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Differential lineage-specific regulation of murine CD45 transcription by Oct-1 and PU.1

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Abstract

Although it has been established that CD45 expression is regulated at the transcriptional level, neither the regulatory elements that are responsible for its unique expression pattern nor the relevance of its three distinct transcriptional start sites (P1a, P1b, and P2) has been fully characterized. We studied the contribution of the three start sites to CD45 mRNA production in haematopoietic cell lines and primary haematopoietic cells. In myeloid and lymphoid cells and cell lines most CD45 transcripts originate from P1b with the exception of the thymoma-derived T cell line EL4, in which ~90% of CD45 transcripts originate from P1a. The degree of contribution of P1a is highest in lymphoid cells and increases in T cells following mitogen stimulation. In vitro evaluation of sequence upstream of the start sites shows that the P2 start site is sufficient for CD45 expression in lymphoid but not in myeloid cells, confirms the presence of a PU.1-binding site essential for myeloid expression of CD45, and reveals an Octamer-binding site that interacts with both Oct-1 and Oct-2 and activates CD45 transcription in lymphoid and myeloid cells. These findings are the first evidence that Octamer-binding factors are involved in the control of CD45 expression.

Keywords: CD45; PTPRC; Transcription factor; Transcriptional regulation; Alternative start site; PU.1; Octamer; Oct-1; Oct-2

CD45 (formerly known as the leukocyte common antigen) is an abundant cell surface glycoprotein whose expression is restricted to cells of the haematopoietic system [1,2, p. 56]. Numerous isoforms, ranging in size from 180 to 240 kDa, are generated by alternative exon splicing and are expressed in a cell type-specific pattern on functional subpopulations of lymphocytes [3–5]. CD45 is a transmembrane protein tyrosine phosphatase (PTPase), and is a critical component of the antigen-dependent signal transduction machinery of lymphocytes [6] and also regulates integrin-mediated signalling in neutrophils and monocytes [7]. In addition to these functions, CD45 has recently been shown to suppress Janus kinase (JAK) signalling, thereby acting as a negative regulator of cytokine receptor signalling in haematopoietic cells [8]. CD45 expression is typically deficient in leukaemic blasts [9], fueling speculation that the loss of this regulator of proliferation contributes to the leukaemic phenotype.

All nucleated haematopoietic cells express CD45 [1], and the protein is detectable from the earliest identifiable stage of haematopoietic development, [10] permitting its use as a marker of mesodermal commitment to a haematopoietic fate in embryogenesis. Studies of mRNA abundance [4] and nuclear run-on experiments [11] have demonstrated that the expression of CD45 is regulated at the level of transcription, rather than by mRNA stability or by post-translational mechanisms. However, little is known concerning the mechanisms by which the unique expression pattern of CD45 is initiated or maintained.

The mouse *CD45* locus spans 120 kb, including a 50 kb intron following exon 2, and comprises 34 exons [12].

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Alternative transcriptional start sites have been mapped [12] for exons 1a, 1b, and 2, and are referred to as P1a, P1b, and P2. The P1b start site is the major initiation site for *CD45* transcription, and substantial nucleotide sequence conservation between the mouse and human loci has been observed upstream of this site [1]. A potential PU.1-binding site has been identified in this region [13], and the absence of CD45 expression in PU.1 null myeloid cells suggests that this transcription factor is an important regulator of myeloid CD45 expression. DiMartino et al. [14] reported functional studies of the minor P2 start site. They identified a cluster of nucleotides, which they named the TC box, that can act as a minimal promoter for the P2 site. This sequence showed activity only in constructs from which the major P1b start site had been deleted, and the physiological relevance of the TC box remains unclear.

The regulation of CD45 expression in the lymphocyte lineage has not been well characterized. Virts and Raschke [15] studied the expression of *CD45* cDNA constructs in lymphocyte-derived cell lines. They found that *CD45* cDNA, driven either by a retroviral LTR promoter or by the T cell-specific *lck* promoter, failed to result in detectable CD45 protein expression in the T cell lines 27J or BW5147. Transient transfections of the *CD45* 3′ untranslated region fused downstream of a β-galactosidase reporter gene revealed no influence of the 3′UTR on reporter expression. A *CD45* minigene, comprising a genomic DNA fragment containing exons 4–8 flanked by cDNA containing exons 1–3 and exons 9–33, expressed strongly in lymphoid cell lines when driven by the LFA-1 promoter. However, when the LFA-1 promoter was replaced by 19 kb of native *CD45* upstream sequence, relatively weak *CD45* expression was detected. These findings suggest that these *CD45* upstream and intronic sequences may not be sufficient to produce high-level *CD45* expression in T cells. Regulatory elements in the upstream region may however be necessary for high-level, tissue-specific *CD45* expression in other cell types.

In summary, three transcriptional start sites of *CD45* have been mapped, although the importance of the P1a and P2 start sites in lineage- or stage-specific expression of CD45 has not been determined. Sequence analysis of the *CD45* locus suggests the presence of important 5′ regulatory elements immediately upstream these sites. No systematic functional analysis of this region has been published. Here we report an analysis of lineage- and stage specificity of CD45 start site choice and the characterization of regulatory elements upstream of the major promoter of *CD45*.

Experimental procedures

Plasmids. The wild-type CD45 promoter and immediate upstream region from position −438 to +251 relative to the P1b transcriptional start site were amplified by PCR, using an upstream primer containing a *Sac*I site and cloned into pPCRScript (Clontech; Mountain View, CA). A *Sac*I–*Nco*I fragment of this promoter clone was subcloned into pGL3B (Promega; Madison WI) such that the ATG codon of the luciferase gene was substituted for that of *CD45*. Deletion mutants were generated using upstream PCR primers at positions −420, −379, −215, −170, −155, −125, −110, and −100. A construct lacking both the P1a and P1b start sites was generated using the *Nhe*I restriction site at position +25. The ΔOct and ΔPU.1 substitution mutants were generated by a two-step PCR method. The DNA sequences of all clones generated by PCR were verified prior to use in luciferase assays.

Cells, cell culture, and flow cytometry. Murine neutrophils and promyelocytes were obtained by culture of bone marrow from C57Bl/6 and hCG-NuMA-RAR transgenic mice [16], respectively. Thymocytes and T cells from thymus and spleen were isolated from 6-week-old C57Bl/6 mice by fluorescence-activated cell sorting (FACS) after staining with CD4 and CD8 antibodies. Similarly, B220-expressing B cells were isolated from spleens of C57Bl/6 mice by FACS.

The RAW264.7 murine macrophage cell line [17] and NIH3T3 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS: GibcoBRL), L-glutamine and antibiotics. HCD57 erythroleukaemia cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 0.05 mM 2-mercaptoethanol (2-ME), 0.5 U/mL erythropoietin, and 20% FBS. WEHI-3 and EL-4 cells were grown in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.05 mM 2-ME and 10% FBS. MEL3 and M12 cells were grown in RPMI (Gibco) with 4 mM L-glutamine and 10% FBS. Rα30G cells were maintained in Opti-MEM (Gibco) supplemented with 4% WEHI-3 conditioned supernatant and 20% FBS.

Cell surface expression of CD45 was determined in primary cells and cell lines by flow cytometry following incubation with an antibody recognizing all isoforms of CD45 (BD Biosciences; San Jose, CA).

RNA extraction and real-time reverse transcription PCR. RNA was extracted from cell lines or primary cells using Trizol reagent and was purified by column chromatography (RNeasy; Qiagen, Netherlands). Total RNA was reverse transcribed from oligo(dT) primer with MMLV reverse transcriptase (Invitrogen; Burlington, ON, Canada). Quantitative real-time PCR was performed with an Applied Biosystems 7700 Analyzer, according to manufacturer's instructions. Data were analysed by the ΔC_T method, with normalization of all transcript levels to levels of the housekeeping gene *β-actin*. The levels of endogenous CD45 transcripts were quantified by amplification of the 5′ region of either the P1a, P1b, or P2 transcript, using the following primers: sense P1a/P2 (5′-CTT GTC ATA TCT TGG GGA GAC-3′); sense P1b/P2 (5′-AAG ACA GAG TGC AAA GGA GAC-3′); sense P2 (5′-TGC AAA GTA TGC GTT CTT TTC TTT TAG-3′); antisense CD45 exon 2/3 (5′-TGT AGG TGT TTG CCC TGT GAC AAA GAC-3′), sense β-actin (5′-TTC CAG CAG CTG TGG CTA CGA-3′), antisense β-actin (5′-AGT CCG CCT AGA AGC ACT TGC-3′).

Transfections. RAW264.7 cells were transfected transiently by electroporation [18]. Cells were suspended in DMEM with 10% FBS at a density of 3.75×10^7/mL, and 200 μL of cells plus 10 μg of luciferase plasmid and 10 μg of β-galactosidase reporter were electroporated in a 0.4 mm cuvette (Bio-Rad, Hercules CA) in a Bio-Rad Gene Pulser at 250 V and 950 μF. Following a 10 min recovery period at room temperature cells were washed with 5 mL PBS and resuspended in 5 mL DMEM plus 10% FBS. Forty-eight to sixty hours after electroporation cell extracts were prepared in 500 μL lysis buffer (Promega, Madison WI). Luciferase activity was determined from a 20 μL aliquot of cell extract, and β-galactosidase activity from 10 μL of cell extract by the manufacturer's protocol (Promega, Madison WI). Transfections were performed in triplicate.

Nuclear extracts and DNA-binding assays. DNA-binding assays were performed as described by Vyas [19]. Cells (5×10^7) were pelleted, washed twice with PBS, and resuspended in 500 μL of buffer 1, comprising 10 mM Hepes, pH 8, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and protease inhibitor (Complete Protease Inhibitor Cocktail, Roche, Mannheim). After 15 min incubation on ice, 35 μL of 10% NP40 was added and the suspension was mixed well by vortex. Cell debris and nuclei were then pelleted by centrifugation and resuspended in 50 μL of buffer (20 mM Hepes, pH 8, 400 mM NaCl, 1 M EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor). After 15 min incubation on ice, the nuclear fraction was isolated by collecting the supernatant after centrifugation.