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The PBX1 lupus susceptibility gene regulates CD44 expression

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Abstract

PBX1-d is novel splice isoform of pre-B-cell leukemia homeobox 1 (*PBX1*) that lacks its DNA-binding and Hox-binding domains, and functions as a dominant negative. We have shown that PBX1-d expression in CD4⁺ T cells is associated with systemic lupus erythematosus (SLE) in a mouse model as well as in human subjects. More specifically, PBX1-d expression leads to the production of autoreactive activated CD4⁺ T cells, a reduced frequency and function of Foxp3⁺ regulatory T (Treg) cells and an expansion of follicular helper T (Tfh) cells. Very little is known about the function of PBX1 in T cells, except that it directly regulates the expression of miRNAs associated with Treg and Tfh homeostasis. In the present study, we show that PBX1 directly regulated the expression of *CD44*, a marker of T cell activation. Two PBX1 binding sites in the promoter directly regulated *CD44* expression, with PBX1-d driving a higher expression than the normal isoform PBX1-b. In addition, mutations in each of the two binding sites had different effects of PBX1-b and PBX1-d. Finally, we showed that an enhanced recruitment of co-factor MEIS by PBX1-d over PBX1-b, while there was no difference for co-factor PREP1 recruitment. Therefore, this study demonstrates that the lupus-associated PBX1-d isoform directly transactivates CD44, a marker of CD44 activation and memory, and that it has different DNA binding and co-factor recruitment relative to the normal isoform. Taken together, these results confirm that PBX1 directly regulates genes related to T cell activation and show that the lupus-associated isoform PBX1-d has unique molecular functions.

Keywords

Lupus; PBX1; CD44; T cells

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Conflicts of interest

The authors have no conflict of interest to declare.

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1. Introduction

The pre-B-cell leukemia homeobox 1 (*Pbx1*) gene has been identified as the lupus susceptibility gene corresponding to the *Sle1a1* locus in the MZM2410 mouse model (Cuda et al., 2012). This locus leads to the production of activated autoreactive histone-specific CD4⁺ T cells in a T-cell intrinsic manner (Chen et al., 2005); Cuda et al., 2012); Cuda et al., 2007). *Sle1a1* also leads to a reduction of the frequency and function of Foxp3⁺ regulatory T cells (Tregs) (Choi et al., 2016); Cuda et al., 2007) as well as an expansion of the follicular helper T cell compartment in the mLN (Tfh) (Choi et al., 2016). These three phenotypes are key features of lupus pathogenesis (Craft, 2012); Mohan et al., 1993); Ohl and Tenbrock, 2015). It is therefore important to understand how Pbx1 is responsible for these events and what molecular interactions drive the observed phenotypes in CD4⁺ T cells.

Pbx1 is a member of the TALE family of homeodomain-containing transcription factors that modulates the DNA-binding function of Hox proteins (Mann and Chan, 1996). (Berkes et al., 2004) (Longobardi et al., 2014). Pbx1 plays a central role during development and organogenesis by integrating multiple signals through its interaction with numerous partners (Laurent et al., 2008), including Meis and Prep1 TALE proteins that regulate chromatin remodeling and co-activator access (Berkes et al., 2004) (Longobardi et al., 2014). In the development of the immune system, Pbx1 is required to maintain the self-renewal of hematopoietic stem cells (Ficara et al., 2008), restraining myeloid maturation to preserve the differentiation potential of lymphoid progenitors (Ficara et al., 2013). *Pbx1*-deficient embryonic stem cells fail to generate common lymphoid progenitors, resulting in the absence of B and NK cells, as well as an impaired T cell development (Sanyal et al., 2007). Accordingly, we have shown that mesenchymal stem cells (MSC) from mice expressing *Sle1a1* show an accelerated lineage differentiation and expression of innate inflammatory genes, as well as an impaired immunoregulatory capacity (Lu et al., 2015), which corresponds to defects reported in MSCs from lupus patients as well as lupus-prone mice (Collins and Gilkeson, 2013). These complex regulatory networks regulated by reflect the ability of Pbx1 complexed with either Meis or Prep1 to recruit a wide array of Hox and non-Hox co-factors, many of which vary between cell types, and to direct either gene activation or repression (Laurent et al., 2008).

Contrary to B cells (Sanyal et al., 2007), the function of Pbx1 in T cells is unknown, except for the requirement of Prep1/Pbx1 heterodimer DNA binding for thymic development of double negative T cells (Penkov et al., 2008). Out of the seven protein-coding splice isoforms known for Pbx1 in mice, CD4⁺ T cells express *Pbx1-b*, which lacks exons 7 and 9 that are present in the full-length *Pbx1-a* (Cuda et al., 2012). In addition, NZM2410 and *Sle1a1* CD4⁺ T cells express a novel splice isoform, *Pbx1-d*, which lacks exons 6 and 7 that encode the DNA and a HOX binding domains, respectively (Cuda et al., 2012). Pbx1 amino acid sequence is identical between human and mouse, and we found that *PBX1-d* is expressed at a significantly higher frequency in the CD4⁺ T cells of lupus patients as compared to healthy controls, validating the role of this susceptibility allele in lupus (Cuda et al., 2012). *Pbx1-d* is the only known Pbx1 isoform lacking the DNA binding domain, suggesting that it functions as a dominant negative (DN) binding partner with reduced transcriptional function. We have validated the DN function of Pbx1-d showing that it is

equivalent to a Pbx1 knock-down construct in activating MSC differentiation (Sengupta et al., 2012). At the single gene level, the DN function of Pbx1-d was also validated, both as a transcriptional activator of Sox3 in MSCs and a transcriptional repressor of CD44 in Jurkat T cells (Sengupta et al., 2012). We have started to characterize the molecular mechanism by which Pbx1 regulates T cell function, and shown that it directly transactivates the expression of miR-10a, miR-21, and miR-155, three miRNAs that have been implicated in Treg and Tfh homeostasis (Choi et al., 2016). We performed the current study to further define the role of Pbx1 in regulating T-cell specific genes and to assess the ability of the Pbx1-d isoform to recruit the Meis and Prep1 co-factors.

2. Materials and methods

2.1. ChIP-Seq and ChIP-qPCR

Chromatin immunoprecipitation was performed on Jurkat T cells with a ChIP assay kit (Millipore, Billerica, MA, USA), according to manufacturer's instructions with an anti-PBX1 polyclonal antibody (P-20, Santa Cruz, CA, USA) or rabbit IgG (Santa Cruz) as negative control in triplicates. The final libraries (average size 350 bp) were quantitated with the Kapa SYBR Fast qPCR reagents (Kapa Biosystems, Wilmington, MA, USA) with an ABI7900HT real-time PCR system (Life Tech., Carlsbad, CA, USA). In preparation for sequencing, barcoded libraries were pooled equimolarly, and diluted to 9 pM for cluster generation on the cBOT (Illumina, San Diego, CA, USA). Samples were sequenced on a single flowcell lane on the HiSeq2000 instrument, using a 2x101 cycle multiplex, paired-end configuration. A typical sequencing run in the HiSeq2000 produced 300–400 million paired-end reads per lane. For ChIP sequencing 50–100 million reads provided sufficient depth for analysis. Peak calling was performed using CisGenome (www.biostat.jhsph.edu/~hji/cisgenome/). Data was averaged between the three replicates precipitated with anti-PBX1 or IgG control and compared by *t* tests. For ChIP-PCR experiments, 10⁷ Jurkat cells were washed 2 times with cold PBS, and chromatin immunoprecipitation was performed following the cross-linking chromatin immunoprecipitation protocol (<http://www.abcam.cn>) with the same antibodies as for the ChIP-Seq experiment. Putative PBX1 binding sites in the *CD44* intron 1 and promoter were identified using Jaspar3 (<http://jaspar.genereg.net/>) set at a 75% score threshold. PCR primers are listed in Table S1 as CD44_ChIP P1, P2 and I1. SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) was used for quantification. Results were expressed as fold enrichment relative to the IgG control. *POL2* binding to *GAPDH* was used as positive control.

2.2. Luciferase assays

The *CD44* promoter region containing both P1 and P2 putative PBX1 binding sites (Fig. 1) was amplified with the CD44-PROM primers listed in Table S1, and the 912 bp fragment was ligated into the pGL4.23 luciferase reporter vector (Promega, Madison, WI, USA). HEK 293 cells were transfected with the CD44_PROM-pGL4.23 along with 0–1000 ng DNA of PBX1-b or PBX1-d expression plasmids using Lipofectamine® 2000 (Life Tech.). After 48 h, cells were lysed and the firefly and Renilla luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's

instruction. The results were presented as the ratio of Renilla to firefly luciferase activities normalized to the values for cells that were not transfected with PBX1 plasmids.

2.3. CD44 message expression

RNA was extracted from HEK 293 cells transfected with 1 ug PBX1-b or PBX1-d expression plasmids using the RNeasy mini kit (Qiagen, Germantown, MD, USA). After cDNA synthesis from 5 ug total RNA using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), real-time qPCR analysis of *CD44* expression was performed using Bio-Rad' SYBR[®] Green Supermix and its expression was normalized to *GAPDH*. Primer sequences are listed in Table S1 as CD44_qRT-PCR. Relative expression was calculated using the 2^{-C_t} method and normalized to the *RQ* expression in cells transfected with an empty vector.

2.4. Flow cytometry

Jurkat T cells stably transfected with a lentiviral vector (LV) co-expressing either PBX1-b or PBX1-d and GFP (Sengupta et al., 2012) were stained with an anti-human CD44-Phycoerythrin (PE)-conjugated antibody (IM7, eBioscience, San Diego, CA, USA) or rat IgG2b-PE isotype control. At least 50,000 events were acquired per sample on a FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA). Dead cells were excluded based on forward and side scatter characteristics. Analysis was conducted with the FCSEXPRESS software (De Novo Software, Glendale, CA, USA) comparing CD44 expression in GFP⁺ and GFP⁻ cells.

2.5. Site directed mutagenesis

The PGL4.23 plasmid containing the two PBX1 P1 and P2 binding sites in the *CD44* promoter was amplified by the Q5[®] high fidelity DNA polymerase (New England Biolabs, Ipswich, MA) with either the Q5CD44M1 primers introducing a BafI restriction site in P1, or the Q5CD44M2 primers introducing a HpyCH4V restriction site in P2. An analysis with JASPAR3 predicted that the introduced mutations abrogated PBX1 binding. The primer sequences are listed in Table S1. The PCR fragments were cloned into pGL4.23 and mutations were confirmed by restriction enzyme analysis and Sanger sequencing.

2.6. Immunoprecipitation and Western blotting

Immunoprecipitation (IP) and Western blotting (WB) was performed on two types of cells: 1) HEK 293 cells transfected with 1 ug PBX1-b or PBX1-d expression plasmids, along with FLAG-tagged MEIS or PREP-1 (a kind gift from Dr. F. Blasi, IFOM, Milano, Italy) and 2) Jurkat T cells transfected with either LV-PBX1-b-GFP or LV-PBX1-d-GFP (Sengupta et al., 2012). Cells were lysed in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl, pH 8.0) containing a protease inhibitor mixture (Roche, Branford, CT, USA) for 20 min on ice. Lysates were centrifuged at 12,000 rpm for 5 min at 4°C. Supernatants were pre-absorbed with 10% (v/v) of protein A-sepharose beads (Pierce, Waltham, MA, USA) for 1 h, then incubated with 3 µg/ml of IP antibody overnight at 4°C followed by incubation with 10% (v/v) of protein A-sepharose beads for 1 h at 4°C. The beads were then rinsed three times with the lysis buffer with protease inhibitor at 4°C before elution with an equal volume of

SDS loading buffer by boiling for 5 min. 15 µl of each sample eluate was run on a 10% SDS-PAGE gel. The gel was then transferred to nitrocellulose membrane. Membranes were blocked with 20 mM Tris, pH 8.0, containing 4% (w/v) milk and 0.05% (v/v) Tween 20. WB antibodies were incubated for 1 h at room temperature. The antibodies used for IP and WB were as follows: anti-PBX1 (P-20), anti-MEIS1/2 (N-17, Santa-Cruz), and anti-FLAG (Sigma, St. Louis, MO, USA).

2.7. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 7 software. Unless indicated, graphs show mean and standard deviation of the mean (SEM) for each group. For comparisons between two groups, paired and unpaired Student's *t*-tests were used. 2-way ANOVA with Dunnett's post-tests were used for multiple comparisons as appropriate. Each experiment was performed at least three times.

3. Results and Discussion

3.1. PBX1 binds to the CD44 promoter

We performed a ChIP-Seq analysis of Jurkat T cells and identified 125 binding sites for which the fold difference (FD) with the anti-PBX1 antibody over the IgG control was at least 2 fold and the *p* value less than 0.05 (Table S2). Pbx1 expression is low in primary T cells (Cuda et al., 2012) as well as in Jurkat T cells (Sengupta et al., 2012). A much higher number of PBX1 binding sites have been reported in cell lines in which PBX1 expression is high, such as > 20,000 binding sites in MCF7 breast cancer cells (Magnani et al., 2011) and > 2,000 promoter sites reported in OVCAR3 ovarian cancer cells (Thiaville et al., 2012). A high number of binding sites were also identified in mouse embryonic cortex primary cells expressing high levels of PBX (Golonzhka et al., 2015). Fewer binding sites could be expected in cell types such as T lymphocytes that express low levels of PBX1, as this protein may play a regulatory rather than an essential role. The PBX1 binding sites in Jurkat T cells included the PBX1 gene itself (FD = 2.12, *p* = 0.00519), as well as PAX3 (FD = 2.16, *p* = 0.012), which is a known target of PBX1 (Chang et al., 2008), and MEF2B (FD = 16.65, *p* = 0.0158), a gene involved in myogenesis, a process in which PBX1 plays a central role (Sagerstrom, 2004). Interestingly, PBX1 was also bound to the *ESRRG* locus (FD = 2.55, *p* = 3.67×10^{-5}), and we have found that in the NZM2410 mouse model, reduced expression of *Esrrg* in CD4⁺ T cells was associated with an autoimmune phenotype (Perry et al., 2012). None of the genes revealed in this analysis were T cell-specific or associated with a specific T cell function, except for the activation marker CD44 (FD = 2.92, *p* = 0.015). We have previously shown that transfection of Jurkat T cells with either PBX1-D or PBX1 shRNA transactivated *CD44* expression (Sengupta et al., 2012). For these reasons, we focused on characterizing the mechanisms by which PBX1 regulated CD44 expression. The PBX1 binding site identified by ChIP-Seq was located in intron 1 (Fig. 1a). In addition, two PBX1 binding sites, P1 and P2, were identified in the promoter (Fig. 1b). Only a modest binding was detected by ChIP-PCR at the I1 and P2 sites (Fig. 1c), but a strong binding was detected at the P1 site (Fig. 1c and d).

3.2. PBX1-d binding to the CD44 promoter enhances CD44 transcription

We performed luciferase assays to evaluate whether PBX1 binding to the *CD44* promoter affected transcription, and to compare the activity of the prevalent PBX1 isoform in T cells, PBX1-b, to that of the SLE-associated DN PBX1-d isoform. The *CD44* promoter containing the two PBX1 binding sites directing the expression of a luciferase construct transfected into 293 HEK cells in the presence of PBX1-b or PBX1-d. Both PBX1 isoforms transactivated the *CD44* promoter (Fig. 2a). Evidence of the higher activity of PBX1-d was shown in the dose-response analysis of transcriptional yields to increasing amounts of PBX1-b and PBX1-d expression plasmids (Fig. 2b). In addition, PBX1-d transfection significantly enhanced the transcription of the endogenous *CD44* gene in HEK 293 cells (Fig. 2c). Finally, we compared CD44 protein expression in Jurkat T cells transfected with PBX1-b-GFP or PBX1-d-GFP. Transfected cells with either isoform expressed more CD44 than their non-transfected GFP-negative counterparts, but cells transfected with PBX1-d expressed significantly more CD44 than cells transfected with PBX1-b (Fig. 2d). These results confirm that PBX1-d transactivates *CD44* expression significantly more than the prevalent isoform, and demonstrate that PBX1 binding to the *CD44* promoter contributes to this transactivation.

3.3. PBX1-b and PBX1-d have different requirements for the P1 and P2 promoter binding sites

To define the relative requirements of the P1 and P2 binding sites by the PBX1-b and -d isoforms, each binding site was mutated individually (Fig. 3a). Luciferase assays with 200 ng of either PBX1 expression plasmid showed that the mutation in P1 significantly reduced transactivation by PBX1-b but not PBX1-d (Fig. 3b). At this plasmid concentration, the mutation in P2 had no effect with either isoform. A dose-response analysis confirmed that PBX1-b transactivation was significantly reduced in the absence of P1 binding, while it had little effect on PBX1-d transactivation (Fig. 3c). The same dose response analysis confirmed that the abolition of P2 binding has little effect on PBX1-b transactivation, but it significantly increased PBX1-d transactivation in the presence of higher doses of expression plasmid (Fig. 3d). It is possible speculated that the mutation in the P2 promoter prevented PBX1-b binding to fully potentiate transactivation by PBX1-d. It should be noted that at all doses that were tested, transactivation by PBX1-d was significantly higher than transactivation by PBX1-b, for either P1 or P2 mutants as well as for the unmutated WT sequence (Fig. 3c and d). Although PBX1-d does not have a DNA binding domain, it can still bind DNA as a heterodimer complex with MEIS, since MEIS and PBX1 share an identical consensus binding site (Penkov et al., 2013). The PBX1 binding sequence requirement is complex and entirely depends on its co-factors, as the sequence for PBX1 binding alone has not been defined (Longobardi et al., 2014). PBX1-b and PBX1-d share the same sub-optimal HOX-binding ability (Di Rocco et al., 1997), but the reliance of PBX1-d on cofactors for DNA binding may direct PBX1-d complexes to different binding sites than PBX1-b complexes, which is suggested by our results with the *CD44* promoter.

3.4. PBX1-d enhances MEIS recruitment

We compared the interactions of PBX1-b and -d isoforms with their binding partner MEIS and PREP1. Both PBX1-b and PBX1-d were associated with MEIS in Jurkat T cell lines

stably transfected with either isoform. However, PBX1-d pulled down more MEIS than PBX1-b did (Fig. 4a) and conversely, MEIS pulled down more PBX1-d than PBX1-b (Fig. 4b). PREP1 was not detected in Jurkat T cells by WB (data not shown). This is consistent with PREP1 being preferentially involved in the transcriptional regulation of house-keeping genes, while MEIS/PBX1 complexes transactivate developmentally regulated genes (Penkov et al., 2013), if we postulate that effector T cell differentiation programs share some features of the embryonic developmental programs.. To better compare recruitment of MEIS and PREP1 to the PBX1-b and -d isoforms, we co-transfected HEK 293 cells with PBX1-b or PBX1-d, and either MEIS-FLAG or PREP-FLAG. Consistent with the results obtained in Jurkat T cells, there was more PBX1-d/MEIS than PBX1-b/MEIS complexes (Fig. 4c). In contrast, there was no difference between the amount of PREP complexed with either PBX1 isoform (Fig. 4d). These results suggest that PBX1-d enhances the recruitment of MEIS as compared to the PBX1-b isoform. Drastically different recruitments of co-activators and co-repressor have been found between the Pbx1a and Pbx1-b isoforms (Asahara et al., 1999). Our results suggest that PBX1-b and PBX1-d not only differ by their binding site preferences but also by the ability to recruit co-factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- PBX1-d is novel splice isoform of PBX1 that is expressed in lupus CD4+ T cells.
- PBX1 binds to the CD44 promoter and directly regulates its expression.
- PBX1-d enhances CD44 expression relative to PBX1-b, the normal PBX1 isoform
- PBX1-d and PBX1-b have different DNA binding and co-factor recruitment requirement.

Fig. 1. PBX1 binds multiple sites in the *CD44* gene

(a) Map of the *CD44* gene with the location of the putative PBX1 binding sites in intron 1 (I1) and the promoter (P). (b) Details of two putative promoter PBX1 binding sites (P1 and P2) as well as their corresponding PBX1 binding sequences in upper case. (c) Chromatin from Jurkat T cells was immunoprecipitated with antibody against PBX1 or IgG control, and amplified by qPCR with primers flanking each of the 2 promoter sites and the intron 1 site. A positive control (Ctrl) was provided by immunoprecipitation with a POL2 antibody and amplification of the *POL2* binding site in the *GAPDH* promoter. ChIP-qPCR results are expressed in fold enrichment relative to the IgG negative control (N = 3). (d) Representative ChIP-PCR product at the P1 site.

Fig. 2. PBX1-d binding to the CD44 promoter enhances transcription

(a) Relative luciferase activity units (RU) in 293 HEK cells transfected with CD44_PROM PGL4.23 alone (Ctrl) or with 200 ng of PBX1-b or PBX1-d expression plasmids (N = 3, Dunnett's multiple comparisons test). (b) Relative luciferase activity units in 293 HEK cells transfected with the CD44_PROM PGL4.23 and 0–1000 ng of PBX1-b or PBX1-d expression plasmids (N = 3, 2-way ANOVA). The same experiment was performed 3 times with similar results. (c) Endogenous *CD44* gene expression in the HEK 293 cells transfected with CD44_PROM PGL4.23 alone (Ctrl) or with 1000 ng of PBX1-b or PBX1-d expression plasmids (N = 5, Dunnett's multiple comparisons test). (d) Representative FACS plots showing CD44 expression in Jurkat T cells transfected with PBX1-b -GFP or PBX1-d-GFP. The graph shows the quantitation for four assays for each cell type. Either PBX1-b or PBX1-d GFP⁺-transfected cells expressed more CD44 than their non-transfected GFP⁻ counterparts (paired t tests, $p < 0.01$). Comparison between GFP⁺ cells was performed with an unpaired t test *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$.

Fig. 3. Effects of mutations in the P1 and P2 promoter binding sites on *CD44* transactivation by PBX1-b and PBX1-d

(a) From top to bottom: consensus PBX1 binding site sequence, promoter P1 WT and mutated (M1) sequences, and promoter P2 WT and mutated (M2) sequences. The arrows indicate the location of the restriction sites (BafII and HpyCH4V) created by the mutations. The predicted binding sequences are in upper case and the mutated nucleotides are in bold.

(b) Relative luciferase activity units (RU) in 293 HEK cells transfected with WT, M1 or M2 mutated *CD44_PROM* PGL4.23 in the presence of 200 ng of PBX1-b or PBX1-d expression plasmids (N = 3, Dunnett's multiple comparisons test). Relative luciferase activity units in 293 HEK cells transfected with the M1 (c), M2 (d) or the WT *CD44_PROM* PGL4.23 and 0–1000 ng of PBX1-b or PBX1-d expression plasmids (N = 3, 2-way ANOVA between WT and M1 or M2 for each PBX1-b or PBX1-d transduced cells). *: p < 0.05; **: p < 0.01.

Fig. 4. PBX1-d enhances MEIS recruiting

(a) Lysates from Jurkat T cells either untouched, or transfected with PBX1-b or PBX1-d were immunoprecipitated with anti-PBX1, then immunoblotted with anti-MEIS, or anti-PBX1 as control. (b) Lysates from Jurkat T cells either untouched, or transfected with PBX1-b or PBX1-d were immunoprecipitated with anti-MEIS, then immunoblotted with anti-PBX1, or anti-MEIS as control. 293 HEK cells were transduced with MEIS-FLAG (c) or PREP1-FLAG (d), along with either nothing else, PBX1-b or PBX1-d. Lysates were immunoprecipitated with an anti-FLAG antibody, then immunoblotted with either anti-PBX1 or anti-FLAG as control. Each experiment was performed as least three times.