Structural and Functional Characterization of the Leukocyte Integrin Gene CD11d

ESSENTIAL ROLE OF Sp1 AND Sp3

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CD11d encodes the latest α-subunit of the leukocyte integrin family to be discovered, and it is expressed predominantly in myelomonocytic cells. We have isolated a genomic clone that contains CD11d and showed this gene to be 11,461 bp downstream and oriented in the same direction as the related CD11c gene. CD11d transcription begins 69–79 nucleotides upstream of the ATG codon. Transfection analysis of CD11d-luc reporter constructs revealed that the −173 to +74 region is sufficient to confer leukocyte-specific expression of luciferase in myelomonocytic cells (THP1 and HL60), B-cells (IM9), and T-cells (Jurkat). Transfection analysis showed that down-regulation of CD11d expression by phorbol ester was myelomonocytic-specific and is mediated by one or more cis-elements within the −173 to +74 region. In vitro DNase I footprint analysis and electrophoretic mobility shift analysis showed that Sp1 and Sp3 bind at −63 to −40. Deletion of the Sp-binding site significantly reduced CD11d promoter activity. Overexpression of either Sp1 or Sp3 in THP1 cells led to activation of the CD11d promoter even in the presence of phorbol ester, whereas down-regulation of either factor by antisense oligonucleotides decreased CD11d promoter activity. In contrast, overexpression of Sp3 in IM9 and Jurkat cells down-regulated CD11d promoter expression. In vivo genomic footprinting revealed that the −63 to −40 region is bound by a Sp protein in unstimulated HL60 cells but not in phorbol ester-stimulated HL60 cells. In contrast, this site is bound in both unstimulated and phorbol ester-stimulated IM9 and Jurkat cells. Together, these results show that myelomonocytic-specific phorbol ester down-regulation of CD11d is mediated through both Sp1 and Sp3.

The integrins are a large family of membrane glycoproteins composed of noncovalently associated α- and β-subunits that mediate cell-cell and cell-matrix interactions (1, 2). The β2 integrin, encoded by CD18 (3), heterodimerizes with four distinct α-subunits, encoded by CD11a (4), CD11b (5), CD11c (6), and the recently identified CD11d gene (7), which together comprise the leukocyte integrin subfamily. Immune and inflammatory responses including leukocyte migration, tumor cell killing, phagocytosis, and the respiratory burst are highly dependent on leukocyte integrin expression (8–12). Genetic mutations in the β2-subunit lead to loss of surface expression of these integrins in patients with leukocyte adhesion deficiency (13), and affected individuals characterize present with severe and recurrent life-threatening bacterial infections.

Surface expression of the leukocyte integrins differs with the particular cell type. CD18 and CD11a are expressed in all leukocytes (4, 12), whereas CD11b and CD11c are predominantly myeloid (monocytes and granulocytes)-specific (5, 6). CD11d is expressed moderately on myelomonocytic cell lines and subsets of peripheral blood leukocytes and strongly on tissue-specialized cells, including macrophage foam cells within atherosclerotic plaques, and on splenic red pulp macrophages (7). The functions of CD11d have not been determined in any detail; however, its expression in these two specialized cell types suggests that CD11d may play a role in the atherosclerotic process such as clearing lipoproteins from plaques and in phagocytosis of blood-borne pathogens, particulate matter, and senescent erythrocytes from the blood. Differentiation of myelomonocytic cell lines along the monocytic/granulocytic pathway with phorbol esters results in increased transcription and subsequent surface expression of all of the leukocyte integrins (14–19) except CD11d, which undergoes down-regulation from the surface (7). Analysis of the genomic sequences for CD11a, CD11b, and CD11c showed these genes to be controlled by transcription factors including c-Jun/c-Fos (20), PU.1 (21, 22), Ets (20), and CCAAT/enhancer-binding protein (23). Of particular interest was the observation that the ubiquitous transcription factor Sp1 binds the CD11b (24) and CD11c promoters (25) in a cell-specific manner. Only a partial genomic clone of the CD11d gene has been reported (26), and by comparison with the structural organization of the closely related CD11c gene, this clone contains only exons 15–30. Forward and reverse oligonucleotide primers specific to the 3′-untranslated region of CD11d and the 5′-coding region of CD11d were used in the polymerase chain reaction (PCR)1 to demonstrate that the CD11c gene lies approximately 11.5 kb upstream and in the same orientation as CD11d (27). To understand the molecular mechanisms responsible for expression of CD11d, we isolated a human genomic clone containing the 3′- and 5′-ends of CD11c and CD11d, respectively, and show that these genes are separated by 11,461 bp of DNA and confirm their orientation to be the same. We show that the −173 to +74 region of the CD11d gene is sufficient to confer leukocyte-specific expression in transfected cells that is dependent on both Sp1 and Sp3. Lastly, we provide evidence that the Sp binding site within the −173 to +74 region is bound in

1 The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s); PMA, phorbol 12-myristate 13-acetate; luc, firefly luciferase gene; EMSA, electrophoretic mobility shift analysis.

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probes were synthesized by extension of a 22-nucleotide-long primer on the luciferase reporter RNA, 25 mM polyacrylamide, 8 M urea gel. (Ambion), extracted with phenol/chloroform, and analyzed on a 5% polyacrylamide, 8 M urea gel. The cells were harvested 24 h post-transfection, and luciferase activity was assayed. Firefly luciferase light output was measured in a LB96V-2 Wallac Berthold plate luminometer and normalized against Renilla luciferase from the cotransfected vector or against the total protein concentration in the cellular extract.

Cotransfections of Sp Expression Plasmids—Mammalian cells were transfected as described above with 15 μg of a firefly luciferase reporter plasmid; 5 μg of pCMV-Sp1fluc, pCMV-Sp2fluc, or pCMV-Sp3fluc; and 5 μg of pRL-SV40. The total volume of the plasmid transfection mix was adjusted to 25 μg with the control plasmid pCDNA I. DNA was introduced into Drosophila cells by calcium phosphate-mediated transfection as described previously (25). Approximately 3 × 10^6 Drosophila cells were transfected with 15 μg of a specific luciferase reporter plasmid and 5 μg of PaecSp1, PaecSp2, or PaecSp3. The total volume of the plasmid transfection mix was adjusted to 30 μg with the control plasmid pCDNA I. The calcium phosphate-DNA precipitates were left on the cells for 48 h prior to harvesting and assaying for luciferase activity. Most transfections were performed in triplicate and repeated two to three times to ensure reproducibility. Experimental data was pooled and analyzed using Microsoft Excel and expressed as the mean ± S.D.

In Vitro DNAse I Footprinting Analysis—The PCR was performed to produce double-stranded DNA fragments containing the 173 to 74 region of the CD11d promoter. The probe was labeled with [γ-32P]ATP. The probe was purified by electrophoresis through a 5% agarose gel onto NA45-DEAE paper according to the manufacturer’s instructions (Schleicher and Schuell). Approximately 1–2 × 10^5 cpM of probe (1–2 ng) and either 50 μg of nuclear extract protein prepared as described (28) or 1 or 4 footpointing units (concentration determined by the manufacturer (Promega Corp., Madison, WI) of purified Sp1 protein were incubated in a total volume of 50 μl of binding buffer. The binding buffer contained 0 or 5 μg of poly(dI-dC), 6.25 mM MgCl2, 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, and 25 mM Tris-HCl, pH 8.0. After 15 min at room temperature, 50 μl of 5 mM CaCl2/10 mM MgCl2, and 0.2–2 units of DNase I were added. After 1 min at room temperature, the reaction was stopped with 50 μl of 0.2 M NaCl, 0.03 M EDTA, 1% SDS, 10 μg of Escherichia coli tRNA; phenol/chloroform-extracted; ethanol-precipitated; and analyzed on a sequencing gel.

Electrophoretic Mobility Shift Analysis (EMSA)—EMSA was performed as described previously (20). Nuclear extracts to be used in EMSA were prepared as described (28). The following double-stranded labeled probe was used: 5'-CCAATATATTTAATACACGCTTCCCTTCACCACCTTGCTGCTCTTCCTTGCTTTG-3', corresponding to the −81 to −31 region of the CD11d promoter. The probe was labeled with [γ-32P]ATP and 2 × 10^5 cpM of probe was incubated for 30 min on ice with 5 μg of nuclear extract from either unstimulated or PMA-stimulated THP1, IMB, or Jurkat cells. For supershift analysis, 1 μg of antibody specific for Sp1, Sp2, or Sp3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added after incubation of the probe with protein.
The reaction products were analyzed by polyacrylamide gel electrophoresis as described (20).

Sp1 and Sp3 Knock-out with Antisense Oligonucleotides—The following phosphorothioate-modified nucleotides were prepared and HPLC-purified: 5'-GTGCTGGTGGAGGCTCACTCCTGTT-3', a Sp1-specific antisense oligonucleotide positioned at Thr83 of the Sp1 mRNA (32); 5'-GGACCTCACTACGGATTATA-3', an Sp3-specific antisense oligonucleotide positioned at the Ile translational initiation codon of the Sp3 mRNA (33); and 5'-ATTGCATCTATCGGCTTGATTTACCT-3', an nonsense oligonucleotide. THP1 or HL60 cells were incubated in complete medium with either nonsense or antisense oligonucleotide at a final concentration of 20 μg for 48 h (fresh oligonucleotides were added after 24 h) as described (29). Northern blotting was performed and analyzed on a Storm PhosphorImager (Molecular Dynamics) to determine the extent of down-regulation of mRNAs for Sp1, Sp3, CD11d, and actin.

In Vivo Footprint Analysis—The genomic DNAs from HL60, Jurkat, and IM9 cells were purified by lysed cells by treatment with proteinase K followed by extensive phenol/chloroform extractions as described (30). The genomic DNAs were treated either in vivo or in vitro with dimethyl sulfate, cleaved with piperidine, and analyzed by ligation-mediated PCR as described (25, 30). The genomic DNAs were treated either

RESULTS

Structural Analysis of the 5' Flanking Region of the Human CD11d Gene—The identification of CD11d, a novel leukocyte integrin α-subunit on myelomonocytic cells, prompted us to begin an analysis of the transcriptional regulation of the CD11d gene. We used to our advantage a recent report that showed using PCR, that CD11d is positioned no more than 11.5 kb downstream of the CD11c promoter (32); and we screened a collection of CD11c-positive cosmid clones for the presence of CD11d. One clone was completely sequenced, and it contains exons 1–7 of CD11d. The genes are arranged in the same orientation, with the translational stop codon of CD11c positioned 11,461 bp upstream of the translational start codon of CD11d (Fig. 1). The sequences of the 1.2-kb 5' flanking region and exons 1–7 are shown (Fig. 2).

Determinatoin of the Transcriptional Start Site of the CD11d Gene—Poly(A)° RNA and total RNA from myelomonocytic HL60 cells was subjected to primer extension analysis with primers N379 (antisense primer with 5' end positioned 20 nucleotides downstream of the ATG start site) and N470 (antisense primer with 5' end positioned immediately upstream of the ATG start site), respectively. The longest extension product obtained with N379 was 101 nucleotides long (Fig. 3A), positioned the transcriptional start site 78 nucleotides upstream of the ATG site. Two extension products, 92 and 93 nucleotides long, were observed with N470 total RNA and would position the transcriptional start site(s) 14 and 15 bp further upstream. No extension products were detected when yeast total RNA was analyzed (Fig. 3A). No extension products were detected when yeast total RNA was analyzed (Fig. 3A).

An S1 nuclease protection assay was performed with a 247-
nucleotide single-stranded DNA probe that was generated by extension of the \([\gamma-S]P\)ATP-labeled N470 primer on a double-stranded DNA template with exonuclease-free Klenow polymerase. Hybridization of the probe with myelomonocytic THP1 poly(A\(^+)\) RNA and subsequent S1 nuclease digestion produced four major protected fragments 75–79 nucleotides long and with varying intensities (Fig. 3B). These results would therefore position the start of transcription 75–79 bp upstream of the ATG codon. No protected fragments were produced when the probe was hybridized to yeast total RNA (Fig. 3B). A second antisense DNA probe, 99 nucleotides long with the 5’-end positioned 19 nucleotides upstream of the ATG codon, was chemically synthesized, end-labeled with \([\gamma-S]P\)ATP, and hybridized with HL60 poly(A\(^+)\) RNA (Fig. 3B). S1 nuclease digestion produced four major protected fragments 49–52 nucleotides long, and the most intense fragment was 51 nucleotides long (Fig. 3B). This second S1 analysis would therefore position the start of transcription 69 bp upstream from the ATG codon.

An RNase protection assay was performed with a 562-nucleotide-long RNA probe prepared by \textit{in vitro} transcription and uniformly labeled with \([\alpha-S]P\)UTP. The probe included the 455 nucleotides immediately upstream from the ATG codon. Hybridization of the RNA probe with total RNA from HL60 cells and subsequent digestion with RNase A/T1 produced four protected fragments 71–74 nucleotides long (Fig. 3C). The length of the two most intense fragments would position the transcriptional start site 72 or 74 bp upstream from the ATG codon. No protected fragments were produced when the probe was hybridized to yeast total RNA.

Taken together, these results position two transcriptional start sites 69–79 bp and 91–92 bp upstream from the ATG codon. RNase protection assays, which provide for the most stringent hybridization and digestion conditions and which were repeated five times, consistently confined transcription to within 71–74 bp upstream of the ATG codon. No TATA box is present, and transcription is most probably determined by an initiator (Inr) control element (35) that is found in the \(CD11a\) (36) and \(CD11c\) genes (37, 38). Since the 69–79-bp region shows homology to the classical Inr and the largest RNase-protected fragment is 74 nucleotides long, we have assigned the thymidine 74 bp upstream from the ATG codon as the major site (+1) of \(CD11d\) transcription.

**Functional Analysis of the CD11d Promoter**—CD11d is expressed predominantly on myelomonocytic cells, and exposure to phorbol ester led to its down-regulation from the cell surface (7). Since \textit{cis}-elements for basal and cell-specific activity for the other three \(CD11\) \(\alpha\)-subunit genes lie within 500 bp upstream of the ATG codon (18, 21, 22, 24, 25, 29, 39), we initially focused on the −946 to +74 region of \(CD11d\). THP1 cells were transfected with construct \(CD11d\)-(−946/+74)-\textit{luc}, which contains the −946 to +74 region of \(CD11d\) fused to the luciferase gene, and 24 h post-transfection, cells were exposed to PMA for varying times (Fig. 4). Transfected THP1 cells exposed to PMA for up to 10 h showed no decrease in luciferase expression; however, after 24 h, luciferase activity decreased 55% (Fig. 4). For comparison, expression from the \(CD11a\) promoter, which is active in all leukocytes, and \(CD11c\) promoter, which is predominantly active in myelomonocytic cells, was monitored following transfection of the \(CD11a\)-\textit{luc} and \(CD11c\)-\textit{luc} constructs, respectively. In the presence of PMA, luciferase activity from \(CD11a\)-\textit{luc} in THP1 cells was increased 4.5-fold over that obtained with the promoterless plasmid pGL3-Basic, and luciferase activity from \(CD11c\)-\textit{luc} was increased 8.3-fold (Fig. 4). These results show that chronic rather than acute exposure to PMA leads to down-regulation of \(CD11d\) expression (and up-regulation of \(CD11a\) and \(CD11c\) expression as expected) and that one or more \textit{cis}-elements within the −946 to +74 region mediates this effect.

The −946 to +74 region was further examined to localize the \textit{cis}-element(s) responsible for PMA-induced down-regulation of...
CD11d and/or other elements that influence either basal or cell-specific expression. A series of CD11d reporter constructs containing progressively larger 5-deletions was prepared and transfected into various cell lines. For comparison, expression from the CD11c promoter was monitored following transfection of CD11c-luc. Luciferase expression from the constructs transfected into THP1 cells varied, but not significantly. CD11d(-173/+74)-luc, which contains only the -173 to +74 region of CD11d, retained all of the activity observed with CD11d(-946/+74)-luc and was 43-fold higher than that obtained with pGL3-Basic (Fig. 5A). The -173 to +74 region is sufficient to confer leukocyte-specific activation of the CD11d promoter, since luciferase activity from CD11d(-173/+74)-luc transfected into IM9 and Jurkat cells was increased 24- and 30-fold, respectively (Fig. 5, B and C). In contrast, luciferase expression from CD11d(-173/+74)-luc in MCF-7 breast cancer cells was increased only 6.4-fold (Fig. 5D).

Luciferase expression from the CD11d-luc 5-deletion constructs transfected into THP1 cells was reduced to approximately the same extent (78-94%) after exposure to PMA (Fig. 5A). A similar response to PMA was confirmed in another myelomonocytic cell line, HL60 (data not shown). This shows that a PMA-responsive cis-element(s) lies within the -173 to +74 region, since CD11d(-173/+74)-luc, which contains only this region, responds to PMA. In contrast, luciferase expression in IM9 and Jurkat cells transfected with CD11d(-173/+74)-luc was not reduced by PMA but instead was increased 2.9- and 1.7-fold, respectively (Fig. 5, B and C). As expected, expression of the CD11c promoter was restricted to PMA-stimulated myelomonocytic cells (Fig. 5). These results show that the -173 to +74 region regulates cell-specific down-regulation of CD11d by PMA.

Sp1 and Sp3 Bind to the -63 to -40 Region in the CD11d Promoter—DNase I footprint analysis was performed to determine whether DNA binding proteins interact with the -173 to +74 region. When nuclear extracts prepared from unstimulated and PMA-stimulated THP1 cells were added to a probe labeled on the coding strand, strong protection of the -63 to -40 region was revealed (Fig. 6A). This same region was also protected by nuclear extracts prepared from unstimulated and PMA-stimulated Jurkat and IM9 cells (Fig. 6A). When a probe labeled on the noncoding strand was used, strong protection of an overlapping region, -72 to -45, was detected with unstimulated and PMA-stimulated nuclear extracts from all three cell lines (Fig. 6B). DNA sequence analysis of the overlapping region revealed the presence of an Sp1 binding site. In vitro DNase I footprint analysis showed that purified Sp1 protein could also protect the -63 to -40 region (Fig. 7).

To confirm that Sp1 can bind within the -63 to -40 region and determine whether other Sp1-related proteins also may bind, EMSA was performed with nuclear extract protein from unstimulated and PMA-stimulated THP1, IM9, and Jurkat cells. Three DNA-protein complexes were seen when nuclear extract protein from each cell line was added to an oligonucleotide probe that spans the -81 to -31 region (Fig. 8). DNA-protein complex 1 formed from all nuclear extracts could be supershifted with an antibody to Sp1, indicating that Sp1 was bound in this complex. Formation of DNA-protein complex 3 was inhibited (rather than supershifted) when an antibody to Sp3 was included in the EMSA. Inhibition of complex 3 formation was seen when nuclear extracts from the three cell lines were analyzed. These results indicate that Sp1 and Sp3 are not co-bound on the same DNA molecule, since anti-Sp3 antibody did not affect the formation of the Sp1-specific complexes and anti-Sp1 antibody did not supershift the Sp3-specific complexes. These results suggest that Sp1 and Sp3 compete for binding to the same or overlapping sites in the CD11d promoter. Neither PMA-dependent nor cell-specific formation of these complexes was seen. The identity of the protein bound in complex 2 has not been determined. Further, antibodies specific to other Sp1-related proteins including Sp2 and Sp4 (33), Egr 1 (40), and Egr 2 (41) did not alter the patterns of DNA-protein complexes (data not shown).

The Sp1/Sp3-binding Region Is Essential for CD11d Promoter Activity—To determine whether the Sp1/Sp3-binding site is important for CD11d expression, this site was deleted from CD11d(-173/+74)-luc, and its effect on expression was monitored in transfected cells. Deletion of the -63 to -40 region in CD11d(-173/+74)Δ-63/−40-luc resulted in a 76% reduction of luciferase expression in THP1 cells (Fig. 9A). When transfected THP1 cells were exposed to PMA, expression from CD11d(-173/+74)-luc was reduced 70%, similar to pre-
vicious observations. Deletion of the -63 to -40 region further reduced luciferase expression from CD11d(-173/+74)Δ-63/-40-luc in PMA-stimulated THP1 cells only an additional 7% (Fig. 9A). Luciferase expression from CD11d(-173/+74)Δ-63/-40-luc in transfected IM9 and Jurkat cells was reduced 80 and 76%, respectively (Fig. 9, B and C). Although PMA did not reduce the expression of luciferase from CD11d(-173/+74)-luc transfected into IM9 and Jurkat cells, expression from CD11d(-173/+74)Δ-63/-40-luc was reduced 82 and 80%, respectively (Fig. 9, B and C). These results show that the -63 to -40 region is essential for CD11d promoter activity in both myelomonocytic and nonmyelomonocytic cells. Further, the inability of PMA to reduce luciferase expression from CD11d(-173/+74)-luc in nonmyelomonocytic cells is dependent on the integrity of this region.

The Response of the CD11d Promoter to Sp1 and Sp3 Is Cell-specific—To determine whether Sp1 and Sp3 functionally interact with the CD11d promoter, Drosophila cells, which are deficient in Sp-related proteins, were cotransfected with expression constructs for Sp1 and Sp3 (pPacSp1 and pPacSp3) along with CD11d(-591/+74)-luc (Fig. 10). The role of another member of the Sp family, Sp2, expressed from pPacSp2, was similarly analyzed. Sp1-dependent luciferase activity from the CD11d promoter was shown to increase 4.1-fold in Drosophila cells cotransfected with pPacSp1 and CD11d(-591/+74)-luc (Fig. 10A). In contrast, no induction of luciferase activity was seen when either pPacSp2 or pPacSp3 was cotransfected. Analysis of CD11d(-378/+74)-luc and CD11d(-173/+74)-luc in co-transfection experiments yielded similar results (data not shown).

It is known that Sp1 and Sp3 compete for the same sites on the CD11c promoter (29); however, luciferase activity from CD11d(-591/+74)-luc is maximal in the presence of pPacSp1 alone and was not further increased when both pPacSp1 and pPacSp3 were present (Fig. 10A). Since pPacSp1-dependent expression of luciferase from CD11d(-591/+74)-luc is also not decreased in the presence of pPacSp3, Sp3 does not compete with Sp1 for binding to the same site. This suggests that Sp3 does not function as a repressor of CD11d promoter activity, in contrast to its reported repressor-like activity on other promoters (42, 43). An alternative hypothesis is that the response of
Characterization of the CD11d Promoter

The level of CD11d mRNA in nonsense-treated cells. Further, the level of Sp3 mRNA was reduced 85% following treatment of THP1 cells with Sp3-antisense oligonucleotide resulted in an 88% reduction of Sp3 mRNA but did not affect the level of Sp1 mRNA. The level of CD11d−(591/+74)-luc. Overexpression of Sp2, however, had no effect. Cotransfection of both pCMV4-Sp4/lflu and pCMV4-Sp3/lflu along with CD11d−(591/+74)-luc did not increase luciferase activity above that obtained when either expression construct was transfected separately. This indicates that Sp1 and Sp3 compete for the same site as suggested in the above EMSA of these factors.

To determine whether Sp1 and Sp3 regulate the CD11d promoter in other cell types, the same cotransfection experiments were performed in IM9 and Jurkat cells. Sp1-dependent luciferase activity of the CD11d promoter increased 1.8-fold in unstimulated IM9 cells (Fig. 10C); however, no response of this promoter to Sp1 was observed in unstimulated Jurkat cells (Fig. 10D). Overexpression of Sp2 in either cell line had no effect. In contrast to that observed in transfected THP1 cells, Sp3 exhibited repressor activity in IM9 and Jurkat cells. Luciferase activity decreased 40% in IM9 cells and 56% in Jurkat cells cotransfected with pCMV4-Sp3/lflu and CD11d−(591/+74)-luc (Fig. 10, C and D). Overexpression of Sp3 attenuated the activation of the CD11d promoter by Sp1 in IM9 cells cotransfected with both pCMV4-Sp1/lflu and pCMV4-Sp3/lflu. Analysis of CD11d−(173/+74)-luc in cotransfection experiments yielded similar responses to Sp1, Sp2, and Sp3 in these cell lines (data not shown).

To determine whether the PMA-induced down-regulation of the CD11d promoter is affected by the level of Sp1 or Sp3 in THP1 cells, cotransfection experiments were performed in PMA-stimulated cells. In the absence of pCMV4-Sp4/lflu, luciferase activity from CD11d−(591/+74)-luc was significantly reduced in PMA-stimulated cells. Cotransfection of pCMV4-Sp1/lflu along with CD11d−(591/+74)-luc increased luciferase activity 3.28-fold, completely reversing the response of THP1 cells to PMA (Fig. 10B). Similarly, cotransfection of pCMV4-Sp3/lflu along with CD11d−(591/+74)-luc increased luciferase activity 3.62-fold in PMA-stimulated THP1 cells (Fig. 10B). In the presence of both pCMV4-Sp1/lflu and pCMV4-Sp3/lflu, no further increase in CD11d promoter activity was observed over that obtained when each expression construct was transfected separately. No response to PMA/Sp4/Sp2/lflu was seen. In all experiments, overexpression of either Sp1 or Sp3 in PMA-stimulated THP1 cells did not significantly increase the absolute level of luciferase activity over that obtained in unstimulated cells that were not transfected with either factor. In PMA-stimulated IM9 cells, Sp1 further increased luciferase activity over that obtained in unstimulated cells overexpressing this factor, and Sp3 continued to exhibit repressor activity (Fig. 10C). In PMA-stimulated Jurkat cells, no differences were found in the responses to Sp1 and Sp3 when compared with those observed in unstimulated cells (Fig. 10D).

To confirm whether Sp1 and Sp3 regulate the CD11d promoter in myelomonocytic cells, antisense oligonucleotides to the 5’ portion of the coding regions for these factors were added to unstimulated THP1 and HL60 cells for 48 h to down-regulate their expression (Fig. 11). Northern blot analysis showed that the level of Sp1 mRNA in Sp1-antisense treated THP1 cells was reduced 85% when compared with the level of Sp1 mRNA in nonsense-treated cells. Further, the level of Sp3 mRNA was unaffected by the Sp1-antisense oligonucleotide. The level of CD11d mRNA was reduced 71% following treatment of THP1 cells with Sp1-antisense oligonucleotides. Treatment of THP1 cells with Sp3-antisense oligonucleotide resulted in an 88% reduction of Sp3 mRNA but did not affect the level of Sp1 mRNA. The level of CD11d mRNA was reduced 74% following treatment of THP1 cells with Sp3-antisense oligonucleotides. Essentially identical results were obtained when another myelomonocytic cell line, HL60, was analyzed. Treatment of

FIG. 9. The Sp1-binding site is essential for CD11d promoter activity. A deletion of the Sp1-binding site (Δ−693/+40) was introduced into CD11d−(173/+74)-luc, and both wild-type and deletion constructs were transfected into various cell lines. Luciferase expression from CD11d−(173/+74)-luc in all unstimulated cell lines is set at 100%. Differences in transfection efficiencies were corrected. The mean luciferase activities ± S.D. are indicated.

FIG. 10. Induction of the CD11d promoter with Sp1 and Sp3. Drosophila cells (A) were cotransfected with CD11d−(591/+74)-luc and 5 μg each of the Sp1, Sp2, or Sp3 expression plasmids pPacSp1, pPacSp2, or pPacSp3, respectively. THP1 cells (B), IM9 cells (C), or Jurkat cells (D) were cotransfected with CD11d−(591/+74)-luc and 5 μg each of the Sp1, Sp2, or Sp3 mammalian expression plasmids pCMV4-Sp1/lflu, pCMV4-Sp2/lflu, or pCMV4-Sp3/lflu, respectively. Fold Increase represents the increase in luciferase expression relative to that obtained following cotransfection with the Drosophila control plasmid pPacO (0) or the mammalian control plasmid (0), which do not express Sp proteins. PMA was added for 24 h to the mammalian cell transfections. The mean luciferase activities ± S.D. are indicated.

the CD11d promoter to these Sp proteins is cell-specific and that the activity of the CD11d promoter in Drosophila cells may not accurately reflect the actions of Sp proteins on this promoter in myelomonocytic cells.

To address the latter hypothesis, THP1 cells were cotransfected with mammalian expression constructs for Sp1, Sp2, and Sp3 (pCMV4-Sp1/lflu, pCMV4-Sp2/lflu, and pCMV4-Sp3/lflu) along with CD11d−(591/+74)-luc (Fig. 10B). Sp1-dependent luciferase activity from the CD11d promoter increased 2.2-fold in unstimulated THP1 cells cotransfected with pCMV4-Sp1/lflu and CD11d−(591/+74)-luc. In contrast to the results seen in transfected Drosophila cells, Sp3 could also activate the CD11d promoter. Luciferase activity increased 2.6-fold in unstimulated THP1 cells cotransfected with pCMV4-Sp3/lflu and CD11d−(591/+74)-luc. Overexpression of Sp2, however, had no effect. Cotransfection of both pCMV4-Sp4/lflu and pCMV4-Sp3/lflu along with CD11d−(591/+74)-luc did not increase luciferase activity above that obtained when either expression construct was transfected separately. This indicates that Sp1 and Sp3 compete for the same site as suggested in the above EMSA of these factors.
HL60 cells with Sp1-antisense oligonucleotides resulted in a 91% reduction of Sp1 mRNA and a 62% reduction of CD11d mRNA. Similarly, treatment of HL60 cells with Sp3-antisense oligonucleotides resulted in a 93% reduction of Sp3 mRNA and a 74% reduction of CD11d mRNA. Together with the Sp1 and Sp3 overexpression experiments, these results indicate that Sp1 and Sp3 are equally effective in activating the CD11d promoter in myelomonocytic cells and are most probably the Sp-type proteins that occupy this promoter in vivo.

**Myelomonocyte-specific Down-regulation of CD11d by PMA**

Is Associated with Loss of Sp Protein Binding in Vivo—The above results show that cell-specific down-regulation of CD11d promoter activity is mediated through one or more cis elements within the −173 to +74 region. The inability of IM9 and Jurkat cells to maintain CD11d expression in the presence of PMA when the Sp-binding site was deleted indicated that an Sp protein was a necessary factor involved in this response. The possibility that loss of Sp binding was linked to down-regulation of CD11d promoter activity in THP1 cells exposed to PMA was suggested when the reduction in luciferase activity from CD11d(−173/+74)-luc in transfected THP1 cells exposed to PMA was found to be about the same as that obtained in unstimulated THP1 cells transfected with the Sp-deleted construct CD11d(−173/+74)Δ−63/−40)-luc (70 versus 76% reduction, respectively; Fig. 9). Further, the PMA-induced down-regulation of CD11d promoter activity was reversed when either Sp1 or Sp3 was overexpressed (Fig. 10B). Previously, we (25) and others (24) have shown that cell-specific up-regulation of CD11c and CD11b by PMA is associated with increased binding of Sp proteins in myeloid cells, which raised the possibility that selective Sp protein binding also occurs on the CD11d promoter. To explore this possibility further, in vivo genomic footprinting was performed. Genomic DNA, methylated in vivo with dimethyl sulfate, was isolated from HL60, IM9, and Jurkat cells that were either unstimulated or PMA-stimulated. DNA was also isolated from HL60 and Jurkat cells, stripped of bound protein, and methylated in vitro as controls. Analysis of the CD11d noncoding strand (Fig. 12) revealed hyposensitive sites in unstimulated HL60 DNA at positions −38, −40, −42, −43, −50, −52, −55, −56, −58 to −61, −63, −65, and −66, which correspond to guanine nucleotides in the Sp-binding site. No protection was seen over these positions on genomic DNA from PMA-stimulated HL60 cells (Fig. 12). In contrast, genomic DNAs from IM9 and Jurkat cells, either unstimulated or PMA-stimulated, were similarly protected over these positions (Fig. 12). From these results, we conclude that occupation of the CD11d promoter by an Sp protein, presumably Sp1 or Sp3, is significantly reduced in PMA-stimulated myelomonocytic cells and that myelomonocytic-specific down-regulation of CD11d expression is mediated through preferential loss of Sp protein binding following PMA stimulation.

**DISCUSSION**

In this study, we have isolated a genomic clone for CD11d that contains the intergenic region between CD11c and CD11d and the 5′-coding portion of CD11d, and we have begun to analyze the regulatory mechanisms for CD11d expression. DNA sequence analysis of this clone showed that the ATG translational codon for CD11d lies 11,461 bp downstream of the translational stop codon for CD11c and that both genes are transcribed in the same direction. This finding confirms a previous brief report that CD11d lies no more than 11.5 kb downstream of CD11c (27). In that report, an 11.5-kb DNA fragment was amplified from genomic DNA using specific oligonucleotide primers to the 3′-end of CD11c and the 5′-end of CD11d. Others (26) have reported the isolation of a partial genomic clone containing the 3′-end of CD11c and the 5′-end of CD11d. Others (26) have reported the isolation of a partial genomic clone containing the 3′-end of CD11d. DNA sequence analysis of that clone revealed 12 exons that are homologous to exons 14–17, 21, and 24–30 of the CD11c gene, and it was predicted that five additional exons homologous to exons 18–20, 22, and 23 of CD11c are probably also present. A comparison of the DNA
sequence of exons 9–13 of CD11c reveals considerable homology beginning with nucleotide 982 in the 3486-nucleotide coding sequence of CD11d, and it is likely that these four exons are also present in the CD11d genomic sequence. The CD11d genomic clone in our study also contains exons 1–7 not previously reported, which correspond to nucleotides 1–704 of the CD11d coding sequence. Exon 8 and possibly another exon is predicted to lie between nucleotides 704 and 982 of CD11d, which would be consistent with an average exon size of 100–150 nucleotides.

The major transcriptional start sites for the CD11a, CD11b, and CD11c genes are located 93, 92, and 67 bp, respectively, upstream of the ATG translational start sites (18, 36–38), and several minor transcriptional start sites are clustered within a 30–50-bp region surrounding these sites. We have determined, using RNase protection analysis, S1 nuclease analysis, and primer extension analysis, that transcription of CD11d begins within the region 69–79 bp upstream of the ATG codon. A second transcriptional start site was also revealed 92–93 bp upstream ATG, but only with primer extension analysis. The 69–79-bp region is homologous to the classical initiator, or Inr, that directs transcription in TATA-less promoters including the other CD11 genes. The thymidine at 74 bp upstream from the ATG was assigned as the transcriptional start site, since this corresponded to the longest RNase-protected fragment observed using the most stringent of hybridization conditions.

The −946 to +74 region of CD11d was chosen for analysis in this study because cis-elements for the other CD11 genes are located within 500 bp upstream of their ATG codons. This region, when fused to luc, promoted high level expression of luciferase in THP1 and HL60 (myelomonocytic cells) but not in MCF-7 (breast adenocarcinoma cells), which demonstrates leukocyte-specific expression of this promoter. Progressive deletion beginning at the 5′-end of the −946 to +74 region had no significant effect on expression of the CD11d promoter in either myelomonocytic cell line, THP1 or HL60. In contrast, deletion of the −591 to −378 region led to significant increases in CD11d promoter activity in the B-cell line, IM9, and the T-cell line, Jurkat. This result suggests that a cell-specific silencer element lies within this region, and we are currently investigating this possibility. Although luciferase expression from constructs containing the −591 to −378 region was significantly less in B-cells and T-cells than in myelomonocytic cells, it was still rather high as compared with expression in the nonleukocyte cell line, MCF-7. It is, therefore, very likely that other cell-specific cis-elements lie upstream of −946. Support for this concept is evidenced in transgenic analysis of the leukocyte integrin genes. Although cell-specific expression in transplanted cell lines of reporter genes fused to around 500 bp of 5′-flanking sequence from the other three CD11 genes has been demonstrated, efficient cell-specific expression of CD11 transgenes in mice has not. For example, the 1.7-kb 5′-flanking region of CD11a (44) and the 1.5-kb 5′-flanking region of CD11b (45) were not sufficient to promote high level expression of reporter genes that paralleled those of the endogenous CD11 genes. Such studies show that additional sequences regulate correct leukocyte integrin transgene expression and that transient transfection assays may not reveal such regions. We are now searching the far upstream region of CD11d for cell-specific elements.

In this study, we have shown that CD11d promoter activity decreased following exposure of myelomonocytic cells to PMA, which most probably accounts for the decrease in CD11d surface expression shown in a previous study (7). Acute exposure to PMA activates and then down-regulates specific protein kinase C isoforms, whereas chronic exposure can lead to differentiation of the cell. Decreased CD11d promoter activity following chronic but not acute exposure to PMA is consistent with a mechanism that involves differentiation of myelomonocytic cells. That CD11d expression is confined to subsets of myelomonocytic cells in situ including macrophage foam cells and splenic red pulp macrophages may be a result of the differentiation of macrophage precursors and subsequent down-regulation of CD11d in the majority of mature macrophages. Although acute exposure to PMA did not affect CD11d promoter activity, it remains to be seen whether altered protein kinase C isoform expression as a result of differentiation directly down-regulates CD11d promoter function.

Down-regulation of CD11d promoter activity by PMA was shown to be cell-specific and is mediated by one or more cis-elements within the −173 to +74 region. Within this region is a binding site for Sp1 and Sp3, and deletion of this site led to decreased CD11d promoter activity in both unstimulated myelomonocytic and nonmyelomonocytic cells. This indicated that the activity of one or both of these Sp proteins at this site mediates basal CD11d promoter activity that is not cell-specific. The role of Sp1 as an activator of the CD11d promoter that is not cell-specific was evidenced by the ability of this factor to activate the CD11d promoter in THP1 and IM9 cells. The lack of response to Sp1 overexpression in Jurkat cells suggests that either a different Sp protein functions in this cell type or, more likely, that the high endogenous level of Sp1 in Jurkat cells obscures or limits the response to additional Sp1. Further analysis, however, showed that the response of the CD11d promoter to Sp3 is cell-specific. The Sp3-dependent activation of the CD11d promoter in myelomonocytic cells contrasts with the inability of Sp3 to activate this promoter in transinfected Drosophila cells and its repressor role on CD11d expression in mammalian cells. Sp3 has been shown to contain both activator and repressor functions, and several studies suggest that the ability of Sp3 to attenuate Sp1-dependent activation is promoter-specific (46). Additionally, its ability to repress or activate gene expression is dependent on cell type (47). Our results are thus consistent with what is known about Sp3 function.

That Sp1 mediates basal activity of the CD11d promoter that is not cell-specific is also supported by in vivo genomic footprint analysis that showed the Sp-binding site to be occupied in unstimulated myelomonocytic and nonmyelomonocytic cells. In contrast, PMA exposure led to decreased occupancy of this site specifically in myelomonocytic cells. This is consistent with the concept that Sp1 and/or Sp3 mediate cell-specific responses to PMA. The mechanism for decreased Sp protein binding in PMA-stimulated myelomonocytic cells is not clear. Glycosylation of Sp1 has been shown (48, 49) and has been proposed to account for enhanced transcriptional activity; however, we have not determined whether post-translational modifications of Sp1 account for differential binding of Sp1 to CD11d. Physical interaction of Sp1 with a protein factor could also affect its ability to bind to the CD11d promoter. Sp1 has been shown to physically interact with a number of proteins including the erythroid transcription factor GATA-1 (50, 51), the parvoviral nonstructural protein NS-1 (52), and the TATA box-associated factor TAF11 (53). An activator protein may interact with Sp1 to increase Sp1 binding, and PMA exposure may lead to down-regulation of such an activator protein specifically in myeloid cells.

An alternative scenario is that an inhibitory protein binds to Sp1 and/or Sp3 to decrease binding to the CD11d promoter.

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2 J. D. Noti, A. K. Johnson, and J. D. Dillon, unpublished observations.
Characterization of the CD11d Promoter

Chen et al. (54) identified a 20-kDa protein fraction referred to as Sp1-I that inhibited binding of Sp1 to the rat c-jun promoter. Murata et al. (55) showed that two proteins, 74 and 110 kDa in size, can associate with the amino terminus of Sp1 in vitro and potentially inhibit Sp1 activity. Recent studies suggested that a negative inhibitor may bind to Sp1 and that the retinoblastoma protein stimulates Sp1- and Sp3-mediated transcriptional activation of the c-fos, c-myc, and TGF-β1 promoters (30, 31, 54) through release of this inhibitor. Conceivably, PMA exposure may lead to binding of a cell-specific inhibitory protein to Sp1 and/or Sp3, which, in turn, decreases their ability to bind.

A third scenario is that the ratio of Sp1 to Sp3 differentially controls CD11d promoter expression. Increased Sp1/Sp3 ratios were suggested to account, in part, for the increased expression of the vascular endothelial growth factor receptor in endothelial versus nonendothelial cells (56). Although we have not determined the relative levels of Sp1 and Sp3 in the cell types examined in this study, an increased level of Sp3 in nonmyelomonocytic cells combined with its differential repressor activity in these cells would attenuate Sp1-mediated basal activity of the CD11d promoter. PMA exposure may increase the Sp1/Sp3 ratio in nonmyelomonocytic cells, leading to decreased Sp3 binding and increased Sp1 binding. That Sp3 does not bind to the CD11d promoter to effect PMA-mediated down-regulation in myelomonocytic cells is evidenced in in vivo footprint analysis, which showed no evidence of any protein bound to the Sp site following PMA stimulation of myelomonocytic cells. EMSA performed in this study did not show differential binding of these factors when nuclear extract protein from unstimulated cells was compared with that from PMA-stimulated cells. However, EMSA often fails to detect differential binding of factors and is not necessarily quantitative for detecting levels of specific factors; therefore, the results of EMSA in this study do not rule out this last concept. In vivo footprint analysis, which more accurately reflects the binding status of promoters under more physiological conditions, is consistent with all these scenarios. The differential binding of Sp1 and/or Sp3 to the CD11d promoter in myeloid cells in response to PMA is analogous to promoting binding of another factor. Chen et al. (24) suggested that PU.1 binding to the CD11b promoter induced such chromosomal alterations that enable Sp1 to bind. In our study of the CD11c promoter, we found that Sp1 increases binding of c-Jun (25).

CD11d is predominantly expressed in specialized subsets of peripheral blood leukocytes; however, its role in such cells is unknown. Of particular interest is its expression on macrophage foam cells, where CD11d may be involved in foam cell-specific functions such as low density lipoprotein removal and secretion of growth factors, cytokines, and superoxide radicals. An understanding of the basic regulatory mechanisms that control CD11d expression may extend our understanding of how macrophage foam cells contribute to the progression and/or regression of atherosclerosis. Identification of cis-elements within the CD11d promoter that are important for macrophage gene expression would also be important in the development of an optimized delivery system using these cis-elements to target a range of molecules involved in atherosclerosis to foam cells and test their function.

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Characterization of the CD11d Promoter

8969

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