Positive Regulatory Domain I Binding Factor 1 Silences Class II Transactivator Expression in Multiple Myeloma Cells*

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The major histocompatibility complex (MHC) class II transactivator (CIITA) acts as a master switch to activate expression of the genes required for MHC-II antigen presentation. During B-cell to plasma cell differentiation, MHC-II expression is actively silenced, but the mechanism has been unknown. In plasma cell tumors such as multiple myeloma, the repression of MHC-II is associated with the loss of CIITA. We have identified that positive regulatory domain I binding factor 1 (PRDI-BF1), a transcriptional repressor, inhibits CIITA expression in multiple myeloma cell lines. Repression of CIITA depends on the DNA binding activity of PRDI-BF1 and its specific binding site in the CIITA promoter. Deletion of a histone deacetylase recruitment domain in PRDI-BF1 does not inhibit repression of CIITA nor does blocking histone deacetylase activity. This is in contrast to PRDI-BF1 repression of the c-myc promoter. Repression of CIITA requires either the N-terminal acidic and conserved PR motif or the proline-rich domain. PRDI-BF1 has been shown to be a key regulator of B-cell and macrophage differentiation. These findings now indicate that PRDI-BF1 has at least two mechanisms of repression whose function is dependent on the nature of the target promoter. Importantly, PRDI-BF1 is defined as the key molecule in silencing CIITA and thus MHC-II in multiple myeloma cells.

One of the essential factors required for the transcription of MHC-II genes is the class II transactivator, CIITA (1, 2). MHC-II expression correlates with CIITA expression for both constitutive expression on B-cells and cytokine-induced expression in other cell types (3–5). With only a few exceptions to the rule CIITA functions as a “master regulator” for MHC-II expression (2). During B-cell development MHC-II expression is turned on at a very early stage and turned off when B-cells terminally differentiate into plasma cells (6, 7). The process of extinguishing MHC-II expression in plasma cells is poorly understood. Cell-cell fusion between MHC-II-negative myeloma cells and MHC-II-positive B-cells have indicated the presence of a dominant repressor in the myeloma cell. (8–12). It was later shown that CIITA mRNA transcripts are absent in myeloma cells, and introduction of CIITA rescues MHC-II expression (13). Thus, the dominant repressor of MHC-II expression appears to act by repressing CIITA expression.

CIITA has four distinct promoters, each transcribing a unique exon 1, with three of the forms predominating (14). CIITA expression in B-cells is predominantly from promoter 3 (14, 15). We have recently characterized the CIITA promoter 3 (CIITAp3) in B-cells (16). In vivo, two elements on the promoter, ARE-1 and ARE-2, are occupied and essential for CIITA expression. However in the myeloma cell line NCI-H929, the CIITAp3 is completely unoccupied suggesting that CIITA transcription in myeloma cells may be mediated through changes in promoter assembly (16).

One of the transcription factors induced when B-cells differentiate into plasma cells is PRDI-BF1. It has been shown that introduction of the murine homologue of PRDI-BF1 (Blimp-1) into B-cell lymphoma cell lines leads to many phenotypic changes associated with differentiation into early plasma cells, (17). Despite its dramatic effect on B-cells, only two targets for PRDI-BF1 have been identified, c-myc (18) and IFN-β (22). In this report we now show that PRDI-BF1 directly suppresses CIITA transcription. This results in the silencing of MHC-II expression is long observed in plasma cells and multiple myeloma and as such may be an attractive target for therapeutic regulation of MHC-II.

EXPERIMENTAL PROCEDURES

DNA Constructs and Cell Lines—The CIITAp3 reporter constructs (16), the c-myc PRF reporter construct (18, 19), pc-PRDI-BF1, pcFLAG-PRDI-BF1(331–789), and pcFLAG-PRDI-BF1(398–789) constructs (20) have been described previously. The pc-PRDI-BF1 vector was FLAG-tagged at the C-terminal end of PRDI-BF1. The three protein deletions, pcPRDI-BF1ΔPR1 (deletes amino acids 36–166), pcPRDI-BF1ΔPro (deletes amino acids 308–399), and pcPRDI-BF1ΔZnF (deletes amino acids 508 to 691), were generated in the pc-PRDI-BF1-FLAG construct. All constructs have been confirmed by sequencing. Raji, IM-9, and CA46 are B-lymphoblastoid cells. U266, 8266, SKO, and NCI-H929 cells are multiple myeloma cells. All cells were grown according to ATCC specifications.

Transient Transfection—Cells were transfected by electroporation as described previously (16). Transfections were normalized by cotransferring a constant amount of pRL-TK construct (Promega). To make direct comparisons between the effect of PRDI-BF1 on c-myc and CIITA promoters, the control lanes were set at 100 for both TSA− and TSA+ samples.

Generation of Antibodies to PRDI-BF1—Two peptide sequences N-terminal 1–17 residues and C-terminal 729–737 residues were selected to raise polyclonal antibodies in rabbits (Research Genetics). The antibodies were affinity purified from the serum.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts were prepared according to Dignam et al. (21). EMSA was performed as described previously (16) using syn-

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‡ The abbreviations used are: MHC, major histocompatibility complex; CIITA, class II transactivator; CIITAp, CIITA promoter; PRDI-BF1, positive regulatory domain I binding factor 1; EMSAs(s), electrophoretic mobility shift assay(s); bp, base pair HDAC, histone deacetylase; ARE, activator response element.

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thetic oligonucleotides, 2 μg of poly(dI-dC), and nuclear extract or in vitro-translated proteins. The PRDI-BF1 oligonucleotide spans from −190 to −158 base pairs of the promoter. Unlabeled oligonucleotide competitors were used in a 50–100-fold molar excess.

RNA Analysis—RNA was isolated using Trizol reagent (Life Technologies, Inc.). Ribonuclease protection analysis was performed using a 32P-labeled probe containing the C-terminal (Kpn-I and Xho-I) portion of PRDI-BF1. The RNA probe was hybridized with 15 μg of sample RNA, and digestion was performed using RNase A/T1 (Ambion) according to manufacturer’s specifications.

Western Blot and Immunoprecipitation—Whole cell extract was prepared in phosphate-buffered saline with 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and Complete EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals). Anti-FLAG M2-agarose affinity beads (Sigma) were used to immunoprecipitate overexpressed FLAG-tagged proteins. Endogenous PRDI-BF1 was immunoprecipitated with protein A-agarose beads and the N- or C-terminal PRDI-BF1 antibody at 4 °C for 4 h. The immunoprecipitated proteins were resolved by a 10% SDS polyacrylamide gel electrophoresis, which was transferred to a polyvinylidene difluoride membrane. The membrane was incubated with the indicated primary antibody for 2 h followed by an anti-rabbit IgG horsearid peroxidase antibody. The secondary antibody was visualized by ECL plus (Amersham Pharmacia Biotech). For direct Western blots or immunoprecipitations 1 × 106 or 5 × 106 cells were used per lane, respectively.

In Vitro Transcription/Translation Reactions—In vitro transcription/translation of PRDI-BF1 and PRDI-BF1APR proteins were performed using the TNT quick-coupled transcription/translation system (Promega) according to manufacturer’s specifications. pCDNA blank vector was used as a control. The 32P-labeled proteins were resolved by an SDS polyacrylamide gel electrophoresis and visualized by autoradiography to estimate the yield of full-length product prior to use in the DNA binding assays.

RESULTS AND DISCUSSION

PRDI-BF1 Represses the CIITA Promoter in Myeloma Cells—We have previously reported that endogenous CIITA mRNA levels in myeloma cell lines are significantly lower compared with B-cells (16). Similarly, we observed that a CIITAp3 promoter construct with 1140 or 545 bp of promoter sequences had low activity in myeloma cells, similar to an SV40 minimal promoter whereas in B-cell lines the CIITA promoter was 2–4 times more active in comparison (data not shown). This suggested that the repression of CIITA transcription might be contained within the first 545 bp of the promoter. Progressive 5′ deletions of CIITAp3 were transiently transfected into two myeloma cell lines and tested for promoter activity. As shown in Fig. 1A deletion of the region from −195 to −151 bp resulted in a 7-fold increase in promoter activity in U266 cells and a 3.2-fold increase in NCI-H929 cells. Further deletion down to position −113 bp removes the previously identified activator element ARE-1 and severely diminished promoter activity. This indicates that the factors necessary for activation of CIITAp3 are present in the myeloma cells, but overall activity is silenced by elements between −195 and −151. Homology searches suggested that the region between −180 and −168 bp could be a putative binding site for the transcriptional repressor, PRDI-BF1. To test whether PRDI-BF1 was able to repress CIITAp3, the protein was transiently overexpressed in B-cells. Introduction of PRDI-BF1 repressed transcription from the CIITA promoter (CIITAp3.195) by 60% as compared with vector control (Fig. 1B). Deletion of the potential PRDI-BF1 binding site (CIITAp3.151) leads to the inability of PRDI-BF1 to significantly repress CIITA transcription. These findings and the observation that the murine homolog of PRDI-BF1 is low or absent in B-cells and increases in plasma cells (17) suggest that endogenous PRDI-BF1 in human myeloma cells could be repressing CIITA transcription by binding to this site on the promoter.

PRDI-BF1 mRNA and Protein Levels Are High in Human Myeloma Cells—The relative amount of PRDI-BF1 mRNA in myeloma cells and B-cells was determined with a ribonuclease protection assay (Fig. 2A) and also confirmed by Northern blotting (data not shown). Each of the myeloma cell lines contained PRDI-BF1 mRNA whereas it was undetectable in B-cell lines. We next examined the level of PRDI-BF1 protein present in myeloma cells by immunoprecipitation. PRDI-BF1-specific antibodies were raised against N- and C-terminal peptides. Both antibodies specifically immunoprecipitated a band of ~100 kDa from myeloma cells. The band comigrates with PRDI-BF1 protein expressed from the cloned cDNA when transfected into a B-cell line (Fig. 2B, lanes 1 and 2). Importantly, we also tested for the presence of PRDI-BF1 in bone marrow samples from myeloma patients. Although these samples contain only 68 to 92% myeloma cells, PRDI-BF1 was clearly visible in 2 of 3 patients (Fig. 2C). Thus PRDI-BF1 is present in myeloma cells concomitant with the loss of CIITA expression.

PRDI-BF1 Binds to the CIITAp3 in Vitro—Identification of direct PRDI-BF1 binding to the region between −195 and −151 bp of the promoter was done with a series of in vitro protein/DNA binding studies utilizing the EMSA. Incubation of an oligonucleotide spanning −190 to −158 bp with nuclear extracts from myeloma cells revealed two specific complexes, which were competed by an unlabeled self-oligonucleotide (Fig. 3A). Competition with consensus binding sites such as an interferon response factor element (lane 3) that have similarity to the PRDI-BF1 site did not alter complexes. To directly identify that PRDI-BF1 is part of the complexes bound to DNA the U266 nuclear extracts were immunodepleted of PRDI-BF1 by using either the N- or C-terminal antibody. Western blots indicate that nearly all of the PRDI-BF1 was removed (Fig. 3B). EMSAs performed using these depleted extracts showed that the upper complex completely disappeared, whereas the lower complex was reduced in intensity (Fig. 3C, lanes 1–3). Confirmation that only PRDI-BF1 was depleted and that the extract was not degraded was achieved using either a B-cell specific activator protein (lanes 4–6) or nuclear factor-1 (not shown) consensus binding site in the EMSA assay. The difference
between the two complexes containing PRDI-BF1 is not known as yet, but it is possible that PRDI-BF1 is found in multiple isoforms or that it can complex with other proteins.

Repressive Domains in PRDI-BF1—The PRDI-BF1 protein had been first identified by its ability to bind and repress the PRDI site within the IFN-β promoter, following viral induction (22). It is a 789-amino acid protein (depicted in Fig. 4A) bearing N- and C-terminal acidic domains, a PR domain homologous to RIZ and MDSI-EVI proteins (23), a proline-rich domain between amino acids 331 and 389, and a C-terminal zinc finger DNA binding motif. It has been demonstrated that the proline-rich domain of PRDI-BF1 is able to recruit the Groucho family of corepressors (20), which in turn can interact with histone deacetylases (24). This suggested that the proline-rich domain was critical for transcriptional repression. To identify the domains responsible for PRDI-BF1-mediated repression of CIITA we constructed three individual domain deletions in the protein and obtained two N-terminal deletions of the protein that maintain DNA binding activity (a kind gift from T. Maniatis). We transiently overexpressed these proteins with the CIITA(3.195 reporter in CA46 B-cells (Fig. 4A and B). Several observations have been made. First, deletion of the zinc finger DNA binding motif abolishes PRDI-BF1 activity (lanes 7 versus 2). Second, deletion of the acidic and PR domains does not affect repressive ability (lanes 3 versus 2), but deletion of the acidic, PR, and proline-rich domains completely ablates PRDI-BF1 function (lanes 4 versus 2). Finally, internal deletion of only the proline-rich domain does not affect repression (lanes 6 versus 2), whereas internal deletion of the PR domain partially (30%) abrogates PRDI-BF1 function (lanes 5 versus 2). These findings indicate that a complex interplay of multiple repression domains exists within the N terminus of the protein. The proline-rich alone (lanes 3 versus 4) or the acidic and PR domains alone (lanes 6 versus 2) are sufficient for full activity. Because internal deletion of the PR domain maintains only partial function, this suggests that PR domain is a key contributor to repressive activity of PRDI-BF1. However, which domain is most critical for CIITA repression remains unclear.

Because deletion of the PR domain partially ablates the ability of the protein to repress CIITA, it is essential to know whether this effect is because of deletion of the PR domain or mis-folding of the recombinant protein. One way to address the structure of the recombinant protein is to determine whether it maintains DNA binding activity. EMSA was performed using in vitro-translated PRDI-BF1 and PRDI-BF1ΔPR proteins to make a quantitative comparison between the DNA binding activity of the two. Similar levels of both proteins of the expected molecular weight were expressed by in vitro translation (Fig. 4C). As observed in Fig. 4D both proteins had comparable DNA binding activities. This finding provides evidence that the loss of activity is not because of catastrophic mis-folding although minor alterations are possible. In addition, all the recombinant proteins were abundantly expressed when transfected into CA46 B-cells. Detection of the protein expression levels was performed by Western blot (Fig. 4B).

PRDI-BF1-mediated Repression of CIITA(3 does Not Involve Histone Deacetylase Activity—It has been recently shown that the murine homologue of PRDI-BF1 (Blimp-1) is able to repress c-myc expression by recruitment of histone deacetylase (25). This recruitment was reported to be through the proline-rich domain. Our data in Fig. 4A indicates that the deletion of the proline-rich domain did not affect the repression of CIITA. Although the proline-rich domain might have multiple functions, this suggests that histone deacetylases might not be required for CIITA repression. To test the importance of HDAC activity more directly we inhibited HDAC activity and exam-
ined the effects on c-myc and CIITA transcription. Addition of a histone deacetylase inhibitor, TSA, had no effect on transcriptional repression of CIITA; however, consistent with the previous study it completely abolished repression of c-myc (Table I).

### Table I

<table>
<thead>
<tr>
<th>Cells</th>
<th>Reporter</th>
<th>Control</th>
<th>No TSA</th>
<th>-Fold repression</th>
<th>TSA</th>
<th>-Fold repression</th>
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<tr>
<td>CA46</td>
<td>CIITAp3</td>
<td>100</td>
<td>48.9 ± 4.0</td>
<td>2.0</td>
<td>42.8 ± 8.7</td>
<td>2.3</td>
</tr>
<tr>
<td>CA46</td>
<td>c-myc</td>
<td>100</td>
<td>42.6 ± 11.6</td>
<td>2.3</td>
<td>122.8 ± 15.4</td>
<td>0.8</td>
</tr>
<tr>
<td>U266</td>
<td>CIITAp3</td>
<td>100</td>
<td>153.7 ± 27.6</td>
<td></td>
<td>143.0 ± 25.9</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 4. Repressive domains of PRDI-BF1. **A**, CA46 B-cells were transiently transfected with CIITAp3.195 (15 μg) and cotransfected with control, wild type, or various deletions of PRDI-BF1 (7 μg). Results are an average of four experiments. **B**, CA46 cells were transfected with 15 μg of the PRDI-BF1 deletion constructs and harvested 36 h later. In the left panel whole cell extracts were subjected to immunoprecipitation with anti-FLAG antibody followed by Western blot using the same antibody. In the right panel a direct Western blot was performed using antibody to the N terminus of PRDI-BF1. Arrows indicate the wild type and mutant PRDI-BF1 proteins. **C**, in vitro transcription/translation of PRDI-BF1 (lanes 3 and 4) and PRDI-BF1ΔPR (lanes 5 and 6) proteins. Lanes 1 and 2 are control (empty expression vector was used for the in vitro transcription/translation reaction). Detection of the 35S-labeled proteins was performed by SDS polyacrylamide gel electrophoresis. In the odd numbered lanes 2 μl of protein has been loaded, and in the even numbered lanes 4 μl protein has been loaded. **D**, PRDI-BF1ΔPR retains DNA binding activity. EMSA was performed using a 32P oligonucleotide containing the PRDI-BF1 binding site and the in vitro-translated proteins. Lanes 1–3, control; lanes 4–6, PRDI-BF1; lanes 7–9, PRDI-BF1ΔPR. SP, cold specific competitor.
RIZ and MDSI-EVI1, has been reported (26) to significantly alter the function of the protein and mediate protein-protein interaction (27). The PR domain shares significant sequence identity to the yeast SET domain of proteins that play an important role in determining chromosomal structure and telomeric gene silencing. However the precise mechanism by which PR domains bring about transcriptional repression has not yet been characterized. It has been shown that RIZ1 has tumor suppressor ability, and deletion of the PR domain promotes oncogenesis. A similar observation was noted in the MDS1-EVI1 gene (28). The PR domain of MDS1-EVI1 is a common target of viral insertions and chromosomal translocations in leukemogenesis (29–31) suggesting it may have an important biological function. Other regions within PRDI-BF1 can partially compensate for loss of the PR domain. This now defines the mechanism of MHC-II silencing in myeloma cells. This now defines the mechanism of MHC-II silencing in plasmacytomas first observed over 14 years ago (9, 12). By use of histone deacetylase inhibitors and specific deletions of PRDI-BF1 we have clearly demonstrated that the proline-rich domain is a good candidate for such a compensatory effect. In addition, deletion of the N-terminal acidic domain of Blimp-1 significantly reduced its ability to repress c-myc (25). Thus PRDI-BF1 (Blimp-1) appears to utilize multiple repression domains whose function is dependent on the target promoter.

In summary, we have identified that PRDI-BF1 binds to CIITA promoter and silences CIITA expression in myeloma cells. This now defines the mechanism of MHC-II silencing in plasmacytomas first observed over 14 years ago (9, 12). By use of histone deacetylase inhibitors and specific deletions of PRDI-BF1 we have clearly demonstrated that the proline-rich domain and histone deacetylase activity are not required for PRDI-BF1-mediated repression of CIITA in contrast to their action on c-myc. The findings also suggest that the highly conserved PR domain may have an important role in repression of CIITA.

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