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Human CD1D Gene Expression Is Regulated by LEF-1 through Distal Promoter Regulatory Elements

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CD1d-expressing cells present lipid Ag to CD1d-restricted NKT cells, which play an important role in immune regulation and tumor rejection. Lymphoid enhancer-binding factor-1 (LEF-1) is one of the regulators of the Wnt signaling pathway, which is a powerful regulator in cellular growth, differentiation, and transformation. There is little evidence connecting Wnt signaling to CD1d expression. In this study, we have identified LEF-1 as a regulator of the expression of the gene encoding the human CD1d molecule (CD1D). We found that LEF-1 binds specifically to the CD1D promoter. Overexpression of LEF-1 in K562 or Jurkat cells suppresses CD1D promoter activity and downregulates endogenous CD1D transcripts, whereas knockdown of LEF-1 using LEF-1–specific small interfering RNA increases CD1D transcripts in K562 and Jurkat cells but there are different levels of surface CD1d on these two cell types. Chromatin immunoprecipitation showed that the endogenous LEF-1 is situated at the CD1D promoter and interacts with histone deacetylase-1 to facilitate the transcriptional repressor activity. Knockdown of LEF-1 using small interfering RNA potentiates an acetylation state of histone H3/H4, supporting the notion that LEF-1 acts as a transcriptional repressor for the CD1D gene. Our finding links LEF-1 to CD1D and suggests a role of Wnt signaling in the regulation of the human CD1D gene. ***The Journal of Immunology*****, 2010, 184: 5047–5054.**

CD1d molecules are cell-surface glycoproteins that are noncovalently associated with β_2-microglobulin to form a heterodimeric three-dimensional structure similar to that of class I MHC molecules (1, 2). CD1d is broadly expressed in different cells including T cells, B cells, monocytes, and epithelial cells (3–5). CD1d is also expressed on immature cortical thymocytes and downregulated on mature thymocytes in parallel with the expression of group I CD1 molecules (6–8). CD1d can present α-galactosylceramide to a subset of NKT cells (9, 10) that play an important role in immune regulation (1), tumor rejection (11, 12), and the development of autoimmune diseases (13–16).

We recently showed that the gene encoding the human CD1d (CD1D) is regulated by at least two cell-type–specific promoters that contain multiple putative *cis*-regulatory elements, including T cell factors (TCFs) and lymphoid enhancer-binding factor 1 (LEF-1) (17), or TCF/LEF-1 elements. As the ultimate mediators of the Wnt signaling pathway, LEF-1 and the three TCF proteins (TCF-1, TCF-3, and TCF-4) bind virtually the same DNA sequence in humans (18–20) and act as transcriptional repressors in collaboration with Groucho-related gene family members and with histone deacetylases (21). However, such a transcriptional repressor can be converted to a transcription activator when Wnt signaling is activated. An activation of Wnt signaling leads to the inhibition of glycogen synthase-3β, which binds to the adenomatous polyposis coli/β-catenin complex and regulates the complex assembly (22), resulting in the accumulation of the unphosphorylated β-catenin in the nucleus to form a complex with TCF/LEF-1 and activate the downstream target gene expression (23, 24).

The evolutionarily conserved Wnt signaling pathway plays pivotal roles in the development of many organ systems, and dysregulated Wnt signaling has been associated with tumors, including hematological malignancies (21). In contrast, CD1d-restricted NKT cells play an important role in immune regulation and surveillance of tumor cells (1). Dysregulated CD1d expression has been associated with hematological malignancies (25). However, there is little evidence of a relationship between Wnt signaling and NKT cells. A demonstration of a regulatory relationship between LEF-1 and the CD1D gene would help to reveal whether Wnt signaling is involved in CD1D gene expression or ultimately whether there is a relationship between Wnt signaling and the function of NKT cells. Accordingly, we looked into two leukemia cell lines, K562 (myelogenous leukemia line) and Jurkat cells (T cell leukemia line), and found that LEF-1 does bind specifically to the CD1D promoter and regulates CD1D gene expression.

Materials and Methods

Cell lines and reagents

K562 and Jurkat cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in IMDM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Anti–LEF-1 Ab was obtained from Active Motif (Carlsbad, CA) and Santa Cruz Biotechnology (Santa Cruz, CA). Abs to LEF-1, histone deacetylase-1 (HDAC-1), and acetyl-H3 or acetyl-H4 for chromatin immunoprecipitation (ChIP) assays were obtained from Upstate Biotechnology (Charlottesville, VA). Ab to CD1d (clone CD1d42) was purchased from BD Biosciences (Franklin Lakes, NJ). The LEF-1 cDNA expression vector was kindly provided by Dr. M.L. Waterman (University of California at Irvine, Irvine, CA).

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Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; HDAC-1, histone deacetylase-1; LEF-1, lymphoid enhancer-binding factor-1; PCK, phosphoenolpyruvate carboxykinase; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; TCF, T cell factor.

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EMSA

EMSA was performed to examine the binding of LEF-1 protein to the putative LEF-1 elements using methods as previously described (17). In brief, 10 μg nuclear extract derived from Jurkat cells was incubated with 1.75 pmol (~100×) unlabeled oligonucleotide or 2 μl mouse mAb specific to LEF-1 or an isotype control in EMSA binding buffer for 10 min at room temperature. The γ-[^{32}P]ATP–labeled oligonucleotide probe (20,000 cpm) was subsequently added to the mixture and incubated for an additional 20 min. The mixtures were electrophoresed in a prerun, nondenaturing 4% polyacrylamide gel. The signals were detected using autoradiography. The sequence of CD1D cDNA (26) and a genomic DNA sequence obtained from the Genbank database (Accession no. AL138899) were used as the reference throughout this study. The nucleotide +1 was defined as the "A" of the ATG translation initiation codon, and the nucleotide 5′ to the +1 is designed as −1 (27). Two regions (−423 to −396 and −304 to −285) containing the putative LEF-1 elements were selected for the study. The sequences of the double-stranded oligonucleotides were 5′-ccaaagtctccttttgaaaca-3′ for the region between 304 and −285 and 5′-ggactttgatcctttttttcccttttgcat-3′ for the region between −423 and −396. Three mutant oligonucleotides corresponding to the region between −423 and −396 were also used in the test. These include 5′-ggactttgatc*at*gttttccct*gta*cat-3′ (−423/−396m2/3), and 5′-gga*a*ttt*a*atc*at*gttttcccttttgcat-3′ (−423/−396m1/2), 5′-gga*a*ttt*a*atccttttttccc-t*gta*cat-3′ (−423/−396m1/3), where the underlined letters were the mutations introduced in the oligonucleotide to remove a putative LEF-1 and two putative non–LEF-1 transcriptional binding elements within the oligonucleotide. mAb specific for LEF-1 was used to supershift LEF-1 in the EMSA.

Construction of luciferase reporter gene vectors and transient transfection

The pGL3-basic luciferase reporter gene vectors (Promega, Madison, WI) were used in the study. The pGL3-basic vector was inserted with a genomic DNA fragment (−665 to −202) spanning the distal CD1D promoter or a DNA fragment (−665 to +24) covering both the distal and the proximal promoter (17). The transient transfection and report assay were routinely carried out similarly to those described in a previous study (17). To test the effect of LEF-1 on CD1D gene expression, K562 or Jurkat cells were transiently transfected with the reporter vector (0.5 μg) containing the distal CD1D promoter (−665 to −202) and with the presence of LEF-1 expression vector (0.5 μg) or a control vector (0.5 μg) (mock) using Lipofectamine 2000 (Invitrogen) for K562 cells or TransIT Jurkat Transfection Reagent (Mirus Bio, Madison, WI) for Jurkat cells following the manufactures' instructions. The control vector (mock) is the same vector as the LEF-1 expression vector but without the insertion of the LEF-1 gene. Twenty-four hours after the transfection, reporter activity was detected using a Dual-Glo Luciferase Assay System (Promega), and the comparative specific luciferase reporter activities were measured after normalization with the internal control *Renilla* luciferase according to the manufacturer's instructions.

Site-directed mutagenesis analysis

The GeneTailor Site-Directed Mutagenesis System (Invitrogen) was used to generate mutant constructs following the manufacturer's instructions. The pGL3-basic vector inserted with the CD1D promoter region between −665 and −202 or between −665 and +24 was used as a template to generate the construct that had either one of the two putative LEF-1 binding elements mutated (Fig. 2*A*). The two pairs of primers used were 5′-ctgctaaagcaaggaatttaatcatgttttcccttttgcat-3′ and 5′-tccttgctttagcagtacatgtctc-3′ or 5′-acgttgacccaaagtctcctctgtaacaggaaattga-3′ and 5′-aggagactttgggtcaacgtgtgg-3′.

Detection of CD1d expression by flow cytometry

Cells were seeded at 1×10^6 cells per well 1 d before transfection. The expression of the surface CD1d was detected with a mAb to CD1d (CD1d42) or with an isotype control Ab (Ab control) using flow cytometry as described previously (17).

Small interfering RNA knockdown of LEF-1

Control small interfering RNA (siRNA) or siRNA specifically targeting the human LEF-1 gene sequence were purchased from Santa Cruz Biotechnology. Jurkat cells were transfected with either control or LEF-1 siRNA (100 nM) using TransIT Jurkat Transfection Reagent; K562 cells were transfected with either control or LEF-1 siRNA (100 nM) using Lipofectamine 2000 (Invitrogen). The transfected cells were cultured for 48 h and collected for RT-PCR, quantitative RT-PCR (qRT-PCR), Western blot, and flow cytometric analyses.

RT-PCR and real-time quantitative PCR

Human LEF-1 and CD1D mRNA in Jurkat or K562 cells were detected using RT-PCR and real-time qRT-PCR. RNA was isolated from the transfected cells using the TRIzol reagent (Invitrogen). The isolated RNA was treated with DNase I (Promega) and precipitated using isopropanol. A total of 2 μg of the isolated RNA was processed for reverse transcription using a cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The primers used for CD1D are 5′-gctgcaaccaggacaagtggacgag-3′ and 5′-aggaacagcaagcacgccaggact-3′. The primers used for LEF-1 are 5′-aataaagtcccgtggtgc-3′ and 5′-atgggtagggttgcctgaatc-3′. The primers used for the housekeeping gene β-actin are 5′-agagctatgagctgcctgac-3′ and 5′-ctgatccacatctgctggaa-3′. PCR conditions were 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C, and extension at 72°C for 30 s. The PCR products were separated on 2% agarose gel. Alternatively, 1 μl reverse-transcribed product obtained as described above was used in a 25 μl reaction mixture, which includes SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) and 1 μl primer pair (1.0 μM) to amplify the gene of interest. Actin was used as an internal control in quantitative PCR (qPCR). The cycling conditions were hold at 95°C for 5 min, followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The fluorescent signals were analyzed using an iCycler IQ multicolor real-time detection system (Bio-Rad).

Western blot analysis and coimmunoprecipitation

Cell lysates were prepared using protein lysis buffer (20 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.5% NP-40, and 0.1% Triton X-100). Cell extracts were fractionated by 12% gel and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked at room temperature for 1 h in TBST (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk. Membranes were then incubated overnight at 4°C in TBST containing 5% nonfat dry milk with either mouse anti–LEF-1 Ab (1:1000; Santa Cruz Biotechnology) or rabbit anti-actin Ab (1:5000; Sigma-Aldrich, St. Louis, MO). The membranes then were exposed to a secondary anti-mouse or rabbit IgG Ab coupled with HRP (1:2500; Bio-Rad) at room temperature for 1 h. After being washed, the membranes were then developed using a SuperSignal West Pico chemiluminescent kit (Pierce, Rockford, IL). For coimmunoprecipitation, cell lysates derived from K562 or Jurkat cells were reacted with an anti–LEF-1 Ab or an irrelevant IgG control overnight at 4°C. The immune complexes were precipitated using protein G–agarose beads, washed, and subjected to an SDS-PAGE separation. The separated proteins were transferred onto nitrocellulose membrane and processed as described above for Western blotting using an anti–HDAC-1 Ab to detect the coprecipitated HDAC-1 protein that bound to LEF-1.

ChIP assay

ChIP assays were performed to detect a physical binding of LEF-1, HDAC-1, acetyl-H3, or acetyl-H4 to the CD1D promoter in Jurkat and K562 cells. A kit (Upstate Biotechnology) was used in the ChIP assay to prepare DNA templates immunoprecipitated with Ab specific to LEF-1, HDAC-1, acetyl-H3, or acetyl-H4 following the manufacturer's instructions. Briefly, proteins that bind to the genomic DNA in the cells were cross-linked using a final concentration of 1% formaldehyde in the tissue culture media. Fixation proceeded at 37°C for 10 min. The fixed cells were rinsed twice with ice-cold PBS and collected by centrifugation, then resuspended in SDS lysis buffer (supplemented with protease inhibitors). After incubation for 10 min on ice, the cell lysates were sonicated on ice to break the genomic DNA to an average length of 200–1000 bp and were microcentrifuged at 15,000 rpm at 4°C for 12 min. The sonicated samples that contained chromatin were precleared with the addition of protein A beads for 1 h at 4°C. The precleared chromatin solution was incubated with anti–LEF-1, anti–HDAC-1, anti–acetyl-H3, anti–acetyl-H4, or normal control IgG. Input chromatin solution was used as a positive control. No Ab was used as a negative control. After overnight incubation with each of the corresponding Abs, the Ab/chromatin mixtures were precipitated with protein A beads. The beads were sequentially washed with ChIP wash buffer and TE buffer. The complexes were eluted twice with 250 μl elution buffer (1% SDS and 100 mM $NaHCO_3$) for each ChIP reaction. Cross-links were reversed by adding 5 M NaCl and incubated at 65°C for 4 h. The DNA was deproteinized by adding proteinase K at 45°C for 2 h. The DNA was recovered by phenol/chloroform extraction, ethanol precipitation, and dissolved in TE buffer. The immunoprecipitated DNA was then subjected to PCR. The PCR primers specific for the detection of the CD1D promoter region were 5′-cacctagagacatgtactgc-3′ and 5′-cgctcacttcagtaggtttc-3′. A DNA sequence derived from the promoter region of the gene encoding phosphoenolpyruvate carboxykinase (PCK) was used as a nonspecific

control. The PCR primers used for the amplification of the PCK promoter region were 5′-tgcatccccacctgcgtcct-3′ and 5′-tggccaccagagcgacgatg-3′. PCR conditions were 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C, and extension at 72°C for 30 s. The PCR products were separated in 2% agarose gel. Alternatively, the immunprecipitated DNA was subjected to a real-time qPCR reaction as described above, with an input genomic DNA used as a positive control.

Data analysis

The data are representative of three separate experiments or presented as the mean with error bars where appropriate to indicate one SD derived from three separate experiments.

Results

Specific binding of LEF-1 to the regulatory region of the CD1D gene

EMSA was performed to examine whether the transcription factor LEF-1 can bind specifically to the CD1D promoter region that contains two putative LEF-1 binding elements, 5′-CTTTGAA-3′ (−294 to −288) and 5′-CTTTGAT-3′ (−420 to −414) (Fig. 1*A*). Oligonucleotides, −304 to −285 and −423 to −396, separately covering these two putative LEF-1 elements, were tested in the assay (Fig. 1*B*). A signal was detected when the ^{32}P-labeled oligonucleotide −304 to −285 was reacted with nuclear proteins, and such a signal could be blocked with unlabeled oligonucleotide (Fig. 1*C*). A supershifted signal was detected when a mAb specific to LEF-1 was added into the reaction, suggesting that LEF-1 protein bind to the oligonucleotide. A smear signal was detected when the oligonucleotide −423 to −396 was tested (data not shown), suggesting that multiple nuclear proteins bind to the oligonucleotide. To remove the binding of the irrelevant nuclear proteins to the oligonucleotide, a mutant oligonucleotide −423/−396m2/3 was used in the reaction (Fig. 1*B*). The results showed that a signal was detected and such a signal could be supershifted with the Ab to LEF-1 (Fig. 1*D*). In addition, the detected signal for the mutant oligonucleotide −423/−396m2/3 can be blocked with the unlabeled oligonucleotide −304 to −285 and vice versa (data not shown), indicating that these two oligonucleotides share a same functional element for LEF-1 to bind.

To examine whether the binding signal supershifted by the mAb to LEF-1 was associated with the element 5′-CTTTGAT-3′ within the oligonucleotide −423 to −396, the element 5′-CTTTGAT-3′ was mutated together with one of the other two putative transcriptional elements within the oligonucleotide (Fig. 1*B*) and tested in the assay. As shown in Fig. 1*E* and 1*F*, binding signals were detected, but no signal was supershifted by the Ab to LEF-1, suggesting that the mutation of the element 5′-CTTTGAT-3′ demolished the binding of LEF-1 to the oligonucleotide. It also suggests that the mAb to LEF-1 was specifically reactive to LEF-1 in the supershifted signal seen in Fig. 1*C* and 1*D*. A similar result was obtained for the oligonucleotide −304 to −285 when the element 5′-CTTTGAA-3′ was mutated (data not shown).

The proximal LEF-1 element, containing 5′-CTTTGAA-3′, downregulates CD1D promoter activity

To evaluate the functional role of these two LEF-1 binding elements in regulating the activity of the CD1D promoter, we mutated either one of the LEF-1 binding elements in the CD1D promoter region. Constructs that cover the distal region (Fig. 2*B*) or both the distal and the proximal promoter regions (Fig. 2*C*) of the CD1D gene were tested for luciferase activity in K562 and Jurkat cells. When the element 5′-CTTTGAA-3′ (−294 to −288) was mutated, the luciferase activity increased more than twice that of the wild type in both of the cell lines (Fig. 2), suggesting that this element plays an inhibitory role in CD1D promoter activity. In contrast, the luciferase activity did not change significantly when the element 5′-CTTTGAT-3′ (−420 to −414) was mutated, suggesting that this element has a limited effect on the function of the CD1D promoter.

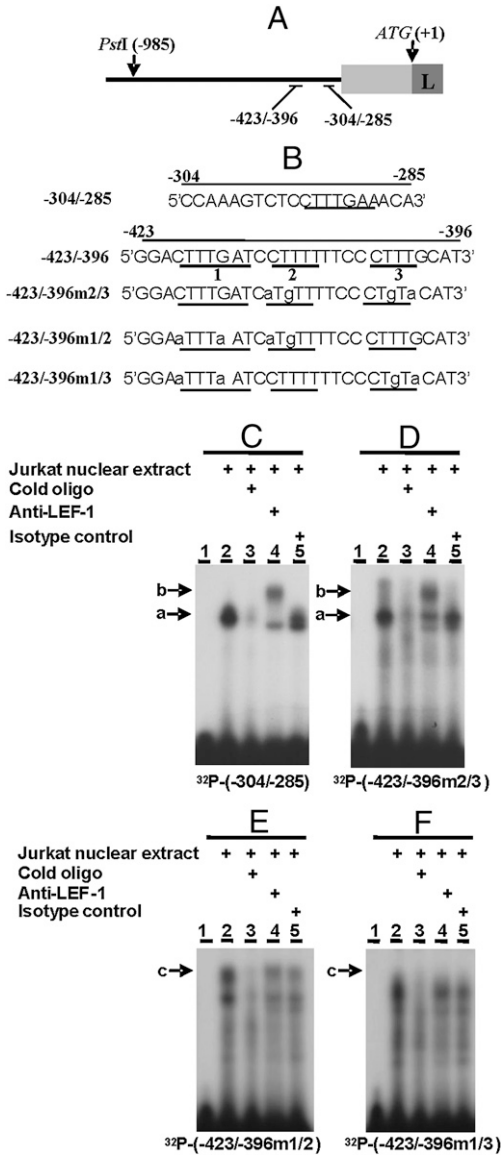

FIGURE 1. Identification of LEF-1 binding elements using EMSA. *A*, The positions of the two oligonucleotides, −304/−285 containing 5′-CTTTGAA-3′ and −423/−396 containing element 5′-CTTTGAT-3′, were marked within the distal CD1D promoter. *B*, Sequence of the two oligonucleotides. To avoid smear signals, three mutant oligonucleotides were also used in the study. Mutations were introduced to remove the putative LEF-1 binding element 5′-CTTTGAT-3′ marked as 1 and two other putative transcriptional elements marked as 2 and 3. These oligonucleotides were subjected to EMSA analysis. *C* and *D*, Arrow "a" points to the binding signal, and arrow "b" points to the signal supershifted by the mAb specific to LEF-1. *E* and *F*, Arrow "c" points to the position where no specific signal was supershifted by the Ab to LEF-1. Unlabeled oligonucleotides or cold oligonucleotides were tested at 100× molar excess over the [^{32}P]ATP–labeled oligonucleotides.

Overexpression of LEF-1 suppresses the human CD1D promoter activity and the endogenous CD1D transcript

We examined whether overexpression of LEF-1 can have an effect on CD1D promoter activity using luciferase assays. We first looked at whether transfection of the LEF-1 cDNA expression vector in

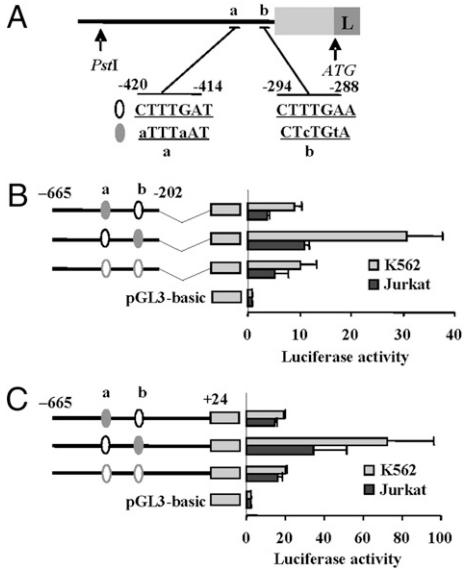

FIGURE 2. Evaluation of the functional effects of two LEF-1 elements on CD1D promoter activity. *A*, Location of the two LEF-1 elements (open oval) and the mutant sequences (shaded oval). *B* and *C*, The mutated LEF-1 elements on the CD1D promoter either with the distal promoter alone (*B*) or with both the distal and the proximal CD1D promoter (*C*) were transfected into Jurkat or K562 cells for promoter activities. The results show that mutation of the element 5′-CTTTGAA-3′ enhances CD1D promoter activity indicating that this element plays a suppressive role in the CD1D promoter.

Jurkat or K562 cells could enhance the expression of LEF-1 protein in these cells by Western blotting. The results showed that endogenous LEF-1 protein is present in these two cell lines because LEF-1 protein is detected in the cells transfected with the control vector (Fig. 3*A*, mock). However, the transfection of the cells with the LEF-1 expression vector enhanced the expression of the LEF-1 protein (Fig. 3*A*, LEF-1V).

We then examined whether an enhanced expression of LEF-1 could affect CD1D promoter activity. The human CD1D promoter construct inserted with the genomic DNA fragment of −665 to −202 was cotransfected with the LEF-1 cDNA expression vector into either Jurkat or K562 cells and then tested for luciferase activity. As shown in Fig. 3*B*, enhanced expression of LEF-1 reduced CD1D promoter activity up to 30%, as seen either in Jurkat or K562 cells, suggesting that LEF-1 has a suppressive effect on CD1D promoter activity.

In addition, we evaluated whether overexpression of LEF-1 can have an effect on CD1D gene transcription using RT-PCR. The results showed that overexpression of LEF-1 is associated with a reduced level of the endogenous CD1D transcripts (Fig. 3*C*). Such results were reproduced when the samples were tested using qRT-PCR (Fig. 3*D*), which is consistent with the results seen in the reporter assay, supporting the notion that enhanced LEF-1 expression has a suppressive effect on CD1D gene transcription.

We then examined whether overexpression of LEF-1 can affect the expression of surface CD1d protein in these two cell lines using flow cytometry. The results showed weak suppression of the surface CD1d expression on K562 cells but not so obvious suppression on Jurkat cells (Fig. 3*E*). The weak effect of LEF-1 overexpression on the surface CD1d could be due to the fact that both cell lines have already expressed LEF-1 abundantly, a further suppression of CD1D level is less prominent.

Silencing of LEF-1 elevates CD1D expression

To confirm that LEF-1 can regulate CD1D gene expression, we evaluated whether a decreased level of LEF-1 can affect CD1D gene expression in Jurkat and K562 cells. We transiently transfected LEF-1–specific siRNA or control siRNA into the cells, then tested the cells for comparative LEF-1 expression and CD1D gene expression. As shown in Fig. 4*A1* and 4*B1* with Western blot analysis to detect endogenous LEF-1 protein, the intensity of the band derived from the cells transfected with LEF-1–specific siRNA is much lower than the intensity of the band derived from cells transfected with control siRNA in either Jurkat cells (Fig. 4*A1*) or K562cells (Fig. 4*B1*). Actin was used as an internal control in the test. The result suggests that the LEF-1–specific siRNA effectively

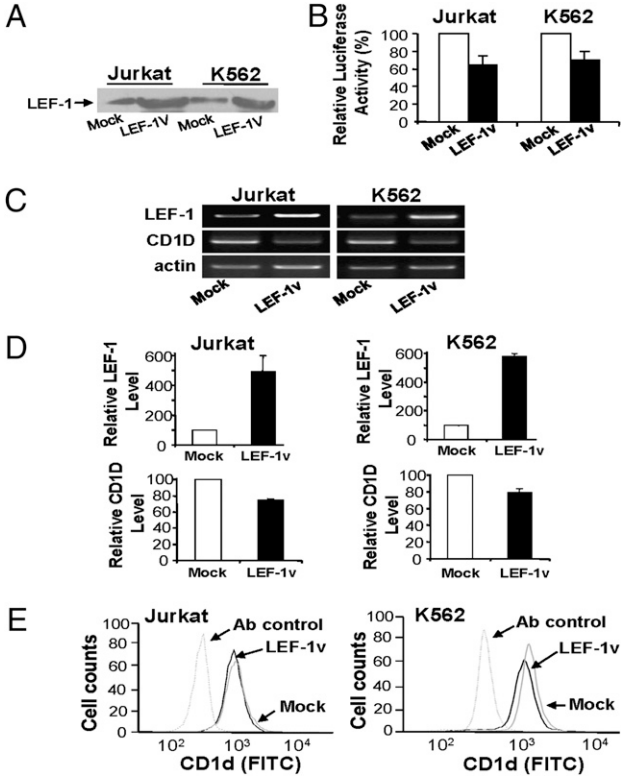

FIGURE 3. Overexpression of LEF-1 suppresses CD1D gene expression in Jurkat and K562 cells. *A*, Western blotting to detect LEF-1 protein in cells transiently transfected with control vector (mock) or LEF-1 expression vector. The results showed that both cell lines express LEF-1 proteins, but the transfection of the cells with the LEF-1 expression vector enhances the level of LEF-1 protein in the cells. *B*, Cotransfection of the cells with the LEF-1 expression vector reduced CD1D promoter activity. The cells were transiently transfected with a reporter construct inserted with the distal CD1D promoter region (−665 to −202) and the LEF-1 expression vector or the control vector (mock). The relative luciferase activity was expressed as a percentage of the mock transfection control. An average of three different experiments was shown. *C*, RT-PCR to test the effect of LEF-1 on CD1D gene expression. Cells were transiently transfected with the LEF-1 expression vector or the control vector (mock) and tested for LEF-1, CD1D, or actin expression. The results showed that an increased level of LEF-1 transcripts is associated with a decreased level of CD1D transcripts in both Jurkat and K562 cells. Note that a shadow band on the top of the CD1D band was caused by diffusion of the loading sample in 2% agarose gel. *D*, qRT-PCR to detect CD1D gene expression in cells transiently transfected with the LEF-1 expression vector or the control vector (mock). The results are expressed as a percentage of the value derived from the respective mock transfection controls. Again, increased LEF-1 expression is associated with reduced expression of the CD1D gene. *E*, Flow cytometry to detect surface CD1d on cells transiently transfected with the LEF-1 expression vector or the control vector (mock). In comparison to the mock control, CD1d expression is minimally decreased on K562 cells and not so obvious on Jurkat cells when the cells were transiently transfected with the LEF-1 expression vector. An isotype control Ab (Ab control) was included in the test.

and specifically reduces the expression of LEF-1 protein in both Jurkat and K562 cells.

A proportion of the above cells were tested for whether reduced expression of the LEF-1 gene can affect the expression of the CD1D gene by RT-PCR, qRT-PCR, and flow cytometry. By RT-PCR, the transient transfection of LEF-1–specific siRNA effectively reduces the expression of LEF-1 transcripts compared with that of the cells transfected with the control siRNA in both cell lines based on the comparative intensity of the bands (Fig. 4*A2*, 4*B2*, LEF-1), which is consistent with the results seen for the effects of siRNA on the expression of LEF-1 protein. In contrast, the level of the CD1D transcripts derived from the cells transfected with LEF-1–specific siRNA increased compared with that of the cells transfected with the control siRNA based on the comparative intensity of the bands (Fig. 4*A2*, *B2*, CD1D). Such results were reproduced using real-time qRT-PCR (Fig. 4*A3*, *B3*), suggesting that reduced expression of LEF-1 is associated with enhanced expression of the CD1D transcript. When the cells were tested using flow cytometry for surface CD1d expression, Jurkat cells transfected with LEF-1–specific siRNA had an obviously increased level of the surface CD1d protein expression compared with that of the controls (Fig. 4*A4*), but such an increase was minimal on K562 cells (Fig. 4*B4*). The difference at the CD1d protein level but not at the transcript level between K562 cells and Jurkat cells in response to reduced expression of LEF-1 suggests that these two cell lines may differ in levels of translation or in the processing of the surface CD1d proteins. Overall, these results imply that the endogenous LEF-1 transcription factor plays a role in the suppression of CD1D gene expression.

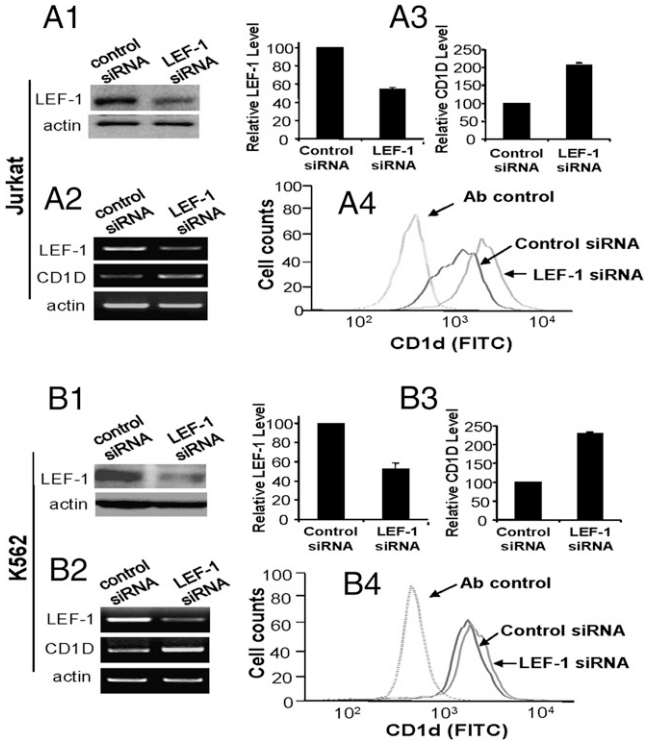

FIGURE 4. Reduced expression of LEF-1 enhances the expression of the CD1D gene in Jurkat (*A*) and K562 (*B*) cells. The cells were transiently transfected with either control siRNA or LEF-1–specific siRNA and processed for the detection of LEF-1 protein expression, comparative levels of LEF-1 and CD1D transcripts, and surface CD1d expression. *A1* and *B1*, Transfection of LEF-1–specific siRNA into the cells effectively reduced the expression of LEF-1 protein detected using Western blot analysis. *A2* and *B2*, By RT-PCR analysis, a decreased level of LEF-1 is associated with an increased level of CD1D transcripts. *A3* and *B3*, By qRT-PCR analysis, decreased expression of LEF-1 (*left panel*) is associated with increased expression of CD1D transcripts (*right panel*). The results are expressed as a percentage of the values derived from the respective control siRNA. *A4* and *B4*, Flow cytometry to detect surface CD1d expression with an Ab specific to CD1d or an isotype control (Ab control) on cells transiently transfected with control siRNA or LEF-1–specific siRNA. The results show that Jurkat cells transfected with LEF-1–specific siRNA express a higher level of surface CD1d than those transfected with the control siRNA, but such an enhanced level of surface CD1d was minimal on K562 cells.

LEF-1 transcription factor binds to the CD1D promoter region and interacts with HDAC-1

We examined, using ChIP assays, whether endogenous LEF-1 is actually bound to the CD1D promoter. The results showed that the CD1D genomic DNA region covering the two LEF-1 binding elements (−451/−242) was detected by PCR using the DNA template precipitated with Ab to LEF-1 (Fig. 5*A*, α-LEF-1) but not so with the irrelevant IgG control (Fig. 5*A*, IgG). In addition, as a negative control, PCK was not detectable using the same DNA template precipitated with Ab to LEF-1 or the IgG controls, although signal was detected in the input samples that were used as positive controls (Fig. 5). The results suggest that LEF-1 binds to the CD1D promoter region that was coprecipitated by the anti–LEF-1 Ab.

We have demonstrated in the above experiments that LEF-1 acts to suppress CD1D gene expression and physically bind to the CD1D promoter region. It is known that human LEF-1 interacts with HDAC-1 and requires HDAC-1 activity to repress transcription (28, 29). To examine whether HDAC-1 interacts with the LEF-1/CD1D promoter region complex, we used an Ab specific for HDAC-1 in the ChIP assay and applied the precipitated DNA as the template in a PCR reaction to detect the presence of the CD1D promoter region. The result showed that the CD1D promoter region can be detected in the PCR reaction (Fig. 5*B*), suggesting that the CD1D promoter region was also coprecipitated with HDAC-1. This could be due to an interaction between

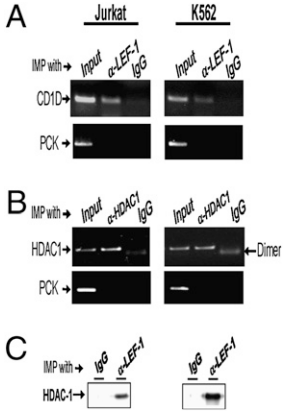

FIGURE 5. ChIP analysis in Jurkat and K562 cells. *A* and *B*, Detection of specific binding of LEF-1 (*A*) or HDAC-1 (*B*) to the CD1D promoter region (−451/−242 bp). The lanes were loaded with PCR products generated from different templates, including template derived from the input chromatin lysates serving as a positive control (input), and the lysates precipitated with Abs specific to LEF-1 (α-LEF-1), Abs specific to HDAC-1 (α-HDAC-1), or an IgG serving as a negative control (IgG). The bands detected in some wells marked with IgG were PCR primer dimers. As controls, the same template samples were also used for the detection of a PCK promoter region. *C*, Western blotting to detect molecular interaction between LEF-1 and HDAC-1. The result showed that HDAC-1 was detected in the sample precipitated with the anti–LEF-1 but not so with the IgG control, suggesting a physical interaction between LEF-1 and HDAC-1 in the cells.

HDAC-1 and LEF-1 that binds to the CD1D promoter region. Such an interaction has already been demonstrated in previous studies (28, 29) and confirmed in ours because HDAC-1 protein can be detected in the immune complex derived from the cell lysates reacted with an Ab specific to LEF-1 but not so with a control IgG by Western blotting (Fig. 5*C*). These results suggest that LEF-1 binds to the human CD1D promoter and interacts with HDAC-1 in these two cell lines.

Reduced binding of LEF-1 to the CD1D promoter is associated with a reduced level of HDAC-1 but an increased level of acetylated histone H3/H4 in the CD1D promoter region

We evaluated whether LEF-1 contributes to the modification of histone acetylation in the CD1D promoter region. We used LEF-1–specific siRNA to knock down LEF-1 expression, then used PCR or qPCR to measure the comparative levels of the CD1D promoter region precipitated with Abs to LEF-1, HDAC-1, acetyl-H3, acetyl-H4, or a control IgG in the ChIP assay. In comparison to the intensity of the PCR bands derived from the cells treated with control siRNA, the intensities of the bands derived from the cells treated with LEF-1–specific siRNA are reduced for the template DNA precipitated with anti–LEF-1 but increased with anti–acetyl-H3 or anti–acetyl-H4 (Fig. 6*A*, CD1D), suggesting that reduced binding of LEF-1 to the CD1D promoter is associated with an increased level of acetyl-H3 or acetyl-H4 in the CD1D promoter. Such a result was reproduced using qPCR (Fig. 6*B*). In addition, a DNA sample precipitated with anti–HDAC-1 was also included in the qPCR and showed a parallel result to that precipitated with anti–LEF-1for either Jurkat or K562 cells (Fig. 6*B*). A primer pair derived from the PCK promoter region was used as a control to verify whether non-CD1D-specific DNA was also present in the template samples precipitated with the above specific Abs. The results showed that no PCK was amplified in template samples precipitated with anti–LEF-1 or IgG control, but minor bands were observed in the samples precipitated with anti–acetyl-H3 or anti–acetyl-H4 (Fig. 6*A*, PCK), suggesting that the PCK promoter could also possess acetyl-H3/H4 proteins. Overall, the results suggest that a decreased binding of the transcription factor LEF-1 to the CD1D promoter due to a reduced level of LEF-1 by LEF-1–specific siRNA is associated with a reduced level of HDAC-1 but an enhanced level of acetylated histone H3/H4 within the CD1D promoter region.

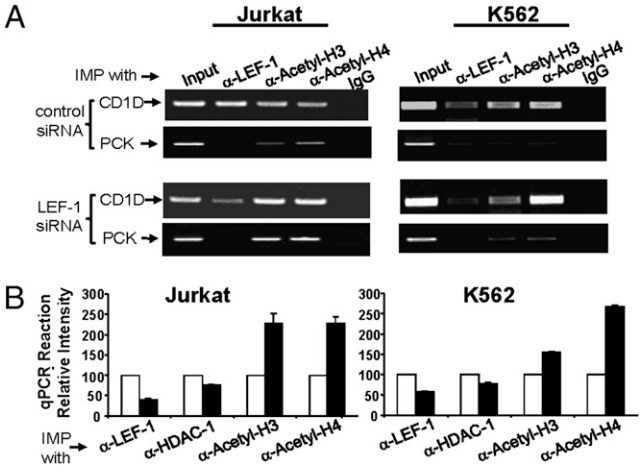

FIGURE 6. Effects of LEF-1 on the differential acetylation status of H3 and H4 within the CD1D promoter region examined using comparative PCR (*A*) or real-time qPCR (*B*) in Jurkat and K562 cells. After treatment of the cells with control siRNA or LEF-1–specific siRNA, the cell lysates were immunoprecipitated with an Ab specific to LEF-1, HDAC-1, acetyl-H3, or acetyl-H4 or with an IgG as a negative control. The precipitated genomic DNA was processed and specifically amplified by PCR or real-time qPCR for the CD1D promoter region. *A*, The bands derived from the cells treated with control siRNA were shown to compare with the respective bands derived from the cells treated with LEF-1–specific siRNA. *B*, The relative intensity of the qPCR reaction for each sample derived from the cells treated with LEF-1–specific siRNA (black) was expressed as the percentage of the intensity of the respective sample derived from the cells treated with the control siRNA (white). The results showed that reduced binding of LEF-1 to the CD1D promoter is apparently associated with a reduced level of HDAC-1 and enhanced acetylation of H3 and H4 within the CD1D promoter region.

Discussion

We demonstrated in this study that LEF-1 regulates human CD1D gene expression. This conclusion is based on the functional effects of LEF-1 on CD1D gene expression in two human leukemia cell lines, K562 and Jurkat. We showed that LEF-1 binds to the CD1D promoter using a gel-shift assay. Such binding was confirmed using a ChIP assay, which showed that LEF-1 protein is physically cross-linked to the genomic DNA covering the two LEF-1 elements within the CD1D promoter region in vivo. LEF-1 was also shown to downregulate CD1D gene expression because cells with increased levels of LEF-1 suppress CD1D promoter activity and decrease the levels of CD1D transcripts. A decreased level of endogenous LEF-1 using LEF-1–specific siRNA enhanced CD1D gene expression, similar to the results in a recent study in which LEF-1 suppresses the expression of the IL-4 gene in T cells (30).

We also showed that, apart from LEF-1, HDAC-1 is also situated on the human CD1D promoter region revealed using the ChIP assay. HDAC-1 can physically interact with LEF-1, as demonstrated using Western blotting, suggesting that LEF-1 is capable of interacting with HDAC-1 when LEF-1 binds to the CD1D promoter. It is known that human LEF-1 interacts with HDAC-1 and requires HDAC-1 activity to repress transcription (28, 29). These together with the repressive activity of LEF-1 on the CD1D gene suggest that the negative effects of LEF-1 on the CD1D gene are associated with the binding of LEF-1 to the CD1D promoter through the 5′-CTTTGAA-3′/5′-CTTTGAT-3′ binding sites with the interaction of HDAC-1 with the same region for histone modification. In addition, our results showed that reduced binding of LEF-1 to the CD1D promoter is associated with a reduced level of HDAC-1 but an increased level of acetyl-H3 and acetyl-H4 within the same region. This suggests that fewer LEF-1 molecules situated on the CD1D promoter interact less HDAC-1 and are accompanied by an increased level of acetyl-H3 and acetyl-H4 in the region, which is consistent with the known facts for the conserved suppressive functional effects of TCF/LEF-1 molecules on other genes (28, 29).

The two identified LEF-1 binding elements within the distal promoter region of the human CD1D gene have different effects in regulating the distal promoter activity of the human CD1D gene. The element 5′-CTTTGAT-3′ (−420 to −414) apparently does not have a functional effect on the function of the CD1D promoter, because the mutation of this element does not change the promoter activity. In contrast, the element 5′-CTTTGAA-3′ (−294 to −288) located closer to the coding region is responsible for the repressive activity, because mutation of this element enhanced CD1D promoter activity. This result is consistent with the results of our previous study showing that the region from −246 to −314 is essential for the function of the distal CD1D promoter (17). Additionally, it is known that the position of the LEF-1 binding site can determine the extent of β-catenin–LEF-1 responsiveness for its target genes (31). Therefore, these results indicate that the

element 5′-CTTTGAA-3′ (−294 to −288) participates in the regulation of the distal CD1D promoter.

The effect of LEF-1 on the CD1D gene is associated with the transcriptional function of the distal promoter but not the proximal promoter of the human CD1D gene. This is due to the fact that the proximal promoter does not harbor a LEF-1 binding element. The two CD1D promoters have a different cell-type–specific activity (17). Consequently, the effects of LEF-1 on the expression of the CD1D gene are limited to the regulation of the distal promoter of the CD1D gene. Together with the known CD1D transcriptional regulators SP1, Ets families, and all-*trans*-retinoic acid (17, 32, 33), our results suggest a complicated regulatory mechanism involved in the regulation of the cell-type–specific expression of the human CD1D gene.

In addition, the effect of LEF-1 on the expression of the surface CD1d protein apparently differs between K562 and Jurkat cells. A reduction of LEF-1 expression using siRNA enhanced the CD1D transcript expression in both K562 and Jurkat cells. However, an increase of the surface CD1d expression was obvious on Jurkat cells but was minimal on K562 cells in response to the LEF-1–specific siRNA. It suggests that these two cell types might differ in translation or posttranslational modification of the CD1d protein, apart from the known fact that these two cell lines have different activities for the two CD1D promoters (17).

The significance of our study that LEF-1 regulates CD1D gene expression is to connect Wnt signaling to the regulation of the CD1D gene. LEF-1 is one of the key regulators of the Wnt signaling pathway (22). LEF-1 participates in the regulation of downstream target genes of the activated Wnt signaling pathway (23, 24). Wnt signaling plays an important role in the normal hematopoietic development and self-renewal process of hematopoietic stem cells (21). Aberrant alterations of the Wnt signaling have been associated with oncogenesis. The alterations include gene mutations or epigenetic modification. Either change can result in the activation of their downstream genes, including oncogene c-MYC and cyclin D1 to promote neoplastic growth (34–37). Aberrant expression of β-catenin, which plays a central role in Wnt signaling, is associated with a poor prognosis in patients with acute myeloid leukemia (38, 39). Interestingly, a recent study showed that a subgroup of patients with acute lymphoblastic leukemia was associated with a poor prognosis when the leukemia cells express CD1d (40). Our data showing that LEF-1 can regulate the CD1D gene would help to establish a relationship between Wnt signaling and CD1D gene regulation, which may reveal the mechanism behind these clinical observations. In addition, it is known that a cross talk between CD1d-expressing cells and NKT cells is essential to the function of NKT cells (1, 2). Our findings thus point in the direction of a physiological or pathological role of Wnt signaling in association with the function, such as immune surveillance, of the CD1d-restricted NKT cells.

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Disclosures

The authors have no financial conflicts of interest.

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