The Zinc Finger Transcription Factor Transforming Growth Factor β-Inducible Early Gene-1 Confers Myeloid-specific Activation of the Leukocyte Integrin CD11d Promoter*

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CD11d encodes the α9 subunit for a leukocyte integrin that is expressed on myeloid cells. In this study we show that the −100 to −20 region of the CD11d promoter confers myeloid-specific activation of the CD11d promoter. Transforming growth factor β-inducible early gene-1 (TIEG1) was isolated in a yeast one-hybrid screen using the −100 to −20 region of the CD11d promoter as bait. Purified GST/TIEG1 protein was able to bind within the −61 to −45 region that overlaps a shorter binding site for Sp1. Transient overexpression of TIEG1 activated the CD11d promoter specifically in myeloid cells, whereas, down-regulation of TIEG1 with small interfering TIEG1 RNA also down-regulated expression of CD11d. In vivo, TIEG1 does not physically interact with Sp1. Cotransfection and electrophoretic mobility shift analyses of TIEG1, Sp1, and Sp3 revealed that TIEG1 competes with these Sp proteins for binding to overlapping sites in the CD11d promoter. Although TIEG1 and Sp1 are ubiquitously expressed in myeloid and non-myeloid cells, chromatin immunoprecipitation assays revealed differential occupancy of the CD11d promoter by these factors. In undifferentiated myeloid and non-myeloid cells, occupancy of the CD11d promoter by TIEG1 is similar. Upon differentiation of myeloid cells and subsequent up-regulation of CD11d expression, TIEG1 occupancy increases. In contrast, occupancy by TIEG1 remains low in non-myeloid cells exposed to phorbol ester. We propose that up-regulation of CD11d expression following differentiation of myeloid cells is mediated through increased binding of TIEG1 binding to the CD11d promoter.

The β2-integrin family of membrane glycoproteins, also known as the leukocyte integrins, is composed of four distinct α-subunits that non-covalently associate with a common β-subunit and mediate a wide range of adhesion-dependent immunological responses (1, 2). The leukocyte integrins are essential for leukocyte migration, tumor cell lysis, phagocytosis, and the respiratory burst (3–6) and are involved in a number of pathological conditions in the vascular system, including ischemic tissue damage in autoimmune diseases (7).

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EXPERIMENTAL PROCEDURES

Cell Culture—THP1 (acute monocytic leukemia, ATCC TIB-202), HL60 (promyelocytic leukemia, ATCC CCL-240), IM9 (B-cell multiple myeloma, ATCC CCL-188), and U937 (human histiocytic lymphoma, ATCC CCL-143) cells were grown in RPMI 1640 and 10% fetal calf serum.

1 The abbreviations used are: TIEG1, transforming growth factor-β-inducible early gene-1; AD, activation domain; BD, binding domain; 3-AT, 3-amino-1,2,4-triazole; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift analysis; RT, reverse transcriptase; ChIP, chromatin immunoprecipitation; PMA, phorbol 12-myristate 13-acetate; RB, human retinoblastoma protein; HRP, horseradish peroxidase; GAPDH, glyceraldehyde-phosphate dehydrogenase; HA, hemagglutinin; PMF, phenylmethylsulfonyl fluoride; CMV, cytomegalovirus; siRNA, small interference RNA; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; TGF, transforming growth factor.
myeloma, ATCC CCL-159). Jurkat (T-cell acute leukemia, ATCC TIB-152), and K562 (highly differentiate progenitors of erythrocytes, granulocytes, and monocytes, ATCC CCL-243) cells were grown in RPMI 1640 medium containing 10% fetal calf serum (IMM cells were grown in 20% fetal calf serum). Schneider’s Drosophila 2 cells (Drosophila melanogaster embryos, ATCC CRL-1693) were grown in Schneider’s medium containing 10% insect-tested fetal calf serum (Sigma). All medium contained 100 units/ml each of streptomycin and penicillin. For certain experimental procedures, cells were stimulated with 10 ng phorbol 12-myristate 13-acetate (PMA) for 24 h, or 100 ng PMA for 48 h.

**Yeast One-hybrid Analysis**—Following the protocol outlined in the MATCHMAKER One-Hybrid System (Clontech, Palo Alto, CA), four copies of the −100 to −20 region of the CD11d promoter were ligated into the Bloom yeast reporter strain and plated onto SD/His−ura−/ura−/ura−/ura−/ura− medium containing 200 μg/ml 5-fluoroorotic acid (5-FOA) and 50 μg/ml 5-fluoro-2′-deoxyuridine (5-FdUrd) as described previously (24). Full-length TIEG1 cDNA was amplified by PCR with primers 5′-ATGCGAGCGCTACGATTGTACCGG-3′ (which responds to the TIEG1 protein with anti-GST monoclonal antibodies (Novagen, cat. #H4054AH), prepared in the plasmid pACT2 to generate fusions of spleen cDNA with the GAL4 Activation Domain (AD), was transformed into the yeast dual reporter strain. Yeast transformants were selected on SD/-Leu−His−/Ura− medium containing 30 or 45 μg 3-amino-1,2,4-triazole (3-AT). The spleen cDNA/Gal4 fusion plasmids were isolated from the transformants and re-transformed into yeast. The yeast reporter pLaCZi to create reporters pHis1-1-CD11d(−100−20) and pLaCZi-CD11d(−100−20). A yeast dual reporter strain was prepared by first integrating pHis−1-CD11d(−100−20) followed by pLaCZi-CD11d(−100−20) into the genome of yeast strain YM4271. The dual reporter strain was selected and maintained on SD minimal medium lacking histidine and uracil. A human spleen cDNA library (Clontech, cat. #H4054AH), prepared in the plasmid pACT2 to generate fusions of spleen cDNA with the GAL4 Activation Domain (AD), was transformed into the yeast dual reporter strain. Yeast transformants were selected on SD/-Leu−His−/Ura− medium containing 30 or 45 μg 3-amino-1,2,4-triazole (3-AT). The spleen cDNA/Gal4 fusion plasmids were isolated from the transformants and re-transformed into the yeast reporter strain and plated onto SD/-Leu−His−/Ura−/ura− medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal). All cDNA/Gal4AD fusion plasmids that retested positive also expressed robust β-galactosidase activity from the integrated LaCZ reporter. The recovered plasmids were sequenced and analyzed by a BLAST search of GenBank (25). Full-length TIEG1 cDNA corresponding to the partial cDNAs in the recovered plasmids were obtained as detailed below.

**Plasmids**—Four copies of the −100 to −20 region of the CD11d promoter were placed upstream of the minimal SV40 promoter in pGL3-Promoter (Promega, Madison, WI). The −173 to +74 region of the CD11d promoter was fused to the transferrin and re-transformed into the yeast dual reporter strain and plated onto SD/-Leu−His−/Ura−/ura− medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal). All cDNA/Gal4AD fusion plasmids that retested positive also expressed robust β-galactosidase activity from the integrated LaCZ reporter. The recovered plasmids were sequenced and analyzed by a BLAST search of GenBank (25). Full-length TIEG1 cDNA corresponding to the partial cDNAs in the recovered plasmids were obtained as detailed below.

**Transfection and Reporter Assays**—Human cells were transfected by electroporation as previously described (15) and analyzed by the Dual-Luciferase Reporter Assay System (Promega). Approximately 1 × 10⁶ cells were cotransfected with 10 μg of each firefly luciferase reporter plasmid, 5 μg of each expression plasmid when used (see figure legends for specific details), and 2 μg of Renilla luciferase plasmid pRL-SV40 (Promega). The total concentration of transfected DNA was adjusted to 20 μg with pCMV-HA. Luciferase activity in cells 24 h post-transfection was assayed using a Promega luciferase assay kit according to the manufacturer’s instructions. Renilla luciferase activity was normalized against Renilla luciferase activity or against the total protein concentration in the cellular extract. The assays were performed in triplicate and repeated three to four times to ensure reproducibility. Statistical analysis was performed by using Microsoft Excel (Microsoft, Redmond, WA), and pooled data from individual experiments were expressed as means ± S.D. The standard error of the mean calculated for each sample was used to determine statistical significance. The p-values calculated were used to determine the level of significance.

**GST Pull-Down Assays**—Varying amounts (50 ng to 2 μg) of purified GST-TIEG1 or unfused GST protein were mixed with 200 μl of GST-Bind resin (Novagen) and incubated for 30 min at 25°C. The resin was washed with phosphate-buffered saline (PBS) and mixed with 50 ng of purified recombinant Sp1 protein (Promega) in 20 μl Tris-HCl, pH 8.0, 1 mM MgCl₂, 2 mM ZnCl₂, 0.1% dithiothreitol, 10% glycerol, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A and incubated at 4°C for 60 min. The resin was collected by centrifugation, washed with wash/sample buffer, and analyzed by Western blotting with anti-Sp1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in the primary reaction and horseradish peroxidase (HRP)-labeled anti-goat immunoglobulins (Amersham Biosciences) in the secondary reaction.

**In Vivo Immunoprecipitation**—Approximately 1 × 10⁷ THP1 or HL60 cells were transfected with 5 μg of pCMV-Sp1/flu, pCMV-HA-TIEG1, or CMV-Rb (human retinoblastoma protein) (24) or combinations of these (see figure legend for details). The transfected cells were harvested 24 h later, washed in cold PBS, and resuspended in 1 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 50 mM Tris-HCl, pH 8.0). The cell lysate was filtered on ice for 20 min and clarified by centrifugation. Anti-Sp1 antibodies were added to achieve a 5 μg/ml final concentration, and incubation was continued for 1 h at 4°C. To precipitate the immune complexes, 50 μg of Protein G-Sepharose 4 Fast Flow (Amersham Biosciences) was added to the lysate with incubation for 1 h at 4°C. The immune complexes were pelleted by centrifugation, washed extensively with lysis buffer and a final wash with 50 mM Tris-HCl, pH 8.0, and resuspended in SDS sample buffer for analysis by Western blot. To detect proteins in the immune complexes, rabbit anti-HA polyclonal antibodies (to detect HA-TIEG1 and HA-Sp1) and rabbit anti-Rb (to detect CMV-Rb) were added in the primary reaction followed by the addition of goat anti-rabbit HRP in the secondary reaction.

**Electrophoretic Mobility Shift Analysis**—Gel-extracted genomic DNA was reamplified as previously described (26) using nuclear extracts that were either purchased (Active Motif, Carlsbad, CA) or prepared as previously described (26). To initially localize the binding site of TIEG1, the following double-stranded oligonucleotide probes were used: 5′-TGTTCCATTATAACACGGCTCCCTACTACCAACTGGCTGCCCTTCTCCTGC-3′, which corresponds to the −80 to −31 region of the CD11d promoter; 5′-TTCATATAACCAATAACTCTCCTACCCACTGTCGTTG-3′, which corresponds to the −78 to −44 region of the CD11d promoter (this probe contains the 5-bp mutation at the −61 site shown in boldface); 5′-AACACGGCCCTCTCCTAAAACCTCTTGCTGCCCTTTCCTC-3′, which corresponds to the −68 to −34 region of the CD11d promoter (this probe contains the +3 bp mutation at the −61 site shown in boldface).

A more precise localization of TIEG1 binding was performed using a series of seven double-stranded oligonucleotide probes corresponding to the −66 to −40 region of the CD11d promoter. The seven probes contained a different 3-bp mutation (shown in boldface) and are as follows: WT, 5′-CCACGGCTCCCCCTACTACCAACTGGCTGCCCTTCTCCTGC-3′, which corresponds to the wild-type −66 to −40 region (the −61 and −51 sites that were mutated in the above probes are underlined for reference); mut1, 5′-AACACGGCTCCCCCTACTACCAACTGGCTGCCCTTCTCCTGC-3′; mut2, 5′-CCACGGCTCCCCCTACTACCAACTGGCTGCCCTTCTCCTGC-3′; mut3, 5′-CCACGGCTCCCCCTACTACCAACTGGCTGCCCTTCTCCTGC-3′; mut4, 5′-CACACGGCTCCCCCTACTACCAACTGGCTGCCCTTCTCCTGC-3′; and mut6, 5′-CCACGGCTCCCCCTACTACCAACTGGCTGCCCTTCTCCTGC-3′. These probes were end-labeled with γ³²PATP to a specific activity of 2–4 × 10⁶ cpm/μg. Each probe (1 × 10⁴ cpm) was incubated for 30 min on ice with either 5 μg of nuclear extract, 100 ng of purified recombinant Sp1 protein, or 100–200 ng of purified GST-TIEG1 as previously described (18). Antibodies to Sp1 or TIEG1 were included in some incubation experiments using supershift analysis. The probes were separated by polyacrylamide gel electrophoresis as described previously (18).
lysate was then diluted 10-fold in ChIP-dilution buffer (0.01% SDS, 1% Triton X-100, 150 mM NaCl, 2 mM Tris-HCl, pH 8.1) containing the above protease inhibitor mix. An aliquot of the diluted lysate was incubated for 4 h at 65 °C to reverse the cross-links and used as input DNA. To reduce the nonspecific background, the remainder of the lysate was incubated with a salmon sperm DNA/protein A-agarose slurry for 30 min at 4 °C, followed by removal of the protein A-agarose by centrifugation. The supernatant fraction was incubated with anti-TIEG1 or anti-SP1 antibodies, or no antibodies overnight at 4 °C. The anti-TIEG1-DNA/SP1 complex was precipitated from the supernatant fractions following incubation with salmon sperm DNA/protein A-agarose for 1 h at 4 °C. The precipitated complexes were extensively washed with buffers from the manufacturer and resuspended in 250 μl of elution buffer (1% SDS, 0.1 M NaHCO3) for 15 min at room temperature to elute the DNA/TIEG1 and DNA-SP1 complexes from the protein A-agarose. After removal of the protein A-agarose by centrifugation, 10 μl of 5 M NaCl was added to the supernatant, which was then heated for 4 h at 65 °C to reverse the cross-links. To purify the immunoprecipitated DNA, proteinase K was added (50 μg/ml final concentration) for 1 h at 45 °C, followed by phenol-chloroform extraction, ethanol precipitation, and resuspension of the DNA in 1 mM EDTA, 10 mM Tris-HCl, pH 8.0. The immunoprecipitated DNA was then amplified by the PCR using primers corresponding to regions of the CD11d promoter. The PCR products were subjected to gel electrophoresis, stained with Vistra Green, and analyzed on a PhosphorImager (Amersham Biosciences).

Expression of TIEG1 and its role in leukocyte integrin CD11d activation

Zinc Finger TIEG1 Confers Leukocyte Integrin CD11d Activation

RESULTS

The −100 to −20 Region of the CD11d Promoter Controls Myeloid Specificity—We had previously shown that Sp1 binds in vitro within the −63 to −40 region of the CD11d promoter and that both Sp1 and Sp3 activate expression of CD11d in myeloid but not non-myeloid cells (15). Furthermore, in vivo genomic footprint analysis revealed that this region can bind one or more proteins, and it was presumed that Sp proteins were bound (15). Because Sp proteins are ubiquitous, we reasoned that myeloid-specific expression of CD11d might be influenced by other transcription factors binding adjacent to the Sp binding site. To define the location of other transcription factor binding sites, site-directed mutagenesis of the region spanning the Sp1 binding site was performed. A series of 5-bp mutations was introduced throughout an extended region contained on a reporter construct CD11d(−173/+74)-luc (which contains the −173 to +74 region of the CD11d promoter fused to the luciferase gene) and analyzed in transient transfection assays (Fig. 1A). Three of five mutations (located at sites −69, −61, and −34) significantly reduced CD11d expression in the myeloid cell lines THP1 and HL60, but not in the B cell line IM9 or the T cell line Jurkat (Fig. 1B). A fourth mutation (located at site −91) significantly reduced expression in HL60, THP1, and Jurkat cells but had no effect on expression in IM9 cells. A fifth mutation (located at site −51) reduced CD11d expression in all cell lines.

Further evidence that myeloid-specific and nonspecific sites are clustered near the Sp1 binding site was demonstrated by transient transfection analysis of a luciferase reporter construct containing four copies of the −100 to −20 region of the CD11d promoter fused upstream of the enhancerless, minimal SV40 promoter in pGL3-Promoter. The −100 to −20 region increased SV40 promoter activity 6- to 7-fold in HL60 and THP1 cells, whereas its activity in Jurkat and IM9 cells was increased 2- to 2-fold (Fig. 2).

Identification of TIEG1 by Yeast One-hybrid Analysis—To identify the transcription factors that interact at the essential sites within the −100 to −20 region, a yeast one-hybrid screen was performed. Four copies of the −100 to −20 region were ligated upstream of the his6+ and lacZ genes in pHS1-1 and pLacZi, respectively, and both constructs were integrated into the yeast strain YM4271 and used as the bait to identify cDNAs that encode putative binding domains capable of interacting within this region. Because CD11d is expressed in splenic red pulp macrophages (11), a cDNA library (3.5 × 106 independent clones) consisting of spleen cDNAs ligated downstream of the GAL4 AD in pACT2 was transformed into the yeast bait strain and Leu+, His+, Ura+ transformants were selected at the higher 3-AT stringency, and over 200 transformants were obtained at the lower concentration. All were shown to express lacZ indicating that the transformants were not merely spontaneous His+ revertants. The pACT2-spleen clones were isolated from these transformants and re-transformed into the bait strain for a second round of selection. Clones enabling robust growth of the bait strain on 45 mM 3-AT media were sequenced and analyzed by BLAST. Seven different clones were found to contain 5′-truncated sequences corresponding to the recently identified transcription factor TIEG1. Full-length TIEG1 was subsequently isolated from spleen cDNA by RT-PCR using appropriate primers.

TIEG1 Activates the CD11d Promoter in Myeloid Cells but Down-regulates CD11d in Non-myeloid Cells—To determine whether TIEG1 functionally interacts with the CD11d promoter in other cell types, Drosophila cells, which are deficient
in TIEG1, were cotransfected with an expression construct for TIEG1 (pCMV-HA-TIEG1) and the luciferase reporter CD11d(-173/+74)-luc (Fig. 3A). However, no significant change in luciferase expression was seen in Drosophila cells that express TIEG1. Because the consensus binding sequence for TIEG1 resembles that for Sp1 and Sp3, transcription factors known to regulate CD11d, it remained possible that TIEG1 might compete with these Sp proteins for binding to the same or overlapping site and affect CD11d promoter activity.

Luciferase activity from the CD11d promoter increased 13.9- and 3.6-fold in Drosophila cells cotransfected with expression constructs for Sp1 and Sp3 (pPacSp1 and pPacSp3) (Fig. 3A). Inclusion of 5 µg of pCMV-HA-TIEG1 in the cotransfections had no effect on activation of the CD11d promoter by Sp1 (Fig. 3A). However, overexpression of TIEG1 attenuated the activation of the CD11d promoter by Sp1 when the concentration of pCMV-HA-Sp1 was lowered to 1 µg (Fig. 3A). This suggests that TIEG1 acts as a passive repressor by competing with Sp1 for the same or overlapping site.

The role of TIEG1 on CD11d expression in leukocytes was next examined. Overexpression of TIEG1 in the myeloid cell lines THP1 and HL60 led to 1.8- and 2.5-fold increases in luciferase expression from CD11d(-173/+74)-luc (Fig. 3B and C). Overexpression of Sp1 or Sp3 increased luciferase expression from the CD11d promoter 3.1- and 3.1-fold in transfected THP1 cells and 1.7- and 1.5-fold in transfected HL60 cells (Fig. 3B and C). Inclusion of pCMV-HA-TIEG1 with pCMV-Sp1/flu in cotransfections of THP1 and HL60 cells did not further increase CD11d promoter activity beyond that observed with either expression construct alone (Fig. 3B and C). Similarly, inclusion of pCMV-HA-TIEG1 in cotransfections with pCMV-Sp3/flu did not increase CD11d promoter activity beyond that observed with either expression construct alone.

2 J. D. Noti, unpublished observations.
alone (Fig. 3, B and C). These results further indicate that TIEG1 and the Sp proteins compete for binding to the same or overlapping site on the CD11d promoter.

To determine whether activation of the CD11d promoter by TIEG1 is myeloid-specific, the same cotransfection experiments were performed in non-myeloid cells. Overexpression of TIEG1 in K562 (undifferentiated lymphoblasts) and IM9 (B cells) cells decreased luciferase expression from CD11d(−173/+74)-luc by 53 and 40%, respectively, and had no effect on CD11d promoter expression in Jurkat (T cells) (Fig. 3, D–F). As was previously observed (15), Sp1 and Sp3 generally down-regulated CD11d promoter activity −50% in these non-myeloid cell lines (Fig. 3, D–F). When pCMV-HA-TIEG1 was included along with either pCMV-Sp1/flu or pCMV-Sp3/flu in cotransfections of these three cell lines, in general, TIEG1 was able to lower luciferase expression from CD11d(−173/+74)-luc further than that observed by either Sp protein (Fig. 3, D–F). Together, these results show that TIEG1 is a myeloid-specific activator of the CD11d promoter but functions as a repressor in non-myeloid cells.

Endogenous CD11d Expression Is Down-regulated by siRNAs Targeting TIEG1—To confirm whether TIEG1 regulates expression of CD11d in vivo, increasing amounts of TIEG1 siRNA were transfected into HL60 and THP1 cells, and PMA (100 nM) was then added for 48 h to induce expression of CD11d. Expression of endogenous TIEG1 decreased in both cell lines as the amount of TIEG1 siRNA was increased (Fig. 4, A and B). Down-regulation of CD11d expression paralleled that of TIEG1 (Fig. 4). Expression of GAPDH was unaffected by siRNA treatment and was used to normalize expression of TIEG1 and CD11d (Fig. 4, C and D). Transfection of HL60 cells with 1 μg of TIEG1 siRNA reduced TIEG1 expression by 88% and CD11d expression by 96% (Fig. 4, A and C). Essentially identical results were obtained in THP1 cells, with maximal reductions of TIEG1 (78% reduction) and CD11d (79%) obtained with 1 μg of TIEG1 siRNA (Fig. 4, B and D). The PCRs were performed within the linear range of amplification as indicated by analyzing samples at 21, 24, 27, 30, 36, and 39 cycles (Fig. 4E).

In contrast, no significant change in expression of either Sp1 or CD11c, another myeloid-specific leukocyte integrin, was evident in either cell line transfected with TIEG1 siRNA (Fig. 4, A and B). Furthermore, no significant differences in gene expression were observed in HL60 and THP1 cells transfected with random siRNAs. These results indicate that expression of TIEG1 is essential for expression of CD11d.

The Binding Site for TIEG1 Overlaps the Sp1/Sp3 Binding Site—We previously showed by DNase I footprint analysis that purified Sp1 protein protected the −63 to −40 region of the CD11d promoter (15). EMSA was performed to determine more precisely the binding sites for TIEG1 and Sp1. Site-directed mutagenesis revealed that nucleotides at −61 and −51 are essential for CD11d promoter activity (Fig. 1). EMSA was initially performed with DNA probes containing the 5-bp substitutions at either −61 or −51 (Fig. 1A). Two major DNA-protein complexes were seen when nuclear extract protein from unstimulated THP1 cells was incubated with a probe containing the wild-type sequences at −61 and −51 (Fig. 5A). Anti-Sp1 antibodies supershifted complex 1 (Fig. 5A). Similarly, the same two complexes formed with the probe mutated at −51, and anti-Sp1 antibodies supershifted complex 1. The single complex that formed with the probe mutated at −61 was not affected by anti-Sp1 antibodies (Fig. 5A) or anti-Sp3 antibodies (data not shown). Nuclear extracts prepared from PMA-stimulated THP1 cells produced identical results (Fig. 5B). These results indicate that the sequences at or near −61 in the CD11d promoter are essential for Sp1 binding. Further confirmation of this finding was shown using purified Sp1 protein in an EMSA with these probes. A single complex containing Sp1 was formed with the probe mutated at −51, but no complex was formed between Sp1 and the probe mutated at −61 (Fig. 6A).

Sp1 protein was then incubated with a series of DNA probes containing 3-bp substitutions throughout the −66 to −40 region of the CD11d promoter (Fig. 7A). Complete loss of Sp1 binding to DNA probes containing mutations spanning −61 to −54 was seen (Fig. 7B). The nucleotides immediately flanking these regions (−66 to −64 and −52 to −50) may not be part of the core binding site for Sp1, because mutations of these nucleotides did not prevent Sp1 from binding.

Purified GST-TIEG1 protein was unable to bind to the probes containing the 5-bp mutations at −61 or −51 but was able to bind efficiently to the wild-type probe (Fig. 6B). On further analysis using the series of 3-bp mutation probes, loss of GST-TIEG1 binding to probes containing mutations spanning −61 to −45 was seen (Fig. 7C). The flanking regions (−66 to −64 and −43 to −41) were either not part of the core binding sequence for TIEG1 or can tolerate nucleotide substitutions.

Competition EMSA was done to confirm that TIEG1 and Sp1 bind to overlapping sites (Fig. 8). Increasing the amount of GST-TIEG1 in bindings reaction containing a fixed amount of Sp1 resulted in loss of the Sp1 complex and appearance of the GST-TIEG1 complex (Fig. 8, lanes 5–8). Likewise, as the
amount of Sp1 was increased in binding reactions containing a fixed amount of GST/TIEG1, the Sp1 complex appeared at the expense of the GST/TIEG1 complex (Fig. 8, lanes 9–12). Increasing the amount of the GST moiety had no effect on the ability of GST/TIEG1 to bind (Fig. 8, lanes 13–15). Taken together, the EMSA results indicate that Sp1 and TIEG1 compete for binding to overlapping sites.

**TIEG1 and Sp1 Physically Interact In Vitro But Not In Vivo**—Although the data indicate that TIEG1 functions by competing with Sp1 for a common binding site, we explored the possibility that in vivo one factor binds directly to the promoter while tethered to the other factor. In vitro, Sp1 was detected in GST-pull-down assays of GST/TIEG1 (Fig. 9). To determine whether these proteins interact in vivo, HL60 cells were transfected with CMV-HA-Sp1 or CMV-HA-TIEG1, or both. Taken together, the expression of each construct was examined by Western blot analysis using anti-HA antibodies to detect HA-TIEG1 and Sp1/flu fusion proteins, and anti-Rb antibodies to detect the Rb protein. The proper expression of each protein in HL60 cells but is not shown. The linearity of the PCR reaction was also confirmed during the analysis of HL60 cells but is not shown.

To determine whether Sp1 and TIEG1 exist as a complex in vivo, cell extracts from transfected cells were incubated with Sp1 antibodies followed by precipitation of the immune complexes. Western blot analysis detected Sp1 but not TIEG1 protein in extracts from cells cotransfected with both expression constructs, or in various combinations was shown (Fig. 10A). No signals were detected in cells transfected with the empty plasmid CMV-HA (Fig. 10A).

The expression of each construct was examined by Western blot analysis using anti-HA antibodies to detect HA-TIEG1 and Sp1/flu fusion proteins, and anti-Rb antibodies to detect the Rb protein. The proper expression of each protein in HL60 cells transfected separately with each expression construct or in various combinations was shown (Fig. 10A). No signals were detected in cells transfected with the empty plasmid CMV-HA (Fig. 10A).

**TIEG1 Is Ubiquitously Expressed in Leukocytes**—CD11d was...
expressed predominately in myeloid cells after exposure to 100 nM PMA for 48 h, whereas expression in non-myeloid cells was undetectable (Fig. 11A). We have shown that TIEG1 functions to activate the CD11d promoter specifically in myeloid cells (Fig. 3). RT-PCR blot and Western blot analyses were next done to determine whether TIEG1 activity in myeloid cells correlates with its expression. The level of TIEG1 mRNA in the myeloid and non-myeloid cell lines was similar and did not change in response to PMA (Fig. 11B). Western blot analysis showed that TIEG1 protein expression was also similar in all cell lines tested (Fig. 11C). Furthermore, there were no significant differences in the level of Sp1 mRNA in these cell lines (Fig. 11D). The results indicate that the myeloid-specific activation of the CD11d promoter is not the result of an increase in either TIEG1 or Sp1 expression in myeloid cells.

Myeloid-specific Expression of CD11d Is Associated with Increased TIEG1 Protein Binding in Vivo—The ability of TIEG1 to activate the CD11d promoter specifically in myeloid cells and the ubiquitous nature of its expression in myeloid and non-myeloid cells suggested to us that interaction of TIEG1 with the CD11d promoter in vivo may differ among cell types. To address this, ChIP assays were done using anti-TIEG1 antibodies and, for comparison, anti-Sp1 antibodies. THP1, HL60, IM9, and Jurkat cells that were either unstimulated or PMA-stimulated for 48 h were treated with formaldehyde to cross-link bound transcription factors to their genomic DNA. Genomic DNA was then isolated and sheared to ~500 bp in length and incubated with anti-TIEG1 antibodies or anti-Sp1 antibodies to precipitate DNA fragments bound by these factors. Cross-linking of the precipitated DNA was reversed, and antibody-precipitated unstimulated THP1 and HL60 genomic DNAs with primers spanning the WT CD11d promoter, or with probes containing either a 5-bp mutation of the −61 region of the CD11d promoter, or with probes containing either a 5-bp mutation of the −61 or −51 region of the CD11d promoter, or with probes containing either a 5-bp mutation of the −61 or −51. The probes were incubated with purified Sp1 protein (A) or purified GST-TIEG1 protein (B). The filled arrowhead shows the single protein-probe complex formed in the absence (−) of specific antibody. The absence (−) or presence (+) of Sp1 antibodies (Sp1 Ab) in the incubation mix is indicated. The unfilled arrow indicates the supershifted complex formed resulting from binding of anti-Sp1 antibody to complex 1. Lanes containing only the probes (probe only) are included.

Fig. 5. Evidence that Sp1 interacts with the −51 region. EMSA was performed with a labeled oligonucleotide probe spanning the wild-type (WT) −80 to −31 region of the CD11d promoter, or with probes containing either a 5-bp mutation of the −61 or −51. The probes were incubated with nuclear extract protein from unstimulated (no PMA) (A) or PMA-stimulated (+ PMA) THP1 cells (B). Filled arrowheads indicate the two protein-probe complexes formed in the absence (−) of specific antibodies. The absence (−) or presence (+) of Sp1 antibodies (Sp1 Ab) in the incubation mix is indicated. The unfilled arrow indicates the supershifted complex formed resulting from binding of anti-Sp1 antibody to complex 1. Lanes containing only the probes (probe only) are included.

Fig. 6. Purified TIEG1 and Sp1 interaction with the CD11d promoter. EMSA was performed with a labeled oligonucleotide probe spanning the wild-type (WT) −80 to −31 region of the CD11d promoter, or with probes containing either a 5-bp mutation of the −61 or −51. The probes were incubated with purified Sp1 protein (A) or purified GST-TIEG1 protein (B). The filled arrowhead shows the single protein-probe complex formed in the absence (−) of specific antibody. The absence (−) or presence (+) of Sp1 antibodies (Sp1 Ab) in the incubation mix is indicated. The unfilled arrow indicates the supershifted complex formed resulting from binding of anti-Sp1 antibodies or anti-TIEG1 antibodies to the single complex 1 formed with specific probes. Lanes containing only the probes (probe only) are included.
50, 250, and 500 ng of GST, respectively. GST/H18528 TIEG1 were added to lanes 9–H18528 500 ng of GST show the protein incubated with the Lanes 5–H18528 show supershift complexes with anti-Sp1 or Anti-TIEG1 antibodies. Varying amounts of purified GST, GST/H11002 throughout the oligonucleotide probes (mut1–6) containing 3-bp mutations (bolded) throughout the −66 to −40 region. The wild-type (WT) probe was included as control. Binding of Sp1 (B) or GST-TIEG1 (C) to each probe is shown.

DNA incubated without antibody (Fig. 12A). When genomic DNA isolated from PMA-stimulated THP1 and HL60 cells was immunoprecipitated with anti-TIEG1 antibodies and amplified with these same primers, there was a substantial increase in yield of the 180-bp PCR product (Fig. 12A). Similar to the siRNA assay, the PCRAs were determined to be performed within the linear range of amplification as indicated by analyzing samples at 24, 27, 30, 36, 39, and 42 cycles (data not shown). Furthermore, the large apparent increases in TIEG1 binding in PMA-stimulated myeloid cells were not simply the result of gross differences in starting input genomic DNAs isolated from unstimulated and PMA-stimulated cells, because the yield in PCR product from these input genomic DNAs was approximately the same (Fig. 12A). In contrast, the binding of TIEG1 in unstimulated IM9 and Jurkat cells did not significantly change when these cells were exposed to PMA (Fig. 12A). Again, no significant differences in starting input DNAs from unstimulated and PMA-stimulated IM9 cells or unstimulated and PMA-stimulated Jurkat cells were found (Fig. 12A). As control, the primers spanning −712 to −520 of the CD11d promoter efficiently amplified all input DNAs, but no PCR product was obtained from any of the DNAs following their immunoprecipitation with anti-TIEG1 or anti-Sp1 antibodies (Fig. 12B).

The same primers spanning the −200 to −20 region were used to assess occupancy of this region by Sp1. DNA from unstimulated THP1 and HL60 cells, when immunoprecipitated with anti-Sp1 antibodies and amplified, produced a significant amount of the 180-bp PCR product corresponding to the −200 to −20 region (Fig. 12A). In contrast to the analysis using anti-TIEG1 antibodies, the yield of the 180-bp PCR product produced using anti-Sp1 immunoprecipitated DNA from PMA-stimulated THP1 and HL60 cells did not increase (Fig. 12A). The extent of Sp1 binding in unstimulated IM9 and Jurkat cells did not significantly change when these cells were exposed to PMA (Fig. 12A). As expected, the control primers spanning the −712 to −520 region produced no amplified PCR product with immunoprecipitated DNAs from all cell lines (Fig. 12B). These results indicate that occupancy of the CD11d promoter by TIEG1 increases specifically in predominately in myeloid cells that have been stimulated with PMA.

**DISCUSSION**

The expression of CD11d appears restricted to myeloid cells and subsets of peripheral blood leukocytes. CD11d is prominently expressed on splenic red pulp macrophages, foam cell macrophages, synovial macrophages, bone marrow cells, minor subpopulations of peripheral blood CD8+ T cells, and splenic red pulp CD4−CD8− cells (11, 14, 15, 27, 28). Furthermore, expression of CD11d is up-regulated during myeloid differentiation of CD34+CD38− and peripheral blood monocytes with GM-CSF and interleukin-3 and during differentiation of myeloid cell lines with PMA (27). The functions of CD11d are not well understood, but its differential expression suggests that it may play roles in the inflammatory response, lipid clearance, and phagocytosis of red blood cells and blood-borne pathogens. Understanding the regulation of CD11d expression may shed light on its roles in these and other processes.

We previously reported that CD11d, like the other leukocyte integrin genes, is regulated by Sp1/Sp3 transcription factors (15). Adjacent to the Sp sites in CD11a−c and CD18 are binding sites for a number of transcription factors, including PU.1, GABPa, GABPB, c-Jun, c-Fos, Ets, and c/EBP (7), and we reasoned that these or other transcription factors may also interact adjacent to the Sp1/Sp3 site in the CD11d promoter. In this report, site-directed mutagenesis revealed sites within the
leukocyte integrin CD11d expression and are essential for CD11d expression. Yeast one-hybrid screening of a spleen cDNA expression library led to the isolation of the Kruppel-like TGF-β-inducible early gene TIEG1 (20, 21). TIEG1 can be classified as a tumor suppressor, because it plays a role in TGF-β-induced inhibition of cell proliferation of human osteoblasts and induces apoptosis in pancreatic, epithelial, and liver carcinoma cells (29–32). A putative role for TIEG1 in myeloid cell differentiation and/or apoptosis has not been addressed, although, interestingly, the TIEG1 gene is located on chromosome 8,q22.2, where genes related to acute myeloid leukemia and osteopetrosis are found (33). The primary role of TIEG1 and its related family member TIEG2 is that of transcriptional regulator, which functions through three repression domains, R1, R2, and R3, contained in the N terminus of these factors (34). The R1–3 repression domains are distinct and exhibit different relative strengths of 92%, 84%, and 51% repression, respectively, and are highly conserved between the TIEG proteins (34). The R1 domain contains an α-helical repression motif (35) that is homologous to the mSin3A-interacting domain found in Mad proteins (36). Interaction of the corepressor mSin3A with the R1 domain of TIEG2 can be disrupted by signaling through the epidermal growth factor-Ras-MEK1-ERK2 pathway, which phosphorylates four sites adjacent to the mSin3A-interacting domain (37). We have, however, not determined whether the repressor activity of TIEG1 on CD11d expression in non-myeloid cells functions through the R1-mSin3A interaction or through either of the other two repression domains, R2 and R3.

The activator function of TIEG1 on the CD11d promoter in myeloid cells is novel, because no transcriptional activation domains were found in TIEG1 or TIEG2 (34). However, recent reports of how TIEG1 enhances TGF-β/Smad signaling suggest that TIEG proteins may act as transcriptional activators. TGF-β induces TIEG1 expression, which, in turn, enhances signaling through the Smad pathway (25, 38). TGF-β initially binds to the type II TGF-β receptor and then forms a heteromeric structure with the type I receptor, which becomes activated by the type II receptor (39). The activated type II receptor then phosphorylates Smad2 and Smad3, which then bind to Smad4 and together translocate to the nucleus to effect transcription. Binding of Smad7 to the type I receptor blocks activation of the Smads resulting in negative feedback to the TGF-β/Smad signaling pathway. TIEG1 enhances Smad signaling by binding to the Smad7 promoter and represses its transcription (25, 38). However, even when Smad7 RNA was first depleted by antisense RNA, overexpression of TIEG1 still enhanced induction of a synthetic Smad binding fused to the luciferase reporter, suggesting that TIEG1 also functions through a Smad7-independent mechanism, which was then shown to occur through up-regulation of Smad2 transcription by TIEG1 (38). It was not determined whether TIEG1 activator function is a direct result of TIEG1 binding to the Smad2 promoter or through an indirect mechanism such as down-regulation of a repressor for the Smad2 gene. In this present study, up-regulation of CD11d expression following differentiation of myeloid cells with PMA was associated with increased binding of TIEG1 to the CD11d promoter in vivo, which supports the concept that TIEG1 contains an activator domain. Whether this is an activator domain distinct from the R1–3 domains or is the result of binding of a coactivator and/or loss of binding of mSin3A or other corepressor to one of the repressor domains remains to be determined.

Typically, yeast one-hybrid screens employ a short (12–36 bp) target sequence as bait to isolate a protein that interacts at this site. Because a number of sites essential for CD11d promoter activity were identified within the −100 to −20 region, we reasoned that multiple transcription factors might be identified in a single screening. Additionally, the use of a longer sequence that more closely mimics DNA folding and protein binding seen with the native promoter may also allow the isolation of proteins with lower affinities for the target sequence. Such a strategy was successfully used to isolate the SpEts4 protein by using a single copy of the −324 to −143 region of the Strongylocentrotus purpuratus hatching enzyme gene, SpHE, as bait in a yeast one-hybrid screen (40). It is interesting that, although the bait sequence −100 to −20 from the CD11d promoter contains an intact Sp1/Sp3 binding site, no Sp clones were identified in any of the screens of this study. EMSA using spleen nuclear-extract protein indicated that both Sp1 and Sp3 are abundantly present in spleen tissue; therefore, the reason why no Sp clones were identified in the one-hybrid screen may reflect a lower affinity of Sp proteins for the overlapping TIEG1 binding site. This is evidenced in our luciferase expression experiments (Fig. 3), which showed that, in transfected THP1 cells, TIEG1 could effectively compete with Sp1 (but not with Sp3) for binding to this common site in the CD11d promoter. TIEG1 was identified in a yeast two-hybrid screen as an interacting partner with GST-Sp1 (41). Although Sp1 was co-immunoprecipitated by GST-TIEG1 in our study, this in vitro interaction may result from enhanced physical associations between the two proteins in some way involving the GST moi-

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3 J. D. Dillon and J. D. Noti, unpublished observations.
and Sp1 expression in unstimulated (−) HL60, THP1, IM9, and Jurkat cells either unstimulated (−) or stimulated with 100 nM PMA for 48 h (+) was sheared, precipitated with anti-TIEG1 (TIEG1) or anti-Sp1 (Sp1) antibodies, and amplified by the PCR (30 cycles) with primers specific to the −200 to −20 region. PCR analysis at 30 cycles was confirmed to be within the linear range by monitoring the PCR through 24–42 cycles of amplification (data not shown). Amplification of cross-linked genomic DNA that was precipitated in the absence of antibodies (No Ab) is shown. Genomic DNA that was not cross-linked (Input) was also amplified by the PCR. The quantity of each input DNA was initially determined by A₂₆₀₋₂₈₀ and adjusted if necessary to yield approximately the same amount of PCR product for all four cell lines for a given set of primers. B, ChIP analysis of the −712 to −520 region was performed with specific primers to this region. Each immunoprecipitation was done in duplicate and two independent experiments were performed. Representative gels stained with ethidium bromide are shown.

FIG. 11. TIEG1 and Sp1 are expressed in myeloid and non-myeloid cells. Northern and Western blot analysis of CD11d, TIEG1, and Sp1 expression in unstimulated (−) HL60, THP1, IM9, and Jurkat cells or cells stimulated with 10 nM PMA for 24 h or 100 nM PMA for 48 h is shown. Expression of actin mRNA and GAPDH mRNA served as controls. A, Northern analysis of CD11d expression in unstimulated or PMA-stimulated cells is shown. B and D, total mRNA from unstimulated or PMA-stimulated cells was amplified by RT-PCR with primers specific to the 3′-coding regions for the TIEG1 and Sp1 genes. Amplification was adjusted to be in the linear range. C, Western blot analysis of TIEG1 protein expression in unstimulated and PMA-stimulated cells is shown.

FIG. 12. ChIP analysis of TIEG1 and Sp1 binding in vivo. A, formaldehyde cross-linked genomic DNA from THP1, HL60, IM9, and Jurkat cells either unstimulated (−) or stimulated with 100 nM PMA for 48 h (+) was sheared, precipitated with anti-TIEG1 (TIEG1) or anti-Sp1 (Sp1) antibodies, and amplified by the PCR (30 cycles) with primers specific to the −200 to −20 region. PCR analysis at 30 cycles was confirmed to be within the linear range by monitoring the PCR through 24–42 cycles of amplification (data not shown). Amplification of cross-linked genomic DNA that was precipitated in the absence of antibodies (No Ab) is shown. Genomic DNA that was not cross-linked (Input) was also amplified by the PCR. The quantity of each input DNA was initially determined by A₂₆₀₋₂₈₀ and adjusted if necessary to yield approximately the same amount of PCR product for all four cell lines for a given set of primers. B, ChIP analysis of the −712 to −520 region was performed with specific primers to this region. Each immunoprecipitation was done in duplicate and two independent experiments were performed. Representative gels stained with ethidium bromide are shown.
location of where proteins bind, they do indicate the specific type of protein that is bound within a region. It appears clear that both Sp1 and TIEG1 are bound to the CD11d promoter in unstimulated myeloid cells, perhaps in a binding equilibrium that accounts for the in vitro observation that the two factors compete for an overlapping site. After 48-h exposure of cells to PMA, TIEG1 binding to the CD11d promoter in myeloid cells substantially increases. Although a direct comparison between the in vitro footprint analysis and the ChIP assays can not entirely be made, because the duration of PMA stimulation varied in the two assays, in general, the ChIP assays are consistent with the initial interpretation that one or more proteins, including Sp1, are bound in vitro. An alternate hypothesis is that in unstimulated myeloid and non-myeloid cells, a repressor protein occupies the CD11d promoter that maintains its expression at low levels particularly in non-myeloid cells.

in vivo footprint analysis thus may reflect the presence of a repressor bound to the CD11d promoter. Additional transcriptional proteins were also identified in the current yeast one-hybrid screen, including gut-enriched Kruppel-like factor 4 (43, 44) and lung Kruppel-like factor 2 (45, 46), both of which have been shown to exhibit repressor function. Thus, the CD11d promoter appears to be controlled by related transcriptional factors that interact at an overlapping binding site, which, depending on cell-type and growth conditions, can either repress or stimulate CD11d expression.

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