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The regulation of TIM-3 transcription in T cells involves c-Jun binding but not CpG methylation at the TIM-3 promoter☆

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ABSTRACT

Tim-3 is an immunomodulatory protein that is expressed constitutively on monocytes but is induced in activated T cells. The mechanisms involved in the regulation of TIM-3 transcription are poorly understood. In the present study, we investigated whether methylation of the TIM-3 promoter is involved in regulatingTIM-3 transcription in T cells, and identified a transcription factor that regulates TIM-3 transcription by associating with the TIM-3 minimal promoter region. Pyrosequencing of the TIM-3 promoter up to −1440 bp revealed 11 hypermethylated CpG sites and 4 hypomethylated CpG sites in human CD4$^+$ T cells as well as in CD11b$^+$ cells. Dimethylation of histone H3 lysine 4 (H3K4), a mark of transcriptional activation, was predominantly found in the proximal TIM-3 promoter −954 to −34 bp region, whereas trimethylation of H3K9 and H3K27, which are markers of transcriptional suppression, were mostly observed in the distal promoter −1549 to −1048 bp region in human CD4$^+$ T cells and CD11b$^+$ cells. However, no change in the methylation status of CpG sites and the histone H3 in the TIM-3 promoter was found during induction of TIM-3 transcription in T cells. Finally, AP-1 involvement in TIM-3 transcription was shown in relation with the TIM-3 minimal promoter −146 to +144 bp region. The present study defines the minimal TIM-3 promoter region and demonstrates its interaction with c-Jun during TIM-3 transcription in CD4$^+$ T cells.

1. Introduction

T cell immunoglobulin domain and mucin domain (Tim-3) is a transmembrane protein involved in the regulation of the activity of cells of the immune system, for example by enhancing phagocytosis of apoptotic cells and by inhibiting cytokine production by monocytes and dendritic cells (DeKruyff et al., 2010; Nakayama et al., 2009; Zhang et al. 2011). Tim-3 also functions as a negative regulator of the Th1 immune response, as evidenced by the prolonged Th1 immune response in Tim-3 knockout mice and the increased production of IFN-γ by CD4$^+$ T cells in the presence of the Tim-3 blocking antibody (Kearley et al., 2007; Sabatos et al., 2003).

Tim-3 is expressed constitutively on monocytes/dendritic cells and is induced in CD4$^+$ T cells (Anderson et al., 2007; Monney et al., 2002). Tim-3 is not detected on the surface of naïve CD4$^+$ T cells, but its expression is induced in T cells by activating stimuli such as anti-CD3/CD28 antibodies, or phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187 (Hastings et al., 2009; Yoon et al., 2011). Tim-3 expressed in differentiated Th1 cells modulates inappropriate Th1 immune responses. Dysregulation of Tim-3 expression has been observed in some pathologic states such as HIV infection and autoimmune diseases (Jones et al., 2008; Koguchi et al., 2006; Vali et al., 2010; Yang et al., 2008). The upregulation of Tim-3 expression in HIV infection or the downregulation of Tim-3 expression in multiple sclerosis is suggested to be associated with insufficient or excessive Th1 immune responses, respectively, thus contributing to disease progression. Thus, a mechanistic understanding of the processes involved in the regulation of Tim-3 expression is important for elucidating the role of this protein in normal and pathologic immune responses; however, only limited information regarding Tim-3 transcriptional regulation is currently available.

☆ Activator protein 1 (AP-1); histone H3 lysine 4 (H3K4); histone H3 lysine 9 (H3K9); histone H3 lysine 27 (H3K27); phorbol 12-myristate 13-acetate (PMA); transcription start site (TSS).

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Transcription is regulated by the coordinate action of transcription factors and epigenetic enzymes that modify histone tails and alter the methylation status of DNA. The critical role of DNA methylation in gene transcription is indicated by the abnormal expression of IL-4 in naïve T cells deficient in DNA methyltransferase Dnmt1, as observed in in vivo studies (Lee et al., 2001). Additionally, demethylation of the −252 CpG site occurs at the IL-2 promoter in human naïve $CD4^+$ T cells after activation, leading to the recruitment of transcription factors and modification of histones and resulting in enhanced IL-2 transcription (Murayama et al., 2006). Histone modifications associated with transcriptional activation include acetylation of histone H3/H4 and methylation of H3K4, whereas those associated with transcriptional suppression include di- or trimethylation of H3K9 and H3K27 (Wilson et al., 2009). Hypoacetylated histone is converted into acetylated histone at the IL-2 promoter of naïve T cells after stimulation (Adachi and Rothenberg 2005). The differentiation of $CD4^+$ T cells into Th1 cells capable of producing high amounts of IFN-γ is concomitant with an increase in the permissive methylation of H3K4 at the IFN-γ promoter (Berger 2007; Chang and Aune 2007). In contrast, suppressive methylation of H3K27 increases but permissive H3K4 methylation disappears at the IFN-γ promoter in Th2 cells that are incapable of expressing IFN-γ (Chang and Aune 2007; Schoenborn et al., 2007). To date, epigenetic modification at the TIM-3 locus has not been reported.

In order to acquire a mechanistic understanding of TIM-3 transcriptional regulation in T cells, the methylation status of CpG sites and histone H3 of the TIM-3 promoter was examined. Additionally, the minimal TIM-3 promoter region was defined and the role of c-Jun in the regulation of TIM-3 transcription was shown.

2. Materials and methods

2.1. Cell isolation

$CD4^+$ T cells and $CD11b^+$ cells were isolated from peripheral blood mononuclear cells of healthy subjects using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells were sorted into $Tim\text{-}3^+$ or $Tim\text{-}3^-$ cells by fluorescence activated cell sorter (FACS Aria II cell sorter, BD Biosciences, Rockville, MD) after labeling with APC-conjugated anti-Tim-3 antibody (Ab) (eBioscience). Naïve $CD4^+$ T cells were isolated from human umbilical cord blood using Naïve $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). The purity of the isolated cells, as indicated by flow cytometry, exceeded 90%. All blood samples were obtained in compliance with the Ajou University Institutional Review Board protocols (AJIRB-GEN-GEN-10-127 and AJIRB-GEN-SMP-11-232).

2.2. Cell culture and activation of T cells

Jurkat T cells and $CD4^+$ T cells were maintained in RPMI 1640 (Invitrogen, Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 U/ml, Invitrogen), and streptomycin (100 μg/ml, Invitrogen). Jurkat T cells and $CD4^+$ T cells were stimulated with PMA (50 ng/ml, Sigma) and ionomycin (0.5 μM, Sigma) for the indicated time.

2.3. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using RNA STAT-60 (Tel-Test, Friendwood, TX) and subjected to real-time RT-PCR for TIM-3 transcription analysis using Sybr Premix Ex Taq (TaKaRa). The TIM-3 primers (Bioneer) used were 5′-TCCAAGGATGCTTACCACCAG-3′; 5′-GCCAATGTGGATATTTGTGTTAGATT-3′. TIM-3 transcription was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcription.

2.4. Pyrosequencing

Genomic DNA (500 ng) was bisulfite-converted using the EpiTect Bisulfite Kit (QIAGEN) according to the instructions of the manufacturer. Sequences adjacent to CpG dinucleotides were amplified by PCR using primers designed with Assay Design Software. Primer sequences are listed in Supplementary Table 1. Pyrosequencing of the resulting PCR products was performed at DisGene (Dajeon, Korea) using a Qiagen PSQ instrument.

2.5. Chromatin immunoprecipitation assay

Cells were resuspended in buffer containing 1% formaldehyde and then 0.125 M glycine was added at room temperature for cross-linking. Next, cells were lysed in buffer containing 1% (wt/vol) SDS and sonicated to obtain DNA fragments of an average length of about 300–500 base pairs. Protein-DNA complexes were immunoprecipitated overnight at 4 °C with Abs against dimethylated histone H3 lysine 4 (H3K4, Millipore), trimethylated H3K9 (Abcam), trimethylated H3K27 (Millipore), C-Jun (Santa Cruz Biotechnology), or c-Fos (Abcam) or with normal rabbit IgG (Abcam) as a control. DNA was purified after reversal of crosslinks at 65 °C and analyzed by real-time PCR. PCR primers are listed in Supplementary Table 2. Dissociation-curve analysis showed a single peak indicating specific amplification. The amount of each product in the input and precipitated DNA was calculated from the cycle threshold.

2.6. Plasmid construction and CpG methylation

We generated luciferase reporter vectors by ligation of human TIM-3 promoter region DNA fragment into pGL3-Basic vector (Promega, Madison, WI, USA). Human TIM-3 promoter DNA was subcloned from T3U(1.8)-luc, T3U(1.0)-luc, and T3U(0.5)-luc (Kim et al., 2012) into a pGL3-Basic vector. Additionally, we amplified the TIM-3 proximal promoter region by PCR using appropriate primer sets and T3U(1.0)-luc as the template. We then inserted the PCR products into pGL3-Basic vector and verified each inserted TIM-3 promoter DNA by sequencing and comparison with the published gene sequence (gene ID 84868). The luciferase reporter vector pCpGL-T3U(330) was constructed by PCR-based amplification of a DNA fragment from the TIM-3 promoter region (−186 bp to +144 bp from the transcriptional start site) and subcloning of the amplicon into the pCpGL-Basic vector (a kind gift from Dr. Rehli, Univ. Hospital, Germany, (Klug and Rehli 2006)). The reporter vector pGL3_T3U_330 (30 μg) was incubated with 60 units of CpG methyltransferase (M.SssI) (New England Biolabs) in methylase buffer supplemented with 160 μM S-adenosylmethionine for 16 h at 37 °C. The efficiency of CpG methylation was tested by digestion of DNA with the 5-methyl-cytosine-sensitive restriction enzyme, SmaI (New England Biolabs). Then, the DNA was ethanol precipitated and used in the luciferase reporter assay.

2.7. Luciferase reporter assay

Jurkat T cells were transfected with the luciferase reporter vector (3.6 μg) together with a pEGFP-N1 plasmid (0.4 μg, Clontech, Mountain View, CA) to normalize transfection efficiency using a Microporator (Digital Bio Technology, Seoul, Korea) with settings of 1800 V, pulse width 20, and pulse number 1. Twenty-four hours after transfection, the cells were stimulated with PMA (50 ng/ml) for 24 h, or with PMA and ionomycin (0.5 μM) for 6 h. The luciferase activity in the cell lysates was measured using a luminometer