Transcriptional Regulation of CD1D1 by Ets Family Transcription Factors

Yanbiao Geng, Peter Laslo, Kevin Barton and Chyung-Ru Wang

*J Immunol* 2005; 175:1022-1029; doi: 10.4049/jimmunol.175.2.1022

http://www.jimmunol.org/content/175/2/1022

References

This article cites 69 articles, 41 of which you can access for free at:
http://www.jimmunol.org/content/175/2/1022.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Transcriptional Regulation of CD1D1 by Ets Family Transcription Factors

Yanbiao Geng,* Peter Laslo,† Kevin Barton,‡ and Chyung-Ru Wang2*

CD1 molecules are MHC class I-like glycoproteins specialized in presenting lipid/glycolipid Ags to T cells. The distinct cell-type specific expression of CD1D1 plays an important role in the development and function of NKT cells, a unique subset of immunoregulatory T cells. However, the mechanisms regulating CD1D1 expression are largely unknown. In this study, we have characterized the upstream region of the CD1D1 gene and identified a minimal promoter region within 200 bp from the translational start site of CD1D1 that exhibits cell-type specific promoter activity. Analysis of this region revealed an Ets binding site critical for CD1D1 promoter activity. Gel shift assays and chromatin immunoprecipitation experiments showed that Elf-1 and PU.1 bind to the CD1D1 promoter. Furthermore, we found that gene disruption of PU.1 resulted in decreased CD1D1 expression on B cells but not other cell types, whereas conditional activation of PU.1 negatively regulated CD1D1 expression in PU.1-deficient myeloid cells. These findings are the first to demonstrate that Ets proteins are involved in the transcriptional regulation of CD1D1 and that they may function uniquely in different cell types. The Journal of Immunology, 2005, 175: 1022–1029.

The CD1 family comprises a third lineage of Ag-presenting molecules that present lipid Ags rather than peptide Ags to T cells. Although CD1 proteins are structurally related to the MHC class I molecules, CD1 genes have diverged significantly from MHC class I genes and are located outside of the MHC (1–4). The segregation of CD1 from MHC during evolution may have facilitated the diversification of these Ag-presenting molecules with regard to their immunological functions and regulatory mechanisms.

CD1 genes have been conserved throughout mammalian evolution, although the number of CD1 genes varies among species (5). The best-characterized CD1 genes are in the human CD1 family. The five CD1 genes in humans (CD1A, -B, -C, -D, and -E) have been cloned and sequenced. Based on sequence homology, CD1 genes can be separated into two distinct groups, with CD1A, -B, and -C in group 1 and CD1D1 in group 2. In contrast to humans, mice lack group 1 CD1 genes and have two highly homologous group 2 CD1 genes, CD1D1 and CD1D2, which are likely the result of a gene duplication event (4). Although CD1D1 and CD1D2 are 95% homologous, the expression patterns are quite distinct. CD1d1 is constitutively expressed by most hemopoietic-derived cells, including all B cells, T cells, macrophages, and dendritic cells (6–8), and at low levels by some nonhemopoietic cells, such as hepatocytes (7). CD1d2 expression can only be detected at low levels on thymocytes (9).

The expression of CD1d1 has been shown to be essential for the development of a unique subset of T cells, the NKT cells, which are characterized by the expression of NK lineage-specific receptors and the preferential usage of the invariant TCR-α-chain (Vα14-Jα18; hereafter referred to as iNKT cells). Despite its sequence similarity, the expression of CD1d2 was not sufficient for the development of iNKT cells (9), possibly due to the low levels of surface expression and limited tissue distribution of CD1d2. In humans, a similar subset of T cells that recognize human CD1d and use the homologous TCR-α-chain (Vα24-Jα15) has been detected (10). The most striking characteristic of CD1d-restricted iNKT cells, in both mouse and human, is their ability to promptly produce large amount of cytokines, such as IFN-γ and IL-4 upon activation. These cytokines can in turn activate a variety of effector cells including NK cells, T cells, B cells, dendritic cells, and macrophages (11–15). Unlike classical, peptide-specific T cells, CD1d-restricted iNKT cells can recognize CD1d-expressing cells in the absence of exogenously added Ags (16, 17). The apparent reactivity to self-Ags by CD1d-restricted iNKT cells suggests that tight regulation of CD1d expression might be necessary to avoid potential autoreactivity. Recent studies have shown that the appropriate pattern and intensity of CD1d1 expression is indeed important for the development of iNKT cells, because CD1d1 expression driven under either MHC class Ia or MHC class II promoters is not sufficient for iNKT cell development in a CD1d1-deficient background (18, 19). In the former model, the overexpression of CD1d1 on dendritic cells leads to excessive negative selection of iNKT cells (19, 20); whereas in the latter model, the lack of CD1d1 expression on thymocytes results in failure of positive selection of iNKT cells (18).

CD1d1-restricted iNKT cells have now been shown to contribute to a variety of immune responses, including control of autoimmune diabetes (21–24), antitumor immunity (25, 26), and protection from infectious diseases (27–29). However, the mechanism of iNKT cell activation in these models remains poorly understood. Recent studies have demonstrated that CD1d1 expression is up-regulated in response to some infections or inflammatory conditions. For example, increased CD1d1 expression can be detected on mucosal-associated B cells after the development of intestinal inflammation (30) and on biliary hepatocytes during hepatitis C virus infection (31). These findings suggest that the levels and cell type-specific expression of CD1d1 may affect the functions of iNKT cells.

The surface expression of CD1d1 is likely to be regulated at many levels. Some recent studies have shown that intracellular...
trafficking behavior of CD1d can influence the surface expression of CD1d. In AP-3-deficient mice, cell surface expression of CD1d is increased due to redistribution of CD1d from lysosomes (32, 33). In contrast, the cell surface expression of CD1d is reduced in cells that lack MTP, a microsomal triglyceride transfer protein, due to the accumulation of CD1d in the endoplasmic reticulum (34). However, very little is known regarding the mechanisms that regulate the transcription of CD1D1. In this article, we have characterized the CD1D1 promoter region by identifying a minimal promoter region within 200 bp from the translational start site of CD1D1. This fragment contains putative C/EBP, GATA, Ets, and other binding sites. Mutation of the Ets site abolished the promoter activity, suggesting that the Ets binding site in the CD1D1 promoter region contributes significantly to the basal promoter activity of the CD1D1 gene. Gel shift assays and chromatin immunoprecipitation (ChIP)3 assays showed that PU.1 and Elf-1 bind to the CD1D1 promoter in vitro and in vivo. Using Elf-1 knockout mice and a PU.1 inducible system, we demonstrated the functional importance of these factors in transcriptional regulation of CD1D1 in B cells and myeloid cells, respectively.

Materials and Methods

Cell cultures

The mouse cell lines used in this study were P388D1 (P388), a macrophage cell line; L929, a fibroblast cell line; ASL 1, a thymoma; WEHI-231, a B lymphoma; and BCLI, a B cell leukemia cell line. All cells were cultured in RPMI 1640 supplemented with 10–15% FBS, penicillin-streptomycin (0.1 mg/ml), l-glutamine (2 mM), and 2-ME (50 μM). BCLI-R cells were established from the selective passage of nonadherent BCL1 cells.

RNA extraction and quantitative real-time PCR

Total RNA isolation was performed using TRizol reagent (Invitrogen Life Technologies). Single-stranded CDNA was generated with Superscript II reverse transcriptase (Invitrogen Life Technologies). Real-time PCR was performed using the ABI Prism 7700 instrument (PE Applied Biosystems). Each of the PCR was run in duplicate, and the level of CD1d1 expression was quantified by the ABI Prism 7700 instrument (PE Applied Biosystems). PCR of cDNA specimens were conducted in a total volume of 20 μl using Universal Master Mix according to the manufacturer’s instruction. Primer and probe sequences are as follows: CD1D1 probe, 5′-CGTTGCTCTCTAGAGCACGGGAGAAGTC-3′; CD1D1 forward primer, 5′-GACACTGCCCCC CTATTGTGT-3′; CD1D1 reverse primer, 5′-TG GCTTCTCCTTGCTTCTGAGGT-3′; GAPDH probe, 5′-TGGACTCTG CACCAACACTGCAGTGCAG-3′; GAPDH forward primer, 5′-TTTAC CACCACTGAGAGG-3′; and GAPDH reverse primer, 5′-GGCATG GACTGTTGCGTCACTG-3′.

Mapping the transcription start sites of mouse CD1D1 gene

The transcription start site of the CD1D1 gene was determined by the method of RNA ligase-mediated rapid amplification of 5′-CDNA ends (RLM-RACE; GeneRacer; Invitrogen Life Technologies). Briefly, 5 μg of total RNA extracted from BCLI cells was dephosphorylated with calf intestinal phosphatase to remove the 5′ phosphates except on full-length, capped mRNA. The RNA was then treated with tobacco acid pyrophosphatase to remove the 5′ phosphates except on full-length, capped mRNA, and 5′-end labeled with [γ-32P]ATP by ligating with the synthetic RNA oligo (5′-CGAUCUGAGCACGGAUACUCGUAU-3′). The labeled mRNA was reverse transcribed using cloned avian myeloblastosis virus reverse transcriptase and random hexamer primers. Fragments containing the transcription start site were amplified using HotStar Taq DNA polymerase (Qiagen) with the following primers: forward primers, 5′-GCACTGCGAGCAGAGGACGTG-3′; CD1D1 forward primer, 5′-CTCGGGACTGTTGTGCGGACTGTTG-3′; and CD1D1 nested primer, 5′-CAACA GCCATGGTGTAGCCAGGAG-3′. The PCR products were purified and cloned into pCR 4-TOPO cloning vector (Invitrogen Life Technologies), and multiple clones were sequenced.

Plasmid construction and site-directed mutagenesis

DNA fragments containing CD1D1 gene were isolated from a 129sv genomic DNA phage library (Stratagene) (35). A 4.4-kb DNA fragment upstream of the translation start site of CD1D1 gene was obtained by EcoRI digestion first, blunt-ended, and then digested by KpnI. The 4.4-kb fragment cloned into Sma1 site of the pGL2 basic vector was used for construction of luciferase reporter constructs using QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instruction. The double-strand oligonucleotides containing Ets binding sites from the CD1D1 promoter region (wild-type, 5′-CCTGCAGGCGCTGTTTCGCTG-3′; and mutant, 5′-CTGCAGGCGCTGTTTCGCTG-3′) were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (New England Biolabs). The labeled probes (1 x 106 cpm) were incubated with 2.5 μg of nuclear proteins at room temperature for 30 min in 30 μl of binding solution (10 mM Tris buffer (pH 8.0), containing 75 mM KCl, 1 mM EDTA, 1 mM DTT, 1 μg of poly(dIdC), and 4% Ficoll). The resulting complexes were resolved on a 5% native polyacrylamide gel and subjected to autoradiography. For competition studies, a 50–100 molar excess of unlabeled competitor oligonucleotides were added to the binding reaction just before adding the probe. For the supershift assays, 1 μg of each Ab specifically directed against a member of the Ets transcription factors was mixed with binding reactions as described above, incubated for 15 min at room temperature, and loaded onto the gel. Specific Abs against PU.1 (SC-352X), Elf-1 (SC-631X), and Ets-1 (SC-350X) were purchased from Santa Cruz Biotechnology.

Mice, T cell, and B cell purification

C57BL/6 were purchased from The Jackson Laboratory. The generation of Elf-1 knockout mice has been described previously (38). Single-cell suspensions from the spleen and bone marrow of Elf-1 and Elf-1−/− mice were prepared by mechanical disruption in HBSS containing 2% FBS. To isolate B and T cells, splenocytes were stained with FITC-conjugated anti-B220 or anti-TCR-β (H57–597) Ab (Pharmingen). The FITC-labeled cells were purified using anti-FITC microbeads and magnetic columns (Miltenyi Biotech) according to the manufacturer’s instruction. Cell purity was >97% as measured by flow cytometry.

Flow cytometry

Cells were prepared and stained for flow cytometric analysis as previously described (20). PE-conjugated anti-TCR-β, B220, and isotype control Abs were obtained from Pharmingen. CD1d-specific mAb, 5C6 (hamster IgM),

3 Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; RLM-RACE, RNA ligase-mediated rapid amplification of 5′ CDNA ends; HPRT, hypoxanthine phosphoribosyltransferase.
CD1D1 promoter region contains Ets family consensus binding site that is essential for the promoter activity of CD1D1

To assess the significance of the 5′-flanking region of CD1D1 for targeted gene expression, a 4.4-kb fragment from the upstream of the CD1D1 gene was cloned into the pGL2 vector and introduced into three different cell types to compare luciferase activity: BCL1 (B lymphoma, CD1high), P388 (macrophage, CD1int), and L929 cell lines (fibroblast, CD1low). These three cell lines were chosen for transient transfection because of their distinct cell types and CD1D1 expression levels. As shown in Fig. 3, the promoter/luciferase activity of the 4.4-kb construct in these cell types is reflective of the relative hierarchy of CD1D1 expression found in these cell types. BCL1 cells (CD1high) displayed the greatest activity (55-fold above control vector), followed by P388 (22-fold above control). The activity in nonhemopoietic L929 cell line (CD1low) is substantially lower (5-fold above background). To identify cis-regulatory elements that may be important for CD1D1 expression, a series of 5′ truncation mutants were generated. Removing the region between positions −4400 to −3131 had no effect on the CD1D1 promoter activity in P388 and L929, but results in 4- to 5-fold increase of promoter activity in BCL1. Nevertheless, a similar hierarchy of promoter activity was observed between the cell
CD1D1 promoter contains Ets family binding site that is essential for driving luciferase reporter gene. A, P388, BCL1, and L929 cells were transfected with a luciferase reporter driven by promoterless pGL2 basic vector. The SD bars represent three independently performed experiments. B, Ets mutants were generated by site-directed mutagenesis, replacing the Ets core binding site TTCC (−105 to −102) with TTAC in 125-bp pGL2 luciferase reporter plasmid from A. Either wild-type (wt) 125-bp pGL2 luciferase plasmid or mutant 125-bp (mEts) pGL2 luciferase plasmid was transfected by electroporation in the cell lines indicated. Cell lysates were prepared 18 h posttransfection. The data shown are expressed as a ratio to luciferase activity of the promoterless pGL2 basic vector. The SD bars represent three independently performed experiments.

To determine which of the Ets family members bind to the −105 Ets binding site, polyclonal Abs directed against specific Ets family members were added to the EMSA binding reaction. We initially focused our attention on PU.1, Ets-1, and Elf-1, because PU.1 and Ets-1 have been implicated in the regulation of various lymphoid- and myeloid-restricted promoters, and Elf-1 is known to selectively interact with Ets sites containing GGAA but not with GGAT core binding motifs (44). As shown in Fig. 4A, anti-Elf-1 Ab altered the mobility of complex A in all four cell lines tested, although the proportion of complex A supershifted by anti-Elf-1 was significantly smaller from L929 cells compared with that from P388, BCL1, and BCL1R cells. The addition of anti-PU.1 Ab completely eliminated the complex C in nuclear extracts from P388, BCL1, and BCL1R cells, but had no effect on L929 cells (Fig. 4C). It is noteworthy that BCL1R, a subline derived from BCL1 that expresses lower levels of CD1D1, appears to have significantly higher levels of PU.1 complexes. PU.1 is not detectable in L929 cells by Western blot (data not shown), which may explain why the nuclear extract from L929 cells does not generate the lower complex and cannot be supershifted by anti-PU.1 Ab. Notably, the addition of anti-Ets-1 did not alter migration of any complexes, although anti-Ets-1 can do so when incubated with nuclear extracts from BW5147 or R1.1 cells and Ets-1 consensus oligonucleotides.

To establish whether Elf-1 and PU.1 bound to the CD1D1 promoter in vivo, ChIP assays were performed. Cross-linked chromatin from P388, L929, and BCL1 cells was immunoprecipitated with Ab specific for PU.1 and Elf-1. Coprecipitated DNA was used as a template for PCR amplification with primers spanning the Ets binding site in the CD1D1 promoter region. As a control for Ab specificity, primers spanning the HPRT promoter region where no Ets consensus site exists were used. As shown in Fig. 5, the CD1D1 promoter was amplified when the immunoprecipitation was performed using Ab specific for Elf-1 in all the three cell lines studied. In contrast, it can only be amplified from the BCL1 and P388 cells, but not L929 cells, when the immunoprecipitation was performed using anti-PU.1. This is consistent with the expression of CD1D1. Continued truncation, which removed E2F and AML-1a, led to the loss of residual promoter activity.

The Ets family of transcription factors is a diverse group of ~30 proteins that bind to a core consensus sequence 5′-GGAA-3′, or the reverse strand 3′-TTCC-5′. To evaluate the functional significance of the Ets binding site for CD1D1 promoter activity, a site-specific mutant, in which the core TTCC site is altered to TTAC, was made in the context of the 125-bp construct and transfected into BCL1, P388, and L929 cells. Mutation of the Ets site reduced luciferase activity by 75% in L929, and by 90–95% in BCL1 and P388 cells (Fig. 3B), confirming that Ets binding sites has an essential role in the regulation of the CD1D1 promoter.

PU.1 and Elf-1 bind to an Ets site in the proximal promoter of the CD1D1 gene in vitro and in vivo

EMSA was performed to establish whether Ets factors could bind to the CD1D1 promoter. Nuclear extracts prepared from BCL1, P388, and L929 cells were incubated with double-stranded radio-labeled oligonucleotide probe spanning the sequence from position −125 to −105. As shown in Fig. 4A, we observed three specific protein/DNA complexes (complex A, B, and C) with nuclear extracts from BCL1 and P388 cells, whereas only the upper two complexes were detected with nuclear extract from L929 cells. All of these complexes were successfully competed for by cold probe, but not by oligonucleotide mutated within the Ets core elements (Fig. 4A). Furthermore, there were no detectable complexes when EMSA was performed with radiolabeled mutated Ets probe (data not shown).

FIGURE 3. CD1D1 promoter region contains Ets family binding site that is essential for driving luciferase reporter gene. A, P388, BCL1, and L929 cells were transfected with a luciferase reporter driven by promoterless pGL2 basic vector. The SD bars represent three independently performed experiments. B, Ets mutants were generated by site-directed mutagenesis, replacing the Ets core binding site TTCC (−105 to −102) with TTAC in 125-bp pGL2 luciferase reporter plasmid from A. Either wild-type (wt) 125-bp pGL2 luciferase plasmid or mutant 125-bp (mEts) pGL2 luciferase plasmid was transfected by electroporation in the cell lines indicated. Cell lysates were prepared 18 h posttransfection. The data are expressed as a ratio to luciferase activity of the promoterless pGL2 basic vector. The SD bars represent three independently performed experiments.

FIGURE 4. Ets factors can bind to the CD1D1 promoter. EMSA was performed to establish whether Ets factors could bind to the CD1D1 promoter. Nuclear extracts prepared from BCL1, P388, and L929 cells were incubated with double-stranded radio-labeled oligonucleotide probe spanning the sequence from position −125 to −105. As shown in Fig. 4A, we observed three specific protein/DNA complexes (complex A, B, and C) with nuclear extracts from BCL1 and P388 cells, whereas only the upper two complexes were detected with nuclear extract from L929 cells. All of these complexes were successfully competed for by cold probe, but not by oligonucleotide mutated within the Ets core elements (Fig. 4A). Furthermore, there were no detectable complexes when EMSA was performed with radiolabeled mutated Ets probe (data not shown).

To determine which of the Ets family members bind to the −105 Ets binding site, polyclonal Abs directed against specific Ets family members were added to the EMSA binding reaction. We initially focused our attention on PU.1, Ets-1, and Elf-1, because PU.1 and Ets-1 have been implicated in the regulation of various lymphoid- and myeloid-restricted promoters, and Elf-1 is known to selectively interact with Ets sites containing GGAA but not with GGAT core binding motifs (44). As shown in Fig. 4A, anti-Elf-1 Ab altered the mobility of complex A in all four cell lines tested, although the proportion of complex A supershifted by anti-Elf-1 was significantly smaller from L929 cells compared with that from P388, BCL1, and BCL1R cells. The addition of anti-PU.1 Ab completely eliminated the complex C in nuclear extracts from P388, BCL1, and BCL1R cells, but had no effect on L929 cells (Fig. 4C). It is noteworthy that BCL1R, a subline derived from BCL1 that expresses lower levels of CD1D1, appears to have significantly higher levels of PU.1 complexes. PU.1 is not detectable in L929 cells by Western blot (data not shown), which may explain why the nuclear extract from L929 cells does not generate the lower complex and cannot be supershifted by anti-PU.1 Ab. Notably, the addition of anti-Ets-1 did not alter migration of any complexes, although anti-Ets-1 can do so when incubated with nuclear extracts from BW5147 or R1.1 cells and Ets-1 consensus oligonucleotides.

To establish whether Elf-1 and PU.1 bound to the CD1D1 promoter in vivo, ChIP assays were performed. Cross-linked chromatin from P388, L929, and BCL1 cells was immunoprecipitated with Ab specific for PU.1 and Elf-1. Coprecipitated DNA was used as a template for PCR amplification with primers spanning the Ets binding site in the CD1D1 promoter region. As a control for Ab specificity, primers spanning the HPRT promoter region where no Ets consensus site exists were used. As shown in Fig. 5, the CD1D1 promoter was amplified when the immunoprecipitation was performed using Ab specific for Elf-1 in all the three cell lines studied. In contrast, it can only be amplified from the BCL1 and P388 cells, but not L929 cells, when the immunoprecipitation was performed using anti-PU.1. This is consistent with the expression...
pattern of PU.1 in these cell lines. In agreement with a previous report, PU.1 is found to associate with c-fms promoter in hematopoietic cells, whereas no binding of PU.1 and Elf-1 to the HPRT promoter was observed (45). These data suggest that PU.1 and Elf-1 associate with the CD1D1 promoter in vivo.

CD1D1 expression in splenic B cells from Elf-1 knockout mice is decreased compared with C57BL/6 mice

Elf-1 is highly expressed in most of the hematopoietic cell types as well as some nonhematopoietic cells (46). Cotransfection of an Elf-1 expression plasmid with the 200-bp CD1D1 promoter construct into L929 and P388 cells did not result in significant differences in the luciferase activity (data not shown), possibly due to the high levels of endogenous Elf-1. Thus, we examined Elf-1 knockout mice to determine whether the absence of Elf-1 could affect CD1D1 expression as compared with C57BL/6 mice in various cell types. Elf-1 knockout mice are viable and exhibit normal hematopoietic cell development and function (38). FACS analysis showed that CD1d1 expression in splenic B cells in Elf-1 knockout mice was decreased compared with wild-type mice (Fig. 6A). Furthermore, as measured by real-time PCR, CD1D1 mRNA level in purified splenic B cells from Elf-1 knockout was reduced to 30% of the control C57BL/6 mice (Fig. 6B). In contrast, CD1d1 expression was equivalent in thymocytes and splenic T cells between wild-type and Elf-1 knockout mice (Fig. 6A). Similar to the surface expression, CD1D1 mRNA level in splenic T cells was not affected by Elf-1 deletion. No significant differences in CD1d1 surface expression were detected in fibroblasts, macrophages, and dendritic cells from Elf-1 knockout mice (data not shown). These results suggest that Elf-1 is required for the appropriate CD1d1 expression in splenic B cells, but it is not essential for the CD1d1 expression in other cell types.

Restoration of PU.1 in PU.1-deficient cells decreases CD1d1 expression

PU.1 is a hematopoietic-specific Ets family transcription factor found in progenitors and in myeloid and B cells (47, 48). Two
independently derived mouse strains lacking PU.1 have been reported (49, 50). Mice harboring mutations in both PU.1 alleles possess profound defects in lymphoid and myeloid development. The PU.1-deficient mice used in our study die during embryonic development (49), precluding an analysis of CD1d1 expression in various hemopoietic cells in adult animals. To determine whether the binding of PU.1 to the Ets consensus site in the CD1D1 proximal promoter region could regulate the CD1d1 expression, a retrovirus vector containing PUER, in which PU.1 was fused with the ligand binding domain of the estrogen receptor, was transduced into a PU.1-deficient myeloid cell line (39). This cell line was IL-3 dependent and originally derived from fetal livers of PU.1-deficient mice. Activation of PU.1 by tamoxifen treatment drives the cell to differentiate into macrophages, manifesting with increased expression of F4/80 marker (data not shown). We extracted RNA from tamoxifen-treated cells at various time points and measured the levels of CD1D1 mRNA by RT-PCR. Consistent with the previous report, the expression of c-fms, a PU.1 target gene, was up-regulated in the tamoxifen-treated cells. In contrast, the level of CD1D1 mRNA was significantly decreased within 9 h of tamoxifen treatment (Fig. 7). The down-regulation of CD1d1 expression by PU.1 was further confirmed by real-time PCR and FACS analysis of protein surface expression (data not shown). Notably, no decrease of CD1D1 mRNA was observed in mock-transfected PU.1-deficient cell line upon tamoxifen treatment, as measured by RT-PCR and real-time PCR (data not shown). Together with the evidence that PU.1 binds to the proximal promoter of CD1D1 gene in vivo, these results suggest that PU.1 can negatively regulate CD1d1 expression in myeloid cells.

Discussion

In this study, we have investigated the transcriptional regulation of CD1D1. We have identified a minimal promoter region within 200 bp from the translational start site of CD1D1, which shows high-level promoter activity in hemopoietic cells, but much lower activity in nonhemopoietic cells, suggesting strong cell-type-specific regulatory elements within this region. We further demonstrated a putative Ets binding site, located −105 bp upstream of the translational start site, as a major cis-regulatory element of CD1D1 promoter activity. Gel shift assay and ChIP assays showed that Ets family transcription factor PU.1 and Elf-1, but not Ets-1, bind to the CD1D1 proximal promoter in vitro and in vivo. Disruption of the Elf-1 gene in mice resulted in decreased CD1D1 expression on B cells but not other cell types. Conversely, enhanced PU.1 activity led to a reduction of CD1D1 expression in macrophages. Our findings underscore the complexity of the regulation of CD1D1 by the Ets family of transcription factors. Because several Ets proteins are often expressed in the same cells, it is conceivable that multiple Ets transcription factors may compete for binding to the Ets binding site in the CD1D1 promoter. Different Ets proteins may influence the CD1D1 promoter in a unique manner, depending upon their expression patterns and biochemical properties.

The Ets family was originally defined by the presence of a highly conserved DNA binding domain, which can recognize purine-rich DNA sequences with a central GGAA/T core consensus (51–53). There are over 30 characterized members of the Ets transcription factor family reported to date. Our finding that the CD1D1 promoter is regulated by PU.1 and Elf-1 but not by Ets-1 demonstrated that CD1D1 promoter does not respond to all Ets proteins but only to a specific subset. This is in agreement with the notion that Ets family members often differ in their exact binding site preference outside of the GGAA/T core, with factor-specific recognition spanning 9–15 bp (53). Nevertheless, our gel shift data suggest the presence of additional Ets family member(s) associated with CD1D1 promoter as residual Ets complexes exist following supershift with anti-PU.1 and anti-Elf-1 Abs. In fact, our preliminary data showed that a faint supershifted band could be detected when anti-Fli-1 Ab was tested in the gel shift assays (data not shown), suggesting a minor proportion of the complexes forming on Ets site contain Fli-1. However, the identity of other Ets factors in the complex and their roles in the CD1d1 expression are currently unknown.

Elf-1 is expressed at low levels in many different cell lineages and at high levels in hemopoietic cells (B cells, T cells, and macrophages) (46). However, the effect of Elf-1 on CD1d1 expression was only observed in B cells. The differential effect of Elf-1 on CD1d1 expression might be due to the presence of other Ets transcription factors that could compensate for the effect of Elf-1. One of such factors might be myeloid Elf-1-like factor, which shares significant homology to Elf-1 and has been shown to have stronger transcriptional activity in several hemopoietic gene promoters than Elf-1 (54). Alternatively, Elf-1 might be required for competition with an Ets transcription factor, which is preferentially expressed in B cells and can negatively regulate the expression of CD1D1. It has been shown that the expression of CD1d1 in B cells is quite heterogeneous, with highest levels of CD1d1 expression detected in marginal zone B cells (55, 56). It is noteworthy that the Elf-1 deficiency does not affect B cell development, and the decrease of CD1d1 expression in Elf-1 knockout mice can be detected in all B cell subsets (data not shown). As Elf-1 is ubiquitously expressed throughout T and B cell development, it is possible that Elf-1 is responsible for a basal level of CD1D1 promoter activity, and that expression can be further modulated by the levels of other Ets family proteins that may negatively regulate the CD1d1 expression.

Unlike Elf-1, PU.1 is predominantly expressed on hemopoietic progenitors, myeloid and B cells. PU.1-deficient mice show a complete absence of morphologically normal B cells and macrophages, along with disrupted granulopoiesis and aberrant T lymphopoiesis (49, 50). This data suggests a nonredundant role of PU.1 in hemopoiesis. In our experiments, when PU.1 is activated by tamoxifen treatment in PU.1−/− PUER cells, macrophage lineage commitment was observed. Meanwhile, the level of CD1 expression was decreased within a few hours. This inverse relationship between PU.1 and CD1d1 expression can also be observed in a B cell line, BCL1, and its nonadherent subline, BCL1R. The parental
BCL1 cells express low levels of PU.1 and high levels of CD1d, whereas BCL1R cells express higher levels of PU.1 and lower levels of CD1d (Figs. 1 and 4C). Because PU.1 lacks an autoinhibitory domain and repression domain, the suppression of CD1D1 expression by PU.1 is likely due to competition from other positive regulators such as Elf-1, or recruitment of other negative regulators. Low levels of CD1d1 expression in nonhemopoietic cells, however, cannot be attributed to the inhibitory effect of PU.1, because PU.1 is not expressed on nonhemopoietic cells. It is noteworthy that a significant amount of Ets protein complex in nuclear extracts from L929 cells cannot be supershifted with the Abs against PU.1 and Elf-1, suggesting the presence of unique Ets family members in nonhemopoietic cells which may down-regulate the CD1d1 expression. It is also possible that the lack of a transactivator or coactivator may limit the expression of CD1d1 in nonhemopoietic cells.

Ets transcription factors usually work in concert with other transcription factors, maximizing specificity and tightly regulating gene expression (57). For example, interactions between PU.1 and GATA family transcription factors have been shown to antagonize each other’s activities, whereas interactions between Fli-1 and GATA proteins have been shown to have a synergistic effect (58–62). Interestingly, there is a potential GATA binding site located in close proximity of Ets binding site in the CD1D1 promoter region. Preliminary study showed cotransfection with a GATA-1-expressing plasmid inhibited the luciferase activity driven by the CD1D1 promoter, suggesting GATA family proteins may modulate the effect of Ets proteins on the regulation of CD1D1 transcription (data not shown). It has also been shown that C/EBP physically interacts with PU.1 to cooperate in the transcriptional activation of targeted genes (63, 64). A putative C/EBP binding site is found 30 bp upstream of the Ets binding site in the CD1D1 promoter. Deletion of this motif had a cell-type specific effect on the CD1D1 promoter activity (Fig. 3), suggesting C/EBP family transcription factors might also play a role in regulating CD1D1 expression.

CD1D genes are conserved in all the mammals examined, including the mouse, rat, and human. Sequence analysis reveals a striking sequence similarity (70%) in the first 200 bp upstream of the translation start site between the mouse and rat CD1D gene. The Ets binding site that is critical for CD1d1 expression is well conserved in rat. In contrast, there is no apparent sequence conservation between the upstream promoter regions of human CD1D (hCD1D) and mouse CD1D1. If the upstream CD1D promoter activity is in fact conserved between human and mouse, it might be functionally defined by a short stretch of sequence not detectable in strict homology-based searches. However, it is noteworthy that four potential Ets binding sites can be identified within the 500 bp upstream of the translation start site of hCD1D. It would be of interest to verify whether these Ets binding sites were involved in hCD1D regulation. A recent study has demonstrated that Sp1 can bind to the promoter region of hCD1D in vitro and may contribute to the promoter activity of hCD1D (65). Although there are several potential Sp1 binding sites within the 200-bp promoter region of CD1D1, our preliminary studies showed that anti-Sp1 Ab failed to supershift any DNA-protein complexes from nuclear extracts incubated with oligonucleotides containing the putative Sp1 sites from this region (data not shown). This data suggests that Sp1 may not bind to CD1D1 promoter appreciably. Human CD1d is expressed not only on hemopoietic cell types but also on epithelial cells from a variety of tissues (66–69). It is possible that unique transcription factors may account for a broader tissue distribution of hCD1d.

In summary, this is the first study to characterize the molecular mechanisms underlying the regulation of CD1D1 gene expression.

We have shown that 200-bp 5′-flanking region of the CD1D1 gene contains cis-regulatory elements required for the constitutive expression of CD1D1. A functionally important binding site for members of the Ets family transcription factors was identified in the CD1D1 promoter region. Binding of two of the Ets family transcription factors, Elf-1 and PU.1, has differential effects on the promoter activity of CD1D1 in B cells and myeloid cells, respectively. Our findings provide a starting point for investigating how the different combinations of Ets proteins may affect the CD1D1 expression in various cell types. Further examination of distinct expression patterns of these Ets proteins and their coregulatory protein partners, and the interplay between these factors should provide the foundation for understanding how the CD1D1 gene is regulated under normal and pathological conditions.

Acknowledgments

We thank A. Colomne and Dr. M. Zimmer for critical reading of the manuscript, and Dr. Harinder Singh for the advice in the PuEr system.

Disclosures

The authors have no financial conflict of interest.

References
