. The coactivator CBP, a histone acetyltransferase, binds to PU.1 and promotes activation of the promoter (39). In contrast, PU.1 can also recruit TLE4, a corepressor which in turn recruits the histone deacetylases HDAC1 and HDAC2 (38). We observed TLE4 recruitment to the PRDM1 promoter at the region of PU.1 binding. This recruitment was significantly diminished upon activation by anti-IgM, whereas CBP binding was largely unaffected. This indicates that the components of the PU.1 complex bound at the PRDM1 promoter are modulated by BCR cross-linking to promote gene activation. Conversely, knockdown of PU.1 in unstimulated B cells did not induce PRDM1 expression, suggesting that the PU.1-TLE4 complex is not acting as a dominant repressor.

BCL6 can repress PRDM1 expression (46). Vasanwala et al. (37) showed that AP1 and BCL6 could interact and suggested that the two AP1-like sites, which we have designated as P.J and P.G, may mediate BCL6 repression of PRDM1. Bach2 has also been suggested to repress murine PRDM1 though association with v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog k (avian) bound at the site homologous to site P.G (47). Our in vivo data support the hypothesis that the sites are bound by transcription factors, but the region conferred activation, not repression, in the BCL6-positive CA46 cell line. This suggests that BCL6 does not repress through these elements, although cell line differences between the reports may have an effect. Recent direct evidence of BCL6 binding to intron 5 of human PRDM1 or introns 3 and 5 of murine PRDM1 is consistent with the absence of a dominant repression domain detected by our promoter studies (34, 35). Our studies revealed that the distal region of the promoter (~1921 to ~2686 bp) partially decreased overall promoter activity in the B cell line. This region contained only two clear in vivo occupied elements, and the site P.D was observed only in the B cell line but not in the myeloma line. The factor binding at site P.D remains to be elucidated, but the sequence does have partial homology to the transcription repressor zinc finger E-box binding homeobox 1 (ZEB1; Ref. 48).

In conclusion, we have demonstrated PRDM1 regulation occurs primarily at the transcriptional level in lymphoma and myeloma cells. We show for the first time that PRDM1 is transcriptionally regulated by PU.1 and the corepressor TLE4. Furthermore, we have shown that two AP1 sites and an Sp1 site within the PRDM1 promoter are occupied in vivo. Importantly, we report that the promoter is poised for activation in lymphoma cells, suggesting that inducing PRDI-BF1 expression in lymphoma cells lacking PRDM1 gene mutations is a viable therapeutic approach to inducing apoptosis in these cells.

Acknowledgments

We thank the H. Lee Moffitt Cancer Center Flow Cytometry and Molecular Biology Core Facilities.

Disclosures

The authors have no financial conflict of interest.

References


