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Activating Transcription Factor/cAMP Response Element Binding Protein Family Member Regulated Transcription of CD1A

Angela Colmone, Sha Li, and Chyung-Ru Wang

CD1a has a unique expression pattern among Ag-presenting molecules, expressed specifically on cortical thymocytes and APCs. As autoimmune disease, infection, and tumors can all result in alteration of CD1a expression, we are attempting to characterize the transcriptional regulation, and thus shed some light on specific expression, of CD1A. In this study, we have identified a minimal proximal promoter region required for CD1A transcription. Computer searches within this region identified numerous potential binding sites for lymphoid-specific transcription factors, including the ETS transcription factors, C/EBP, GATA, and CREB. Deletion and site-specific mutant analysis revealed a critical role of a potential cAMP response element (CRE) 965 bp upstream of the CD1A translation start site. Two activating transcription factor (ATF)/CREB family members, CREB-1 and ATF-2, are able to bind this site in vitro and in vivo. Notably, activation of ATF/CREB family members decreases CD1A transcription, while decrease in ATF-2 expression results in increased CD1A RNA level. The fact that these factors also bind the CD1A promoter in human monocytes strongly suggests a role for ATF/CREB family members in regulation of CD1A expression. The Journal of Immunology, 2006, 177: 7024–7032.

The CD1 family of molecules, similar to MHC, presents Ag to specific T cells (1). A key difference between these two groups of molecules, however, is that CD1 does not present peptide Ag like MHC class I and class II, but instead presents lipid Ag to restricted T cells (2). CD1 molecules can be divided into two groups based on sequence homology. The better characterized group 2 CD1 is found in most mammals and includes CD1D in humans and CD1D1 and CD1D2 in mice. CD1A, in contrast, belongs to group 1 CD1, which also consists of CD1B and CD1C. Although homologs exist in the guinea pig and other vertebrates, mice and rats do not express group 1 CD1 (3, 4).

The expression pattern of CD1a is unique among Ag-presenting molecules. Although MHC class I expression is fairly ubiquitous and both MHC class II and group 2 CD1 are broadly expressed on cells of hemopoietic lineage, CD1a, similar to other group 1 CD1 molecules, is expressed on relatively limited cell types (4, 5). Group 1 CD1 is expressed on cortical thymocytes, with loss of expression upon thymocyte maturation (6). CD1a, b, and c are also expressed on dermal dendritic cells, with CD1a specifically expressed in high levels on Langerhans cells (7). In fact, CD1a has been used widely for identification and classification of human dendritic cell populations both in vitro and in vivo. Thus, understanding the regulation of this expression may not only increase understanding of the activation circumstances of CD1a-restricted T cells, but may also be used to better understand and identify dendritic cell subsets.

CD1a expression can be altered by various cytokine-signaling events. Treatment of monocytes by maturation factors such as GM-CSF and IL-4, as well as IL-3 and IL-4, results in up-regulation of CD1a (8, 9). This induction can be suppressed by IL-10 (8). TNF-α, IL-6, and IL-1β can also induce CD1a expression on monocytes (10). These data suggest a role for factors activated downstream of cytokine signaling in CD1a regulation.

The potential role of CD1a-restricted T cells in autoimmunity, tumor response, and in infection raises the question of whether CD1a expression level may modulate these responses. Increased CD1a expression has been observed in several autoimmune disease models, including allergic bronchial asthma, human atherosclerotic lesions, and psoriatic arthritis (11–13). In addition, the density of CD1a+ dendritic cells has also been directly associated with clinical outcome in a variety of human cancer types (14). CD1a expression is also altered in various human infections. Both bacillus Calmette-Guérin and Mycobacterium tuberculosis infection result in demonstrable change in CD1a, b, and c surface expression (15, 16). In addition, a strong correlation between CD1 expression and effective host immunity in leprosy has been reported (17). The correlation of CD1a expression and effective immunity, as well as the potential for down-regulation by both tumors and specific infections, suggests that expression of CD1a may be tightly regulated.

As is perhaps expected by the different expression pattern and separate chromosomal location, the proximal promoter region of CD1A has little homology to either MHC class I or class II proximal promoter regions (18). The CD1A promoter is TATA-less, which is reflected by multiple transcription initiation sites. Among group 1 CD1 molecules, however, there are two conserved regions in the 5′-untranslated region that could potentially be involved in CD1 transcriptional control (1). Beyond this knowledge, however, little is known about the regulation of CD1A.

In the present study, we specifically focus on studying the transcriptional regulation of CD1A. We have identified a minimal proximal region required for CD1A promoter activity. Computer

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searches revealed numerous potential lymphoid-specific transcription factor binding sites within this region. We chose to examine the function of a potential cAMP response element (CRE)\(^3\) 965 bp upstream of the CD1A translation start site. CREB can be activated downstream of growth factor and cytokine-signaling events, highlighting the potential regulatory role of this site (19). The identified CRE is required for CD1A transcription and is able to bind both activating transcription factor (ATF)-2 and CREB-1 in vitro and in vivo. Indeed, alteration of ATF/CREB transcription function either through activation or depletion results in a change in CD1A RNA level. As ATF-2 and CREB-1 can bind the CD1A promoter in freshly isolated human (h) PBMC, these results suggest a role for ATF-2 and CREB-1 in regulation of CD1A expression.

Materials and Methods

Cell cultures

The human cell lines used in this study were MOLT-4, MOLT-3, and Jurkat T cell lines; U-937 and THP-1-monocyte cell lines; and JY, a human B cell line (American Type Culture Collection (ATCC)). All cells were cultured in RPMI 1640 or DMEM supplemented with 10% FBS, penicillin-streptomycin (0.1 mg/ml), 1-glutamine (2 mM), and 2-ME (50 μM). Forskolin (Sigma-Aldrich) was added to cell culture for 24 h at indicated concentrations.

Flow cytometry

For detecting CD1a surface expression, cells were stained for 30 min on ice with biotin-conjugated anti-CD1a (CB6: Ancell) in HBSS containing 2% FBS and 0.1% sodium azide, followed by allophycocyanin-conjugated streptavidin (BD Pharmingen). All flow cytometric analysis was performed using a FACSCan (BD Biosciences) with FlowJo software (Tree Star).

RNA extraction and quantitative real-time PCR

Total RNA isolation was performed using the RNeasy Mini kit (Qiagen). Single-stranded cDNA was generated with Superscript II reverse transcriptase (Invitrogen Life Technologies). Real-time PCR was performed using the ABI Prism 7700 instrument (Applied Biosystems). Each of the PCR was run in duplicate, and the level of expression was normalized to either β-actin or cyclophilin using Sequence Detector software (Applied Biosystems). PCR of CDNA specimens were conducted in a total volume of 30 μl using SYBR Green PCR Master Mix (Qiagen) with specific primers listed in Table I.

Computational analysis of the CD1A promoter region

The TRANSFAC database (www.gene-regulation.com) (20), Alibaba2 (www.gene-regulation.com), and TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html) programs were used to predict putative transcription factor-binding sites upstream of the CD1A translation start site.

Plasmid construction and site-directed mutagenesis

DNA fragments corresponding to various CD1A promoter regions were amplified from bacterial artificial chromosome clones (RP11-101J8 and RP11-4O4013; Sanger Institute) by PCR with the following primers: forward 5′-GCTTCTTCTCCAGCAGTTG-3′, 1000-bp forward, 5′-CTGTATGGAACTAACACTCCAA-3′, 993-bp forward, 5′-GGAGGCCTTGTITTTATCCA-3′, 731-bp forward, 5′-TTGTGTGTCTACACCTGAT-3′, 534-bp forward, 5′-CAGGCCAGTCTGACGACT-3′, 293-bp forward, 5′-GCCACATGAGGTTCTCCA-3′, 73-bp forward, 5′-GAGCTCTGGTGATTGTGGGAA-3′; and reverse, 5′-TGGAAGTAGCAAAACACGCA-3′. PCR products were gel-purified and cloned into PCR-Script (Stratagene). These vectors were then SacI/XhoI (New England Biolabs) digested, and fragments were cloned to the pGLO vector for luciferase assays. Mutations (lower case, underlined) within the 1000-bp pGL2 CREM promoter region (Table II) was end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (New England Biolabs). The labeled probes (1 ng of ~1 × 10^4 cpm) were incubated with 10 μg of nuclear proteins at room temperature for 30 min in 40 μl of binding solution (1 mM Tris (tris(hydroxymethyl)aminomethane) buffer (pH 8.0), containing 75 mM KCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DTT (dithiorhodophenyltrichloroethane), and 1 μg of poly dI:dC). The resulting complexes were resolved on a 5% native polyacrylamide gel and subjected to autoradiography. For competition studies, a 50-fold molar excess of unlabeled competitor oligonucleotides was added to the binding reaction concurrently with probe addition. For the supershift assays, 1 μg of each Ab specific to a member of the ATF/CREB family of transcription factors was mixed with the binding reactions as described above, incubated for 15 min at room temperature, and loaded onto the gel. Specific Abs against CREB-1 (sc-186X), ATF-2 (sc-187X), CREB-1 (sc-2867X), CAMP responsive element modulator (CREM) (sc-440X), and C/EBPβ (sc-150X) were purchased from Santa Cruz Biotechnology.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to protocol using a commercially available kit (Upstate Biotechnology). Briefly, cells were cross-linked with formaldehyde, and cross-linked chromatin was sheared. Chromatin immunoprecipitation (ChIP) was performed according to protocol using a commercially available kit (Upstate Biotechnology). Briefly, cells were cross-linked with formaldehyde, and cross-linked chromatin was sheared.

Table I. Real-time PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1A forward</td>
<td>CATCCTGATCGCAGCTCCCTTTT</td>
</tr>
<tr>
<td>CD1A reverse</td>
<td>ACCTGAAATTTCTAGGCGGCTAT</td>
</tr>
<tr>
<td>ATF-2 forward</td>
<td>TTCTCGAGGACCATACAAAGG</td>
</tr>
<tr>
<td>ATF-2 reverse</td>
<td>TCTTATCGCTCTGTCACAAA</td>
</tr>
<tr>
<td>CREB-1 forward</td>
<td>CACGATTCTATCAGCGCAAG</td>
</tr>
<tr>
<td>CREB-1 reverse</td>
<td>TGGCGCTGCGGAAATAGGAA</td>
</tr>
<tr>
<td>CREM forward</td>
<td>GACTTGCGGGCAGACCACAT</td>
</tr>
<tr>
<td>CREM reverse</td>
<td>CAATCGTCTGCTCCGACAT</td>
</tr>
<tr>
<td>Cyclophilin forward</td>
<td>CTCCTCGTAGGCTTGGGAC</td>
</tr>
<tr>
<td>Cyclophilin reverse</td>
<td>ACACATCGATGTCTCACCC</td>
</tr>
<tr>
<td>Actin forward</td>
<td>GCAGTTTGTGCGATGTCAGCCACCCC</td>
</tr>
<tr>
<td>Actin reverse</td>
<td>CTCTCTGCAGCATGGCTCC</td>
</tr>
</tbody>
</table>

CTCTTCTC-3′; CD1A_C/EBP mut1, 5′-TTCTTCTTTCATGTTACT GGCTCTTCTC-3′; CD1A_GATAmut1, 5′-GCTTGACAGAAGAAGATGA TAATAGAGATATCGTGGG-3′; and CD1A_GATAmut2, 5′-GCTGCA GAAGAAGTCAGCAAAAGAAGATGAATATCGTGGG-3′. All the constructs were confirmed by DNA sequencing.

Transient transfections and luciferase reporter assays

Transfections were performed with an Electro Cell Manipulator 600 (BTX), using parameters of 270 V, 1050 μF, and 720 Ω. Cells were co-transfected with 30 μg of each luciferase reporter construct and 3 μg of pRL-TK (Promega), which was used to determine transfection efficiency. A total of 5 μg of pCMV-CREB133 (BD Clontech) was used in dominant negative experiments. After 16 h, cells were lysed, and the luciferase activities were measured with a Tropix TR717 microplate luminometer using a dual luciferase assay kit (Promega). Reporter activity was represented by the ratio of firefly luciferase (driven by CD1A promoter region) to Renilla luciferase (driven by HSV thymidine kinase promoter region) activity. Transfection efficiencies were found to be similar across cell types.

EMSA

Nuclear extracts were prepared according to the method of Dignam et al. (21, 22). The double-strand oligonucleotide containing a CRE-binding site from the CD1A promoter region (Table II) was end-labeled with γ-32P]ATP by T4 polynucleotide kinase (New England Biolabs). The labeled probes (1 μg of ~1 × 10^4 cpm) were incubated with 10 μg of nuclear proteins at room temperature for 30 min in 40 μl of binding solution (10 mM Tris (tris(hydroxymethyl)aminomethane) buffer (pH 8.0), containing 75 mM KCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DTT (dichlorodihydrofluridchloroethane), and 1 μg of poly dI:dC). The resulting complexes were resolved on a 5% native polyacrylamide gel and subjected to autoradiography. For competition studies, a 50-fold molar excess of unlabeled competitor oligonucleotides was added to the binding reaction concurrently with probe addition. For the supershift assays, 1 μg of each Ab specific to a member of the ATF/CREB family of transcription factors was mixed with the binding reactions as described above, incubated for 15 min at room temperature, and loaded onto the gel. Specific Abs against CREB-1 (sc-186X), ATF-2 (sc-187X), CREB-1 (sc-2867X), CAMP responsive element modulator (CREM) (sc-440X), and C/EBPβ (sc-150X) were purchased from Santa Cruz Biotechnology.

Table II. EMSA probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Oligonucleotide (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB consensus</td>
<td>AGAGATTGCTGGATCGAGTTAATT</td>
</tr>
<tr>
<td>CD1A</td>
<td>CCAAGATTGCACAACTTGGCTAAATT</td>
</tr>
<tr>
<td>CD1A_C/EBP</td>
<td>CCAAGATTGCACAACTTGGCTAAATT</td>
</tr>
<tr>
<td>CEBP-beta consensus</td>
<td>TGGACAGAAGATGTCAGAC</td>
</tr>
<tr>
<td>Non-specific</td>
<td>AACATCCTGAAATCTCCGCTCCTCCTG</td>
</tr>
</tbody>
</table>

Abbreviations used in this paper: CRE, cAMP response element; ATF, activating transcription factor; h, human; ChIP, chromatin immunoprecipitation; CREM, cAMP responsive element modulator; shRNA, short hairpin RNA; DNBCREB, dominant-negative CREB.
human peripheral blood leukocytes were enriched for monocytes by seeding at a density of 1 × 10⁷ cells/well. After 4 h of incubation, the medium was replaced with 20 mg of transfer vector and 10 mg of packaging plasmids pMDLg/pRRE, pRSV-REV, and pMD.G using a calcium phosphate transfection kit (Sigma-Aldrich). Culture supernatants were collected at 48 and 72 h posttransfection, filtered through 0.22-μm filters (Millipore) and concentrated by ultracentrifugation. Virus titers were then analyzed on 293T cells by FACS for enhanced GFP expression.

Transduciton with lentiviral vectors

One day before transduction, 4 × 10⁵ cells/well were seeded in a 12-well plate. Transductions with the recombinant lentivirus and mock transductions were conducted in the presence of 7 μg/ml polybrene in RPMI 1640-1% FBS. After 4 h of incubation, the medium was replaced with RPMI 10. At 72 h posttransduction, the cells were harvested for sorting of GFP expression.

Preparation of human monocytes

Human peripheral blood leukocytes were enriched for monocytes by seeding at a density of 1 × 10⁷ cells/well in a 6-well plate for 2 h. Adherent cells were then cultured in the presence or absence of hGM-CSF (100 ng/ml) and hIL-4 (50 ng/ml) in RPMI 10 for 5 days, with addition of cytokine every 2 days. At day 5, CD1a expression was confirmed by flow cytometry and cells were used in ChIP assays.

Results

CD1a surface expression directly correlates to RNA level in human cell lines

The importance of studying CD1A transcriptional regulation relies on first determining whether RNA level regulation is the key means by which CD1A expression levels are controlled. To this end, real-time PCR was performed on cell lines expressing high, intermediate, and low levels of CD1a to determine whether mRNA level correlated to surface expression level. In fact, MOLT-4 and MOLT-3, high CD1A-expressing cell lines, contain more CD1A mRNA than Jurkat, an intermediate line, or U-937, THP-1, and JY, negative for surface staining (Fig. 1). Thus, RNA level of CD1a does correlate with, and is likely a key regulator of, CD1a expression in all cell types examined.

CD1A minimal promoter region

To explore transcriptional regulation of CD1A expression, regions upstream of the CD1A translation start site varying in length from 2774 to 73 bp were cloned into a pGL2 reporter construct and promoter activity was examined in a dual-luciferase reporter assay. The ATG was chosen as the downstream boundary for these fragments to incorporate multiple transcription initiation sites for CD1A (Fig. 2A). No tissue-specific differences were observed upon transfection into a CD1a⁺ (MOLT-4) or a CD1a⁻ (U-937) cell line in regions extending 2774 bp upstream of the translation start site. The 1000-bp fragment, however, was both sufficient and required for optimal basal promoter activity (Fig. 2B).
Table III. Potential transcription factor binding sites

<table>
<thead>
<tr>
<th>Binding Site</th>
<th>Location</th>
<th>Mutation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRE</td>
<td>−956</td>
<td>TGAAGTCA−→TGAAATCA</td>
</tr>
<tr>
<td>ETS</td>
<td>−677</td>
<td>GAAGAA−→GAGAAAA</td>
</tr>
<tr>
<td>C/EBPmut1</td>
<td>−595</td>
<td>TTTTGATG−→TTTATAGT</td>
</tr>
<tr>
<td>C/EBPmut2</td>
<td>−593</td>
<td>TTTTGATG−→TTTGGAGG</td>
</tr>
<tr>
<td>GATAmut1</td>
<td>−92</td>
<td>GAAGAA−→GAGAAAG</td>
</tr>
<tr>
<td>GATAmut2</td>
<td>−75</td>
<td>AGATA−→AGATAT</td>
</tr>
</tbody>
</table>

* Mutated bases are lowercase and underlined.

Multiple transcription factor binding sites are required for basal CD1A promoter activity

To further explore the transcription regulation of CD1A, computer search programs were used to determine potential transcription factor binding sites within the CD1A proximal promoter region. Multiple sites were identified, including factors that regulate lymphoid genes, such as ETS, C/EBP, and GATA, and a CRE (Fig. 2A). These sites were then mutated by site-directed mutagenesis in the 1000-bp pGL2 reporter and promoter activity was examined (Table III). Single base pair mutations in multiple sites resulted in a 1000-bp pGL2 reporter and promoter activity was examined (Table III). Single base pair mutations in multiple sites resulted in significant decreases in promoter activity, suggesting cooperative interaction between these factors in gene regulation (Fig. 3).

ETS family members have recently been shown to regulate CD1D1 (25). Mutation in a putative ETS-binding site (ETSmut) resulted in a 5-fold decrease in luciferase activity, suggesting an important role of these family members in regulating CD1 proteins. C/EBP family members can interact with ETS family members through their leucine zipper domains (26). Two separate mutations were made in a putative C/EBP-binding site. The upstream mutation (C/EBPmut1) had no significant effect, while the downstream mutation (C/EBPmut2) resulted in a 5-fold decrease in promoter activity, supporting a potential role for interaction between these factors. GATA factors have been shown to interact with ETS family members in T cells (27). A mutation in a GATA consensus sequence in the 5′-untranslated region (GATAmut2) also resulted in a significant (3-fold) decrease in luciferase activity. Most strikingly, however, mutations in two sites, a second noncanonical GATA sequence within the 5′-untranslated region (GATAmut1) and the CRE (CREmut), resulted in complete abolishment of promoter activity, suggesting these sites are required for CD1A transcription. CRE-binding proteins can interact with both C/EBP and ETS family members, as well as acetylate GATA family members, altering their activity (19, 28). The location of the CRE (−965 bp) within the region required in the 1000-bp pGL2 vector for promoter activity further highlights the importance of this site.

ATF/CREB family members bind CD1A CRE

CRE-binding proteins are activated during various immunological events, including cytokine signaling as well as upon thymocyte maturation and T cell activation (19). To determine whether CRE-binding proteins are involved in CD1A regulation, factor binding to the CRE was examined by EMSA. Nuclear extracts from MOLT-4 and Jurkat cells were incubated with radiolabeled probe that contained both the CRE core-binding sequence of the CD1A promoter and sequences flanking this site (CD1A). Protein binding to this CD1A probe was observed and could be specifically competed by excess of unlabeled probe but not an unrelated binding sequence (Fig. 4A). This binding was also competed by addition of a probe that contained a consensus CRE sequence (CRE consensus), suggesting the protein binds to the CRE and not the CD1A flanking sequence (Fig. 4A). Notably, a single base pair difference in the CD1A probe introduced at the same location as in the luciferase reporter construct resulted in less efficient competition than the unmutated probe (Fig. 4A), suggesting that this mutation prevents protein binding.

Proteins that bind the CRE in mammals belong to the ATF/CREB family of transcription factors, a large family of proteins that either activate or repress gene transcription in a promoter-dependent manner (29). To confirm that the factor(s) that bind the CD1A CRE are indeed ATF/CREB family members, supershift experiments were performed using specific Abs to CREB-1, ATF-2, ATF-1, and CREM. These family members were chosen due to expression in lymphocytes and evidence of phosphorylation during stimulation in the immune system (19). CREB family members are all able to bind the CD1A CRE-binding elements in vitro. Immunoprecipitation with CREM Abs resulted in specific amplification of positive controls (Fig. 4B). This factor is likely nonspecific protein binding as it is not observed with the MOLT-4 nuclear extract. However, no single ATF/CREB family-specific Ab, or even a combination of all four Abs, was completely sufficient to shift all protein associated with the CRE in MOLT-4 cells (Fig. 4B and data not shown). Thus, the possibility of specific binding of other factors cannot be eliminated. The data conclusively show, however, that CREB-1, ATF-2, ATF-1, and CREM are all able to bind the CD1A CRE in vitro.

ChIP was then performed to confirm that individual ATF/CREB family members actually bind the CD1A promoter region in vivo. Interestingly, while immunoprecipitation with all four ATF/CREB family member Abs resulted in specific amplification of positive control c-FOS promoter, only anti-CREB-1 and anti-ATF-2 resulted in amplification of the CD1A CRE-flanking sequence. Thus, although ATF/CREB family members are able to bind the CD1A CRE, not all actually do so in the cellular setting. The binding observed, however, is specific, as the negative control rRNA promoter region with no known CRE resulted in no amplified bands (Fig. 4C). Taken together, these results suggest that the ATF/CREB family members ATF-2 and CREB-1 are able to bind the
CD1A CRE in vivo and may play a role in regulating CD1A transcription.

**Functional regulation of CD1A expression**

To investigate possible functional consequences of ATF-2 and CREB-1 on CD1A expression, MOLT-4 cells were treated with forskolin, a cAMP activator that leads to phosphorylation and activation of ATF/CREB family members. Surprisingly, CD1A promoter activity was significantly decreased (3-fold) in the presence of forskolin (Fig. 5A). CD1A RNA level was also significantly decreased after 24 h treatment in three CD1a + cell lines, suggesting that the forskolin-mediated effect occurs at the transcriptional level (Fig. 5B). rRNA levels were not changed by this treatment, but c-FOS levels were increased, as expected. These data suggest that although protein binding to the CRE is required for CD1A transcription, activation of ATF/CREB family members could have a negative regulatory role on CD1A expression.

**ATF/CREB family members functionally regulate CD1A promoter activity**

By increasing the cellular level of cAMP, forskolin activates multiple downstream-signaling pathways. Thus, the forskolin-mediated decrease in CD1A transcription could occur through ATF/CREB family member-dependent and/or -independent mechanisms. To examine the direct role of ATF/CREB family members in CD1A promoter activity, a vector expressing a dominant-negative CREB (DNCREB), pCMV-CREB133 (BD Clontech), was cotransfected with the CD1A reporter construct into MOLT-4, Jurkat cells. DNCREB cotransfection resulted in increased promoter activity in both cell lines, implying a direct functional role of ATF/CREB family members in regulating CD1A transcription (Fig. 6). These data were reproduced in U-937 cells, suggesting that this effect is not restricted to T cell lines (data not shown). Notably, DNCREB blocked forskolin-mediated decrease of promoter activity as well, indicating a dominant effect in this system.

DNCREB can inhibit activation of multiple ATF/CREB family members. To further elucidate the potential differential regulatory roles of ATF-2 and CREB-1 on CD1A transcription, lentiviral vectors were created that stably expressed shRNA specific for either ATF-2 (ΔATF-2) or CREB-1 (ΔCREB-1) and transduced into MOLT-4, Jurkat, and U-937 cells. Jurkat cells were primarily used in these experiments due to high transduction efficiency, however, similar results were obtained in both MOLT-4 and U-937 cells (data not shown).
In the ΔATF-2 cells, ATF-2 expression was decreased at both the RNA (5-fold) and protein levels with no concurrent change in either CREB-1 or CREM expression (Fig. 7A and data not shown). ΔCREB-1 cells had a 5-fold decrease in CREB-1 expression, but a decrease in ATF-2 expression (10-fold) was also observed in these cells, despite minimal homology between the CREB-1-specific targeting shRNA and the ATF-2 sequence. Notably, this effect was not observed for all ATF/CREB family members, as CREM expression was not affected in these cells (Fig. 7A). A second CREB-1-targeting vector resulted in a similar dual-knockdown phenotype (data not shown). As the proximal promoter of ATF-2 has multiple CRE, it is possible that ATF-2 may be regulated by CREB-1 in a species- and tissue-specific manner (31).

ΔATF-2 Jurkat and U-937 cells express significantly more CD1A RNA than the empty virus-transduced cell line (Fig. 7B and data not shown), suggesting a negative regulatory role of this factor on CD1A transcription. The lower level of CD1A induction in the dual-knockdown ΔCREB-1 cells, however, suggests that CREB-1 and ATF-2 may have a differential effect on CD1A expression. It is possible that CREB-1 functions as a positive regulator, and that its loss compensates for the decrease in ATF-2 expression. Indeed, overexpression of CREB-1 in a cotransfection experiment supports this role for CREB-1 as a positive regulator (Fig. 7C).

**FIGURE 6.** DmCReb blocks forskolin-mediated decrease in CD1A promoter activity. The 1000-bp pGL2 vector was transfected into MOLT-4 or Jurkat cells by electroporation in the presence or absence of either 5 μg of pCMV-CREB133 (DmCReb) and 10 μM forskolin. Cell lysates were prepared 16 h posttransfection. Data are represented as fold difference compared with the untreated 1000-bp pGL2 vector. Data are presented as the mean ± SD from three independent experiments. Statistically significant difference is indicated by an asterisk as calculated by Student’s t test (*, p < 0.05).

**FIGURE 7.** ATF/CREB family members functionally regulate CD1A promoter activity. A and B, Jurkat cells were stably transduced with GFP-expressing lentivirus, which produced shRNA specific for nonrelevent sequence, ATF-2, and CREB-1 (Empty virus, ΔATF-2, and ΔCREB-1) in the presence of 7 μg/ml polybrene. Total RNA was extracted from sorted GFP + cells and knockdown was confirmed by real-time PCR. RNA is normalized to cyclophilin. Data in this figure are representative of three experiments. C, The 1000-bp pGL2 vector was transfected into MOLT-4 cells by electroporation in the presence or absence of 5 μg of pCMV-CREB. Cell lysates were prepared 16 h posttransfection. Data are represented as fold difference compared with the untreated 1000-bp pGL2 vector. Data are presented as the mean ± SD from three independent experiments. Statistically significant difference is indicated by an asterisk as calculated by Student’s t test (*, p < 0.05).

**FIGURE 8.** ATF/CREB family members bind the CD1A promoter in human monocytes. A, Flow cytometric staining of representative human CD1a + monocytes and CD1a + monocyte-derived dendritic cell populations. B, ChIP was performed on formaldehyde cross-linked chromatin from either CD1a + monocytes or CD1a + monocyte-derived dendritic cells using control rabbit IgG or specific Abs against either CREB-1 or ATF-2. Real-time PCR was performed using primers spanning the CRE within the promoter region of CD1A. The rRNA promoter region and c-FOS promoter region were amplified by PCR as negative or positive controls, respectively. Values are representative of two experiments and presented as percent input.

**ATF-2 and CREB-1 bind CD1A promoter in human monocytes**

ATF/CREB family members have a direct effect on CD1A RNA level in human cell lines. In human monocytes, forskolin treatment results in decreased surface expression of CD1a, similar to our observations in cell lines (32). Therefore, we chose to determine whether ATF-2 or CREB-1 bind to, and potentially regulate, the CD1A promoter in vivo. PBMC were enriched for adherent cells and cultured 5 days in the presence or absence of GM-CSF and IL-4, yielding 95% homogenous CD1a− (monocytic) and CD1a + (monocyte-derived dendritic cell) populations (Fig. 8A). ChIP was then performed with Abs specific to CREB-1 or ATF-2 to examine binding to the CD1A promoter in ex vivo human cells. The CD1A-binding region was successfully amplified from both monocytes and monocyte-derived dendritic cells, with notably stronger ATF-2 binding in the CD1a + monocytes, consistent with a negative regulatory role of ATF-2 in CD1A expression (Fig. 8B). These data suggest that the ATF/CREB family member regulatory effects observed in human cell lines may mimic regulation in physiological conditions.
CD1A expression can be altered by various cytokine-signaling events, as well as in autoimmune disease, human cancer, and infection (4, 14). To gain insight into the role that CD1a plays in exacerbation or amelioration of these diseases, we here attempt to characterize the regulation of its expression. As expression was found to be regulated at the RNA level, we further examined the transcriptional regulation of CD1A. A 1000-bp region upstream of the translation start site was identified as necessary for proximal promoter activity. Multiple putative transcription factor sites within this region, include ETS, C/EBP, GATA, and a CRE, were found to be required for optimal basal promoter activity. The role of the CRE in CD1A transcriptional regulation was then further explored. ATF/CREB family members ATF-2 and CREB-1 were shown to bind the CRE both in vitro and in vivo. Alteration of the function of these factors, either through forskolin-mediated activation, overexpression of a DNCREB, or gene-specific inhibition, resulted in direct functional regulation of CD1A transcription. This manipulation of CD1A expression is particularly revealing, as it is in the context of human tumor cells. These results can likely be extended to primary human cells as well. Both CREB-1 and ATF-2 can bind the CD1A promoter region in human PBMC.

The CD1A proximal promoter region did not confer tissue specificity, as no difference was observed upon reporter transfection into CD1a+ and CD1a− cell lines. This sequence was sufficient for expression, however, as extending this region an additional 1774-bp upstream also did not result in tissue-specific expression. Indeed, activity with this upstream region was decreased in both cell types, suggesting the presence of upstream silencing elements similar to those found upstream of the human and mouse CD1D promoters (25, 33). Taken together, these data suggest that tissuespecific regulation of CD1A may occur through distal regulatory elements. It is also possible that in the cellular setting, different concentrations and activation states of proximal regulatory proteins affect basal and, more importantly, inducible CD1A expression.

Of the multiple transcription factors required for optimal CD1A basal promoter activity, ETS family members have been implicated in regulation of CD1D in mice, suggesting a role in regulation of multiple CD1 genes (25). Yet the CD1A promoter region has little sequence homology to CD1D1 or either MHC class Ia or MHC class II (1). The CD1A promoter is TATA-less as well, suggesting an alternate pathway for RNA polymerase recruitment (1). The requirement for multiple factor binding suggests a functional synergy, perhaps as a result of a multiprotein complex with cooperative binding or hierarchical site occupation. Indeed, ETS, GATA, ATF/CREB, and C/EBP family members have been shown to interact both with each other and other factors to form a transcription complex in some lymphoid regulatory regions, including the TCRα enhancer (19, 26–28, 34, 35). Thus, it is not surprising that these factors coordinately bind or regulate the CD1A promoter.

CRE and CRE-binding proteins have been shown to regulate the transcription of MHC class I, MHC class II, the MHC CIIA, and the nonclassical MHC, HLA-G (18, 36, 37). Our finding that CREB-1 and ATF-2 specifically bind the CD1A CRE in vitro and in the cellular context again highlights the similarities in regulation of Ag-presenting molecules. CREB-1 and ATF-2 are broadly expressed, including expression in brain and liver as well as lymphoid organs such as spleen and thymus. They form both homo- and heterodimers and interact with other families of transcription factors. This dimerization ability may lead to tissue-specific activation and repression of CD1A.

ATF/CREB transcription factors can be activated by phosphorylation of a kinase-inducible domain by protein kinase A or MAPK family members downstream of over 300 reported stimuli, including inflammatory cytokine signaling, cAMP signaling, and thymocyte maturation and T cell activation. CBP/p300 interaction with transcription factor IIIB and RNA polymerase II is enhanced upon interaction with activated ATF/CREB family members and other transcription factors, including ETS, C/EBP, and GATA family members, resulting in transcription initiation. Differences in factor-binding affinity, transcription complex composition, and cofactor binding regulate this interaction (19, 29, 34). Thus, regulation of CD1A by CRE-binding proteins allows for tight control in various stimulatory conditions.

Forskolin activates adenylate cyclase, resulting in increased cellular levels of cAMP and downstream ATF/CREB family member phosphorylation (38). Similarly to MHC class II, potential ATF/CREB family member activation through forskolin treatment results in down-regulation of CD1A expression (39). For MHC class II, both negative and positive regulation of expression require the same sequence (39). This is likely also the case for CD1A, suggesting factor binding is required for transcription but phosphorylation has an inhibitory role.

Activity-mediated recruitment of inhibitory proteins may also play a role in this regulation. Yin-Yang-1 (YY-1) is one such factor that can directly interact with ATF/CREB family members and mediate transcriptional repression (40). Indeed, multiple YY-1-specific binding sites have been identified in the basal CD1A promoter. Recruitment of an inhibitory factor could prevent optimal assembly of the transcription complex, and thus block CD1A transcription.

It cannot be ruled out, however, that the forskolin effect observed, although consistent with a direct effect on ATF/CREB family members, could also be mediated through as yet unidentified CRE or through an indirect, CREB-independent mechanism. cAMP induction can alter transcription through a number of factors, including ETS and GATA family members (41). The increase in CD1A promoter activity upon DNICREB cotransfection suggests that ATF/CREB family members do have a direct effect on CD1A transcription. Indeed, this effect is dominant over the forskolin-mediated down-regulation. These data suggest that any additional effect of forskolin on CD1A expression is ancillary to the role of ATF/CREB family members on CD1A expression.

The direct inhibitory role of ATF-2 or ATF-2-interacting proteins on CD1A regulation is supported by the increase in CD1A RNA level observed upon specific inhibition of ATF-2 by RNA interference. This increase occurs in both the CD1a-expressing Jurkat cell line and the CD1a− U-937 cell line. It is not sufficient, however, to induce CD1a surface expression in U-937 cells, as the CD1A RNA level, while detectable, remains well below basal levels in CD1a+ cell lines (data not shown).

Cells specifically targeted for CREB-1 inhibition resulted in no significant change in CD1A expression from empty virus-transduced cells, although a trend toward increased expression was observed. Notably, these cells were not solely deficient in CREB-1. ATF-2 levels were also significantly reduced, even though CREM expression, another ATF/CREB family member, was not affected. This effect was observed in two lines independently generated with different targeting sequences. Although CREB-1-mediated regulation of ATF-2 has not been examined in human T cells, it is significant that the ATF-2 proximal promoter region contains multiple CRE, suggesting that CREB-1 may regulate ATF-2 expression in a species and tissue-specific manner (31). Treating these cells as a double knockdown line, loss of CREB-1 appears to compensate for loss of ATF-2, suggesting a potential positive regulatory role
of CREB-1 in CD1A transcription. Indeed, overexpression of CREB-1 in a cotransfection reporter assay results in increased CD1A promoter transcription. CD1A expression could thus be regulated by competition of CREB-1 and ATF-2 CRE binding resulting from activation by different signals, as has been recently shown to be the case in IFN-β-mediated epithelial NO synthase regulation (42).

The observation that ATF-2 and CREB-1 bind to the CD1A promoter in human monocytes and monocyte-derived dendritic cells, with higher levels of ATF-2 binding in the CD1a− monocytes, suggests that this work may have direct physiological relevance. Interestingly, it has been reported that forskolin treatment of GM-CSF/IL-4-derived dendritic cells have decreased CD1a expression, consistent with our cell line data (32). Similarly to CD1A, ATF/CREB family members can be altered in multiple sclerosis, various cancers, and M. tuberculosis-infected cells (14, 15, 43–46). Thus, examining alteration of ATF/CREB family members could lead to insight into CD1a expression, as well as function and activation of CD1a-restricted T cells.

CD1A, B, and C have little proximal promoter sequence homology. Yet, similar expression of group 1 CD1 on thymocytes and APCs suggest that these genes may be coordinately regulated. Regional control through histone acetylation and chromosomal looping is important in the regulation of other immunological genes, including the Th2 cytokine locus and MHC class II (47, 48). A locus control region could function in the coordinate regulation of the CD1 gene cluster as well. In addition, there are many similar transcription factor-binding sites upstream of group 1 CD1 genes, including ETS, GATA, and C/EBP sites (A. Colombe and C. R. Wang, unpublished data). Indeed, CRE are also found upstream of CD1B. Thus, further characterization of CD1A regulation may provide a foundation for understanding regulation of expression and function of group 1 CD1.

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Disclosures

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