The Zinc Finger Transcription Factor ZBP-89, is a Repressor of the Human β2 Integrin CD11b gene

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ABSTRACT

Integrin CD11b is a differentiation marker of the myelomonocytic lineage and an important mediator of inflammation. Expression of the CD11b gene is transcriptionally induced as myeloid precursors differentiate into mature cells, then drops as monocytes further differentiate into macrophages. Previous studies have identified elements and factors involved in the transcriptional activation of the CD11b gene during myeloid differentiation, but no data exists regarding potential down-regulatory factors, especially in the later stages of differentiation. Using two copies of a GC-rich element (-141 to -110) in the CD11b promoter, we probed a cDNA expression library for interacting proteins. Three clones were identified among 9.1 million screened, all encoding the DNA binding domain of the zinc finger factor ZBP-89. Overexpression of ZBP-89 in the monocyte precursor cell line U937 reduced CD11b promoter-driven luciferase activity when U937 were induced to differentiate into monocyte-like cells using phorbol esters. To identify the differentiation stage at which ZBP-89 repression of the CD11b gene is exerted, the protein level of ZBP-89 was correlated with that of CD11b mRNA in differentiating U937 as well as in normal human monocytes undergoing in vitro differentiation into macrophages. A clear inverse relationship was observed in the latter but not the former state, suggesting that ZBP-89 represses CD11b gene expression during the further differentiation of monocytes into macrophages.

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INTRODUCTION

CD11b is the alpha subunit of the integrin αβ heterodimer CD11b/CD18, one of four members of the β2 integrins. It is expressed on myelomonocytic cells and mediates essential adhesion-dependent functions. These include adhesion, migration, phagocytosis, degranulation, antibody-dependent cytotoxicity, and release of proteolytic enzymes during the inflammatory response. Deficiency of CD11b and the other β2 integrins in the inherited disease leukocyte adhesion molecule deficiency predisposes to life-threatening bacterial infections. Increased expression of CD11b on the other hand, leads to poorly coordinated adhesive functions, thus contributing to the genesis of several pathologic states such as ischemia-reperfusion injury and immune complex diseases.

CD11b is a myeloid specific marker. During hematopoiesis, it is first detected at the myelocytic and monoblastic stages, and its expression is significantly increased as cells differentiate into monocytes and granulocytes. Subsequently CD11b gene expression is down-regulated in cells proceeding towards later stages of monocytic differentiation. The drop in expression of CD11b can be reproduced in vitro in human peripheral blood monocytes as they differentiate into macrophages. Although some of the elements and factors underlying the induction of the CD11b gene are known, those contributing to its down-regulation thereafter remain to be defined.

Transcriptional regulation of CD11b is an important determinant in expression of the receptor. The proximal promoter of CD11b spanning nucleotides –242 to +71 appears responsible for the tissue-specific and lineage-specific expression of the gene in vitro. The CD11b promoter contains near its transcription start site elements for the transcription factors Sp1, Sp3, and PU.1 which have been shown to prime the CD11b gene for expression. Inducible expression of CD11b during monocytic lineage differentiation appears to be mediated by an as yet uncloned nuclear factor, named MS-2. No information is available on the nature of factors that mediate transcriptional repression of the CD11b gene. We have utilized yeast one-hybrid screens to identify a Krüpple-like zinc finger transcription factor, ZBP-89, which binds to the CD11b promoter and acts as a transcriptional repressor of this gene as normal monocytes differentiate into macrophages in vitro. The significance of these findings is discussed.
MATERIALS AND METHODS

Cells. Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The promonocytic U937 cell line (ATCC CRL-1539.2) was cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA) in an atmosphere of 5% CO₂. Treatment of U937 with phorbol 12-myristate 13-acetate (PMA) (Sigma) was used as an in vitro model system to assess early stage monopoiesis; these cells did not express the macrophage marker CD16 for up to 96 hours following PMA treatment (not shown). A pool of U937 cells stably expressing the CD11b promoter (sU937-11b-luc) spanning nucleotides -242 to +71 fused to the luciferase gene were generated by transfection with a CD11blucI-pCMV/Zeo (Invitrogen, Clarsbad, CA) construct using electroporation. Cells were selected and maintained in the presence of 200 µg/ml Zeocin (Invitrogen, Clarsbad, CA). The Drosophila embryo cell line SL2 (ATCC CRL-1963) was grown at 25°C in Schneider's Drosophila medium (GIBCO BRL, Grand Island, NY) supplemented with 10% heat-inactivated FCS specified for insect cells (Sigma). Late stage monocytic differentiation into macrophage was carried out using a technique that relies on mitogenically-activated T cell contact with monocytes, thus mimicking the in vivo conversion of monocytes to macrophages by antigen-activated T cells. Briefly, normal human monocytes were isolated from leukopacks of healthy donors by Ficoll-Hypaque gradient centrifugation, followed by adherence to petri dishes in Iscove’s medium plus 10% human AB serum (Sigma, St. Louis, MO) for 90 min at 37°C and in 5% CO₂. Adherent cells were stimulated with Con A (10 µg/ml; Sigma, St. Louis, MO) for 20 hrs in 60/30 media (60% AIM V, 30% Iscove’s medium; GIBCO BRL, Grand Island, NY) plus 10% human AB serum. The following day, cells were extensively washed, and the adherent monocytes (Day 1) were cultured without any cytokine or mitogen for 12 days. Monocytes were identified by flow cytometric analysis using a FACS Vantage instrument and Cell Quest software (Becton Dickinson, San Jose, CA); > 90% of these cells were CD14 positive and < 3% CD3 positive.

Cloning of ZBP89 by Yeast One-Hybrid Screening. The yeast strain YM4271 (MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4-A512, gal80-A538, ade5::hisG) was used to make a reporter strain. The bait, consisting of two copies of the GC-rich region of the CD11b gene spanning nucleotides -141 to -105 (GC: 5'
GGTCAGGAAGCTGGGGAGGAAGGGTGGGCAGGCTGT 3′) (Fig. 1A, B) was subcloned into the plasmid pHISi-1 (Clontech, Palo Alto, CA). The resulting pHISi-1-GC plasmid was digested with XhoI, then transformed into yeast to create the wild-type reporter strain. The empty vector pHISi-1 and the mutant pHISi-1-GCm (5′ GGGTCCTAATCTGGGGCTTAAATGGTGGGCCCTTTT 3′) plasmids (Fig. 1B) were also transformed into yeast after digestion with XhoI to create a background and a mutant reporter strains, respectively. Plasmid integration into the yeast genome was analyzed by plating on synthetic dropout medium lacking histidine (SD-H) but supplemented with various concentrations of 3-aminotriazole of (3-AT). The resulting reporter strains showed growth inhibition at 45 mM 3-AT. We screened a total of 9.1 millions independent colonies from a human HeLa matchmaker cDNA library (Clontech, Palo Alto, CA). Plasmid DNAs were recovered from those colonies which were able to grow in the presence of 45 mM 3-AT and SD-HL (synthetic dropout medium without histidine and leucine). The plasmid DNAs were then transformed into the wild-type-, mutant- and background yeast strains to confirm the specificity of the interaction between the plasmid encoded protein and the bait DNA.

**Plasmid Construction.** A reporter plasmid, pALbWt, was used, which contains the CD11b promoter spanning nucleotides -242 to +71 fused to the luciferase gene. In order to introduce the transversion mutation (G to T) at nucleotide -117 within the CD11b promoter, a two-step PCR ligation was carried out; The first PCR reaction was performed with primer 1 (5′ CTAGAGTCGACCTCGAGCCAAGC 3′) and primer 2 (5′ CCACAGCCTGCCCAACCTTCTCC 3′), and the second PCR was carried out with primer 3 (5′ GGGGAGGAAGGTTGGGCCAGGCTTG 3′) and primer 4 (5′ TAGAATGGCCGCGGCT 3′). The third PCR fragment was generated with primers 1 and 4, using the first and the second PCR fragments as a template. The third PCR fragment was digested with PstI and NarI and subcloned into pALbWt. After confirming the mutation by DNA sequencing, the resulting plasmid was designated pALbM4a. Plasmids pCMV Sport3-ZBP-89 (which constitutively expresses human ZBP-89 in eukaryotic cell lines) and pGEX5T-ZBP-89 (which expresses ZBP-89 fused to glutathione S-transferase (GST)) were kindly provided by Dr. J. L. Merchant. The plasmid pCMV Sport3-Z (which expresses p25 in eukaryotic cell lines) was generated by subcloning a 0.62-kb PCR product digested with SmaI and XbaI into the SmaI
and XbaI sites of pCMVSport3 (GIBCO BRL, Grand Island, NY). The fusion polypeptide GST-Z was generated from pGEX4T-2-Z created by insertion of a PCR product encoding p25 digested with SmaI and XhoI into the SmaI and XhoI sites of pGEX4T-2 (Pharmacia Biotech, Piscataway, NJ). The plasmid CD11blue-pCMV/Zeo was generated by subcloning a 1247bp fragment of pCMV/Zeo (Invitrogen, Clarsbad, CA) digested with PstI and XhoI into the SalI and PstI sites of pALbWt. The expression plasmid pPacSp1 and the parental plasmid pPacO were kindly provided by Dr. Robert Tjian 24 . Both pPacZBP-89 and pPacZ, which express human ZBP-89 and p25, respectively, were constructed by insertion of BamHI-XhoI fragments of PCR products into the BamHI and XhoI sites of pPacO. The PCR-generated fragments in each plasmid construct were confirmed by DNA sequencing.

**Nuclear Extract and Total Protein Lysate Preparation.** Nuclear extracts were prepared using a method modified from that described by Dignam et al 25 . Briefly, cultured cells (1.2 x 10^7) were harvested and washed twice with cold PBS. Cell pellets were resuspended with 2 ml of buffer A (20 mM Tris-HCl (pH 7.8), 50 mM KCl, 80 mM NaCl, 0.2 mM EDTA, 0.1 mM EGTA, 0.2 mM Phenylmethylsulfonyl fluoride (PMSF), 4% glycerol, and the protease inhibitor mixture "Complete" (Roche Molecular Biochemicals, Indianapolis, IN), and incubated on ice for 10 min.

After addition of 10 µl of 10% Nonidet P-40, the cell suspension was vortexed. Disruption of the cell membranes was monitored under a light microscope by Trypan-blue staining. When more than 90% of cells were Trypan-blue positive, they were spun at 6,000 rpm at 4°C for 5 min to pellet the nuclei. The pellet was resuspended in 500 µl of buffer B (20 mM Tris-HCl, pH 7.8, containing 300 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.1 mM EGTA, 0.2 mM PMSF, 25% glycerol, and the protease inhibitor mixture "Complete") and mixed gently at 4°C for 30 min.

After spinning the suspension in a microcentrifuge at 16,000 g for 15 min at 4°C, the supernatant was aliquoted into 20 µl fractions, snap-frozen in liquid nitrogen, and stored at -80°C. For the preparation of the total protein lysate from human monocytes/macrophages, cells were harvested and washed with cold PBS. Cell pellets were incubated with the lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% triton X-100, 0.5% NP-40, 1 mM DTT, and the protease inhibitor mixture "Complete" (Roche Molecular Biochemicals, Indianapolis, IN) for 10 min at room temperature. After spinning the suspension in a microcentrifuge at 13,000 rpm for 15 min...
at 4°C, the supernatant was aliquoted into 20 µl fractions, snap-frozen in liquid nitrogen, and stored at -80°C.

**Electrophoretic Mobility Shift Assay (EMSA) and Immunoblot Analysis.** EMSA analyses were conducted as previously described 18, using the double-stranded oligonucleotides GC (sense strand: 5’-GGGTAGGGAAGCTGGGGAGGAAGGGTGGGCAGGCTGT-3’) or GC1 (sense strand: 5’-AGCTGGGGAGGAAGGGTGGGCAGGCTGT-3’). For electrophoretic mobility super-shift assay (EMSSA), 1 µl of either anti-ZBP-89 antibody (provided by Dr. J. L. Merchant, 26) or anti-Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was preincubated with nuclear extract for 20 min at 4°C, followed by the addition of the DNA probe and further incubation for 20 min at 4°C. The oligonucleotides used in the analyses are shown in Fig. 3A. The other ds-oligonucleotides used were: Sp1, 5’-ATTCGATCGGGGCGGGGCGAG-3’; OCT-1, 5’-TGTCGAYGCAAATCACTAGAA-3’; hTβ, 5’-GATCTGGGGGTGGGGTGGGGGTGGGGGTGGGGG-3’ (containing four copies of the CACCC recognition sequence of ZBP-89) 27. For immunoblot Analysis, nuclear protein (30 µg) was electrophoretically separated by 6% SDS-PAGE and electro-blotted onto a nitrocellulose membrane (BioRad, Hercules, CA). A chemiluminescence western blot detection kit and protocol (Roche Molecular Biochemicals, Indianapolis, IN) were used for antigen detection. The anti-ZBP-89 antibody was diluted 1000 fold and used as the primary antibody.

**Transfection.** U937 cells were transfected by electroporation in incomplete medium at 280V and 960 µF using "Gene Pulser Cuvettes" (BioRad, Hercules, CA). Cells in the early log phase of growth were harvested by centrifugation, washed once with medium and resuspended at room temperature. About 4 x 10^7 cells were transfected with 25 µg of luciferase test plasmid together with 5 µg of the plasmid pRSV-β (Promega, Madison, WI) which contains the lacZ gene. Each transfection of pALbWt and pALbM4a was performed in parallel with a transfection of the promoterless luciferase plasmid pATLuc. Induction of promoter activity was assessed by treating the electroporated cells for 16 h with PMA at 100 ng/ml prior to harvesting. Cells were subsequently pelleted, washed twice with PBS and lysed in 150 µl of Reporter Lysis Buffer (Promega, Madison, WI). Luciferase and β-galactosidase activities were assessed using assay reagents purchased from Promega (Madison, WI) and 100 µl and 20 µl of lysate, respectively.
Luciferase activity was measured using a Monolight 2010 Luminometer which integrated peak luminescence 10 seconds after injection of assay buffer and the values were normalized against β-galactosidase activity. Fold above background for each transfection was calculated by dividing the luciferase activity of each test plasmid by that of pATLuc. The fold above background derived from transfection of pALbWt was assigned an arbitrary value of 100%.

Trans-activation by ZBP-89 was assessed in cotransfections in which 8 µg of pATLuc or pALbWt or pALbM4a were mixed with 1 µg of pRSV-β and 16 µg of either pCMVSPORT3 or pCMVSPORT3-ZBP-89. After normalization of transfection efficiency against β-galactosidase activity, the level of luciferase activity directed by pALbWt in the presence of pCMVSPORT3-ZBP-89 was divided by that of luciferase activity directed by pATLuc (also in the presence of pCMVSPORT3-ZBP-89). This calculation yielded the fold above background activity of pALbWt in the presence of ZBP-89. Nonspecific effects caused by the backbone of the ZBP-89 were corrected by equivalent calculations of luciferase activity but in the presence of pCMVSPORT3. The resulting figures represented the fold above background activity of pALbWt in the absence of ZBP-89. This value is given as 100% then the fold above background values obtained in the presence of ZBP-89 was calculated to determine the degree of trans-activation. All transfections were repeated independently more than three times with at least two different DNA preparations for each plasmid.

sU937-11b-luc were transfected with 25 µg of either pCMVSPORT3 or pCMVSPORT3-ZBP-89 with 1 µg of the plasmid pRSV-β using electroporation, then treated with PMA at 100 ng/ml for 16 h before harvesting. After normalization of luciferase activity by β-galactosidase activity, the level of luciferase in the presence of pCMVSPORT3 was assigned a value of 100, and the effect of ZBP-89 on luciferase activity was expressed as a percentage of this value.

For transfection of the Drosophila cell line SL2, cells were plated at a density of 3 x 10^6/60-mm dish for 18 hrs and washed with PBS twice before transfection. A total of 2.6 µg of DNA and 10 µl of Superfect (Qiagen, Valencia, CA) were mixed in 150 µl of incomplete Schneider's Drosophila medium. After 10 min at room temperature, 0.6 ml of medium supplemented with 10% heat-inactivated fetal calf serum (tested for use with insect cells, Sigma) was added to the DNA-Superfect complexes. The suspension was then added drop-wise to the culture dishes. After a 3-h incubation at room temperature, cells were washed with PBS twice
and 2 ml of complete medium added. Cells were harvested 24 hrs later, washed two times with PBS and lysed in 150 µl of Reporter Lysis Buffer (Promega, Madison, WI). Generally, 10 µl of cell lysate were used in the β-galactosidase assay (Promega, Madison, WI) and 50 µl of lysate were assayed for luciferase activity. pIE3LacZ, which expresses β-galactosidase, was used in all transfections to correct for transfection efficiency.

**Real-Time Reverse Transcription-PCR (RT-PCR).** Total RNA from U937 cells (1.0 x 10^7) which were treated with PMA (100 ng/ml) for 0 hr, 24 hr, 48 hr and 72hr were isolated using the TRIzol reagent (GIBCO BRL, Grand Island, NY) followed by DNase I treatment (Ambion Inc., Austin, TX) as described by the manufacturer. First strand cDNA was prepared by extension of an oligo-d(T)16 primer with M-murine leukemia virus reverse transcriptase (Clontech, Palo Alto, CA). After incubation at 42 °C for 1 hr, the reaction was stopped by heating to 95 °C for 5 min. Equal amounts of cDNA were subjected to PCR to detect CD11b, ZBP-89, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts 28. PCR primers and TaqMan probes for CD11b and ZBP-89 were designed using Primer Express 1.0 Software program and purchased from Applied Biosystems. TaqMan probes for CD11b and ZBP-89 were labeled with a reporter fluorescent dye, FAM (6-carboxyfluorescein), at the 5’ end and a fluorescent dye quencher TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3’ end. For GAPDH, the Pre-Developed TaqMan Assay Reagent (PDAR) which has forward and reverse primers, and a probe labeled with a fluorescent dye VIC at the 5’end and a fluorescent dye quencher TAMRA at the 3’end, was used (Applied Biosystems, Foster City, CA). PCR conditions were 2 min at 50 °C (for AmpErase UNG incubation to remove any uracil incorporated into the cDNA), 10 min at 95 °C (for AmpliTaq Gold activation), followed by 40 cycles at 95 °C for 15 s and at 60°C for 1 min. Primers and probes for detecting ZBP-89 and CD11b are as follows;

- **ZBP89-For** 5’-CCGAGCCTTAACTTTTGACTGAT-3’,
- **ZBP89-Rev** 5’-ATGGTGGCATAGACCTGTTGT-3’,
- **ZBP89-Probe** 5’-6FAM-CCCAAATCAGCCAGCATTCTCTTCC-TAMRA-3’,
- **CD11b-For** 5’-AGTTGCCGAATTGCATCG-3’,
- **CD11b-Rev** 5’-GGCGTTCCCACCAGAGAGA-3’,
- **CD11b probe** 5’-6FAM-AGCCCCATTGTGCATCGCTCGCCT-TAMRA-3’.
Plasmid DNAs pCDNACD11b<sup>29</sup> and pCMVSport3-ZBP-89 were used to generate a standard curve for the determination of the copy number of CD11b and ZBP-89, respectively. To construct the GAPDH-plasmid, a 604 bp fragment was amplified using the forward primer 5’-TCTGCTCCTCCTGGTCACA-3’ and reverse primer 5’-GACGTACTCAGCGCCAGCAT-3’, and a cDNA from U937 cell total RNA as a template. The PCR reaction was performed with the following conditions; 45 s at 94 °C, 45 s at 60 °C, and 1 min at 72 °C for 30 cycles. This fragment was cloned using TA cloning Kits (Invitrogen, Carlsbad, CA) and the resulting plasmid sequenced to confirm its identity. All reactions were performed in the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Test samples, standards, and no template controls were run in triplicates and the data were analyzed using the Sequence Detector V 1.6 program. A standard curve was plotted in Ct vs. the known copy numbers of the templates from each plasmid. The final copy number of CD11b or ZBP-89 was presented respectively as CD11b/GAPDH or ZBP-89/GAPDH ratios.
RESULTS

Identification of ZBP-89 using yeast one-hybrid screens. The proximal GC-rich element spanning nucleotides -141 to -105 (with respect to the transcriptional start site, Fig. 1A, underlined), is required for expression of the CD11b gene in differentiating U937 cells. Two copies of this GC-rich element were used as 'bait' in a yeast one-hybrid screen to identify interacting factors. A mutant form of the GC-rich element (Fig. 1B) was used as a negative control to assess specificity. After $7.1 \times 10^6$ in a first and $2 \times 10^6$ in a second screen of a HeLa cell cDNA library were analyzed, 174 positive colonies grew upon re-streaking onto 45-mM 3AT SD-H plates. Among these, 43 did not show PCR products, and 19 were redundant (based on the digestion pattern of DNA with HaeIII and AluI.) The rest of the candidates were re-transformed into the yeast reporter strain and 22 grew on the selection medium. Of these, only three grew in the selection medium after transformation into the wild-type reporter strain but not into a background strain (which does not carry the bait), or the mutant reporter strain. Sequence analysis revealed that each of these three clones encodes a 206 amino acid protein of ~25 kDa. This we named p25. Blast searches demonstrated that p25 has 100% amino acid sequence identity, except for the last two amino acids, with the N-terminal half of the zinc finger transcription factor, ZBP-89. As shown in Fig.1C, a single nucleotide insertion in the cDNA encoding p25 caused an in-frame stop codon immediately after the four zinc-finger motifs, resulting in premature termination. In order to test the possibility that this truncated form of ZBP-89 is a physiologic product generated by alternative splicing in vivo, RT-PCR was performed using total RNA extracted from HeLa, U937, and U937 cells treated with PMA for 24 hours. Sequencing of the resulting PCR products demonstrated that none contained the nucleotide insertion, suggesting that the premature termination in p25 represents an in vitro artifact in the cDNA library (data not shown).

ZBP-89 binds to the GC-rich element of the CD11b promoter. To confirm that p25 actually binds to the bait sequence, EMSA analyses were carried out with a radiolabeled probe (GC, the double-stranded GC-rich element used for the yeast one-hybrid screen) and GST-Z which represents the protein p25 fused to GST. The fusion proteins were expressed in soluble form in E.coli BL21 (DE3) and used for EMSA. As shown in Fig. 2A, the GST protein alone does not bind to GC (lane 2) whereas GST-Z formed a complex with GC (lane 3). This complex was
competed with unlabelled GC (lane 4) or with the oligonucleotide htβ (lane 5) which represents the ZBP-89 binding site in the T-cell receptor promoter. A Sp1 consensus oligonucleotide did not compete (lane 6). We also performed EMSA with the fusion protein GST-ZBP-89, which represents the full-length ZBP-89 protein fused to GST and obtained similar results (Fig. 2B). This suggests that ZBP-89 binds to the double-stranded GC-rich element of the CD11b promoter.

To investigate the binding characteristics of ZBP-89 on the CD11b promoter during myeloid differentiation, EMSA was carried out by incubating labeled GC with nuclear extracts prepared from untreated U937 cells or U937 cells treated with PMA for 24 hrs to induce their differentiation into monocyte-like cells. Fig. 2C shows that a DNA-protein complex was formed using extracts prepared from induced cells (lane 4) but not from uninduced U937 cells (lane 2). Unlabelled GC or htβ oligonucleotides competed for the complex (lanes 5 and 6, respectively), however, oligonucleotides Oct-1 (lane 7) and Sp1 (lane 10) did not compete. Incubation with an antibody, which specifically recognizes ZBP-89, resulted in the disappearance of the complex (lane 8), but incubation with pre-immune serum (lane 9) or a Sp1-specific antibody (lane 11) did not. These results indicate that the endogenous ZBP-89 protein is inducible upon differentiation of U937 cells and that it binds to the GC-rich element of the CD11b gene.

Identification of the residues in the CD11b promoter that are critical for ZBP-89 binding.

To identify the minimal region required for p25 binding, competitive gel shift assays were performed using the oligonucleotide GC1, which is 9 nucleotides shorter than GC (Fig. 3A). As shown in Fig. 3B, a GST-Z containing complex was formed with radiolabeled GC1 and this was competed efficiently with its unlabeled equivalent (lane 3). Next, a series of oligonucleotides (M1- M6) representing sequential mutation of three or five nucleotides in GC1 were used as competing oligonucleotides. The oligonucleotides M2 - M4 lost their ability to compete for binding to GST-Z (lanes 5-7) while oligonucleotides M1, M5, and M6 effectively competed (lanes 4, 8, and 9 respectively). We also performed the same competition experiment with the fusion protein GST-ZBP-89 and obtained similar results (Fig. 3C). These results demonstrate that p25 and ZBP-89 bind to the double-stranded oligonucleotide spanning nucleotides -127 to -113 of the CD11b promoter, thus delineating the DNA binding segment for ZBP-89.

MS-2, a putative transcription factor, binds to the GC-rich element of the CD11b promoter and might be involved in induction of the CD11b gene during myeloid cell
differentiation. In Fig. 3E (lane 2), we show that MS-2 is a purine-rich single-stranded DNA binding factor. Since the binding sites for p25/ZBP-89 and MS-2 overlap, it was necessary to identify a mutation which disrupts ZBP-89 but not MS-2 binding in order to assess the specific function of ZBP-89. A competitive gel shift assay was performed using GC1 as a probe, and a series of mutant double-stranded oligonucleotides (M4a-e), were used as competitors, each of which represented a different point mutation within GC1 (Fig. 3A). Figure 3D shows that the M4a mutation which has a transversion of G to T at nucleotide –117 does not compete with the radiolabeled wild-type double-stranded probe GC1 (lane 4), indicating that G at –117 is crucial for ZBP-89 binding. The purine-rich sense strand of GC1 (GC1/for) formed an MS-2 complex (Fig. 3E, lane 2). This complex was competed equally well with unlabelled single-stranded wild-type (GC1/for) and mutant (M4a/for) oligonucleotides (lanes 3 and 4, respectively), indicating that G at –117 is not essential for MS-2 binding. Therefore, the M4a mutation abolishes ZBP-89 binding while retaining the ability to bind MS-2. When GST-ZBP-89 was incubated with radiolabeled purine-rich single-stranded GC1/for (lane 5 to 7), no complex was observed in the absence (lane 5) or in the presence of wild-type GC1/for (lane 6) or mutant M4a/for (lane 7). Thus although ZBP-89 and MS-2 share a common DNA binding site, they are distinct factors with different binding and DNA recognition properties.

**Overexpression of ZBP-89 represses CD11b gene expression.** To study the role of ZBP-89 in regulating CD11b promoter activity, increasing amounts of pCMVSPORT3-ZBP-89 were transfected into U937 in which the wild-type CD11b promoter fused to the luciferase gene was stably incorporated. Transfection with pCMVSPORT3 served as a negative control. As can be seen in Fig. 4A, ZBP-89 induced repression of PMA-induced CD11b in a dose-dependent manner, reaching 75% at the maximal amount of transfected DNA tested (Fig. 4A). The effect was also observed in U937 in which pCMVSPORT3-ZBP-89 was co-transfected with a reporter plasmid containing the CD11b promoter fused to the luciferase gene (Fig. 4B, left panel); ZBP-89 decreased PMA-induced wild-type CD11b gene expression by 55%; the reduced repression seen may be a reflection of the lower amount of plasmid DNA allowable in the co-transfection studies. The effect of repression by ZBP-89 was specific since no such repression took place in U937 cells co-transfected with pCMVSPORT3-ZBP-89 and CD11b-M4a promoter plasmids (Fig. 4B, right panel).
Loss of repression of the M4a-CD11b promoter by endogenous ZBP-89. It remains possible that exogenous ZBP-89 overexpressed in differentiating U937 cells may act indirectly in repressing the CD11b promoter. In order to establish whether endogenous ZBP-89 also acts as a repressor, we compared the promoter activities of the CD11b-wt and CD11b-M4a in PMA-induced U937 cells (Fig. 4C). Expression of the mutant CD11b-M4a reporter in differentiating U937 increased to 160% of the wild-type reporter (Fig. 4C), reflecting loss of repression by endogenous ZBP-89. Thus the level of CD11b gene expression in differentiating U937 cells reflects the activity of endogenous ZBP-89.

Reconstitution of ZBP-89 repression in a heterologous expression system. Expression of CD11b is normally restricted to differentiating myeloid cells. The CD11b-negative Drosophila Schneider L2 cell line is deficient in Sp1-related proteins 24,30,31, but can express a CD11b promoter-directed reporter when supplemented with Sp1 32. We found this cell line to be also lacking ZBP-89 by western blotting as well as by EMSA (data not shown). We cotransfected Schneider L2 cells with 0.2 µg of the construct pPacSp1 (which expresses Sp1), together with increasing amounts of pPacZBP-89 expressing ZBP-89, along with either CD11b-wt or CD11b-M4a-luciferase plasmids. In the presence of Sp1, ZBP-89 repressed wild-type CD11b promoter activity by up to 50% (Fig. 4D). In contrast, CD11b-M4a promoter activity was not affected by expression of ZBP-89 (Fig. 4E). Expression of ZBP-89 alone without Sp1 did not activate the CD11b promoter (data not shown). Therefore, repression of CD11b by ZBP-89 can also be achieved in a non myeloid cellular context, and independently of the confounding effects of treatment with phorbol esters.

Expression of CD11b does not correlate with endogenous ZBP89 protein during phorbol ester-induced differentiation of U937

We next determined the correlation between the endogenous levels of CD11b mRNA and ZBP-89 protein in U937 cells differentiated by treatment with PMA for 72 hrs, a treatment which alters homotypic adhesion in these cells (Fig. 5A). Both ZBP-89 protein and CD11b mRNA increased in parallel during the differentiation of PMA-treated U937 into monocyte-like cells (Fig.5B). Thus whereas over-expressed ZBP-89 represses the CD11b gene, we find no inverse correlation between endogenous ZBP-89 and expression of the CD11b gene in this system.
These data argue against a role for endogenous ZBP-89 in repressing CD11b gene during the early stage of monocytic differentiation. Since over-expression of ZBP-89 represses the CD11b promoter-driven reporter in these cells, qualitative and/or quantitative changes in ZBP-89 protein levels together with the potential inaccessibility of the native CD11b promoter to ZBP-89 during early monopoiesis may account for the lack of correlation between endogenous CD11b mRNA and ZBP-89 protein at this stage.

**Down-regulation of CD11b during monocytes to macrophage differentiation correlates with ZBP-89 protein expression.** As the level of CD11b protein is known to drop as blood monocytes differentiate into macrophages, we next assessed the impact of ZBP89 on CD11b expression in these cells. CD11b mRNA and ZBP-89 protein levels were determined during the *in vitro* differentiation of normal human peripheral blood monocytes into macrophages. Normal blood monocytes assumed the typical features of macrophages after 12 days in culture (Fig. 6A, compare left and right panels) as described. As shown in Fig. 6B, CD11b mRNA dropped significantly (by ~75%) in macrophages compared to monocytes. Importantly, this reduction coincided with an increase of protein expression of ZBP-89 (Fig. 6C, compare lanes 3, 4) that has an identical mobility to the form induced in differentiating U937 cells (Fig. 6C, lanes 1-2; compare lanes 2 and 4). These data suggest that ZBP-89 exerts its repressor activity on CD11b during the late stages of monocytic differentiation.

**DISCUSSION**

The major finding in this paper is that ZBP-89 is a repressor of the CD11b gene. Repression is dose-dependent, sequence-specific, and is demonstrable in non-myeloid insect cells. In the U937 model of early stage monocytic differentiation, no inverse correlation was observed between ZBP-89 levels and CD11b gene expression. However, in the model of late stage monocyte to macrophage differentiation, such as inverse correlation was readily detected. Consequently, these findings suggest that the effect of ZBP-89 on CD11b gene expression occurs primarily at the later rather than the early stages of monocytic differentiation. This observation may underlie the observed drop in the levels of CD11b as monocytes differentiate into tissue macrophages *in vivo*. 
ZBP-89 is a 794 amino acid ubiquitously expressed factor\textsuperscript{26,34}, originally cloned in a truncated form, htβ, that mediates mild transactivation of the human T cell receptor gene\textsuperscript{27}. ZBP-89 belongs to the Krüppel zinc finger family\textsuperscript{35}; other members include WT1, GATA1, Sp1, and Egr1. A breakpoint in the chromosomal region housing ZBP-89 is associated with hematologic malignancy\textsuperscript{23,36} and ZBP-89 is known to stabilize p53 resulting in the arrest of cell growth\textsuperscript{37}. In fact, many of the target genes of ZBP-89 are regulated by mitogens or developmental signals\textsuperscript{26,30,34,38-40}. The consensus binding site for ZBP-89 is also found in the promoters of the genes encoding PU.1\textsuperscript{41} and CEBP–α\textsuperscript{42} which play important roles in myeloid differentiation. Thus ZBP-89 appears to be involved in regulation of cellular proliferation as well as differentiation.

The structural basis for ZBP-89-mediated repression of the CD11b gene remains to be determined. Five to ten percent of zinc finger DNA binding motifs share a conserved 120 amino acid domain termed the POZ or BTB domain\textsuperscript{43}. Many POZ domain-containing proteins are transcriptional repressors, although some, such as the GAGA factor, can counteract repression by chromatin remodeling\textsuperscript{44}. Charged, alanine-rich, alanine/proline, and alanine/glutamine rich motifs have also been found in transcriptional repressor domains\textsuperscript{45-47}. However, homology searches reveal that ZBP-89 contains no such domains. A previous study demonstrated that a transferable region in ZBP-89 spanning amino acids 136 to 184 repressed β enolase gene transcription during embryonic muscle cell differentiation\textsuperscript{34}. However, the expression of the truncated form of ZBP-89 called p25 which encompasses this repressor region, did not repress CD11b gene activation (data not shown). This suggests that the carboxyl-terminal domain of ZBP-89 may contribute to CD11b transcriptional repression. Since this segment contains no known repressor domains, repression may be mediated by a yet to be discovered protein domain within this region.

Functionally, four general mechanisms may give rise to selective transcriptional repression of genes. First, a repressor protein can mask a transcriptional activator domain. Second, the repressor may block interaction of an activator with other components of the transcription machinery\textsuperscript{48}; third, it can displace an activator from its DNA element by competition\textsuperscript{49}, and fourth, the DNA element itself may exert allostERIC effects on transcriptional regulators, such that regulators may activate transcription in the context of one gene, yet repress transcription in another\textsuperscript{50,51}. In regulating expression of the β enolase and gastrin genes, ZBP-89
acts as a bona-fide transcriptional repressor; it inhibits basal as well as activated transcription, an effect not mediated by squelching \(^{26,34}\). Recent data have shown that ZBP-89, can compete with Sp1 in regulating expression of the vimentin gene \(^{39}\). Sp1 is also required in CD11b transcriptional activation \(^{32}\), suggesting that some of the repressor activity may be mediated through this mechanism. ZBP-89 and Sp1 do not, however, cross compete in EMSA analyses (lanes 9, 10 in Fig. 2C), suggesting that interaction may take place at the protein-protein level. It is possible that the promoter context and cell type could also contribute to ZBP-89 repressor activity. In this situation, ZBP-89 might act through interaction with an adjacent transcriptional activator, modulating its functional state or local concentration.

The ZBP-89 element overlaps with but is distinct from that of MS-2, a single-stranded DNA binding factor of the CD11b gene whose identity remains to be determined \(^{18}\) (Fig. 3). ZBP-89 may repress by competing with MS-2 for DNA binding \(^{48}\). Supporting this notion is our observation that the activity of the mutant promoter CD11b-M4a is higher than that of CD11b-wt (Fig. 4B). This increase may also be due to the blocked binding of endogenous ZBP-89. However, the binding of adjacent proteins such as MS-2 may also affect ZBP-89 activity by modifying its association with co-modulators or the basal transcription machinery. There are examples of double- and single-stranded DNA binding proteins binding to overlapping DNA segments, and thus regulating transcription. Within the vascular smooth muscle \(\alpha\) actin gene, TEF-1 (Transcriptional Enhancer Factor-1) binds to double-stranded DNA, whereas Pur\(\alpha\), Pur\(\beta\), and MSY-1 bind to single-stranded DNA and act as repressors \(^{52-54}\). Similarly a 42-base pair regulatory element in the 5’-flanking region of the L1 transcript of the murine growth hormone receptor gene interacts both with MSY-1 and the double stranded DNA binding protein NF-1 \(^{55}\). In the proximal promoter of the NMDAR1 gene, a GC-rich element contains the binding sites for the double-stranded DNA binding proteins, Egr-1 and Sp1, and both sense and antisense strands of this element also form complexes with yet to be identified factors \(^{56}\). Considering that the DNA macromolecule is a dynamic structure capable of assuming a single-stranded conformation under certain conditions, changes in the secondary structure of the CD11b promoter may be critical in controlling CD11b gene expression.

The CD11b/CD18 heterodimer is expressed on peripheral blood monocytes, neutrophilic polymorphonuclear leukocytes (PMN), and natural killer cells, but is generally expressed at reduced amounts on tissue macrophages such as Kupffer cells, bone marrow stromal...
macrophages, and alveolar macrophages\textsuperscript{10,13,57-59}. The mechanism(s) that underlie this reduction is not known. \textit{In vitro} differentiation of freshly isolated human monocytes into macrophages, was accompanied by a significant drop in CD11b mRNA and induction of the ZBP-89 protein. These findings suggest that ZBP-89 acts at a later stage in monocytic differentiation as monocytes further transform into macrophages. CD11b/CD18 is known to be important not only in leukocyte recruitment into tissues but also in the many pro-inflammatory adhesion-dependent functions mediated by these cells in tissues. Quantitative as well as qualitative down-regulation of this receptor once monocytes transmigrate into tissues as part of their normal differentiation and maturation may be necessary to avert harmful inflammation. The data presented in this work suggest that ZBP-89 may act at the transcriptional level to elicit such a protective effect during the resolution of an inflammatory response.

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REFERENCES


FIGURE LEGENDS

Figure 1: (A) Nucleotide sequence of the proximal human CD11b promoter. The major transcription initiation site (arrow) is numbered +1. The GC-rich region is in bold and underlined. (B) Nucleotide sequence of GC (wild-type) and GCm (mutant) oligonucleotides (sense strand). Mutations are indicated in bold underlined letters. (C) Schematic of the primary structure of human ZBP-89 and p25 (see text for details). The vertical numbers indicate the amino acid numbers. The acidic domain, two basic domains, four zinc finger domains, and a PEST sequence are shown as a shaded bar, solid bars, hatched bars, and a dotted bar, respectively. A single nucleotide insertion in p25 results in an in-frame premature termination codon after amino acid 284 of ZBP-89.

Figure 2: EMSAs showing the DNA binding activity of recombinant GST-Z (A), GST-ZBP-89 (B) and endogenous ZBP-89 (C).

(A) Lane 1, radiolabeled GC oligonucleotide alone; Lanes 2 and 3, labeled GC in the presence of GST or GST-Z, respectively. In this and the other figures, the arrow indicates the position of the complex. This complex was competed with the unlabelled GC oligonucleotide (lane 4), or oligonucleotide htβ (lane 5). Lane 6 shows the lack of competition by a consensus Sp1 oligonucleotide.

(B) Lane 1, radiolabeled GC alone; Lanes 2 and 3, labeled GC in the presence of GST or GST-ZBP-89 fusion protein, respectively. The resulting complex was competed with unlabelled GC (lane 4), or with htβ (lane 5); Lanes 6 and 7 show the effect of anti-ZBP-89 antibody (lane 6) and pre-immune serum (lane 7), respectively, on the ZBP-89 complex.

(C) Lane 1, radiolabeled GC alone. In lanes 2 and 3, binding reactions containing uninduced U937 nuclear extract. Lanes 4 to 11 show binding reactions containing PMA-induced U937 nuclear extract. ZBP-89 binding to DNA was competed with a 250 fold molar excess of unlabeled GC (lanes 3 and 5) or htβ (lane 6). A consensus Oct-1 oligonucleotide (lane 7) and a consensus Sp1 oligonucleotide were ineffective (lane 10) in competition. Lanes 8, 9 and 11 represent binding reactions pre-incubated with anti-ZBP-89 antibody (lane 8), pre-immune sera (lane 9), and anti-Sp1 antibody (lane 11), respectively.

Figure 3: Characterization of the ZBP-89 binding site within the CD11b gene.
(A) Sequence of the double- or single-stranded oligonucleotides used in EMSA are shown. Mutations incorporated into the GC1 oligonucleotide are shown in lowercase letters, whereas wild-type nucleotides are represented by dashed lines. (B) Lane 1, radiolabeled GC1; lanes 2 to 9, binding reaction with GST-Z competed with the following oligonucleotides: unlabeled GC1 (lane 3), M1 (lane 4), M2 (lane 5), M3 (lane 6), M4 (lane 7), M5 (lane 8), M6 (lane 9). (C) Same as in (B) except that GST-ZBP-89 was used instead of GST-Z. (D) Competition analysis of GC1 binding to GST-Z using mutant oligonucleotides. Lane 1, radiolabeled GC1 alone. Competition by the M4b-e oligonucleotides (lanes 6, 7, 8, and 9) was approximately equivalent to that of WT (lane 3), whereas competition by M4 (lane 4) and M4a (lane 5) was minimal. (E) Binding of the purine-rich single-stranded GC1/for oligonucleotide to MS-2. Lane 1, radiolabeled GC1/for single-stranded oligonucleotide alone; lane 2, the MS-2 complex produced by mixing the labeled GC1/for oligonucleotide with nuclear extract from U937 cells treated with PMA for 24 hours; lanes 3 and 4, competition in the presence of unlabeled GC1/for (lane 3) or M4a/for (lane 4). Lanes 5 to 7, lack of binding of recombinant GST-ZBP-89 to radiolabeled GC1/for in the absence (lane 5) or presence of unlabeled GC1/for (lane 6) or unlabeled M4a/for (lane 7).

Figure 4: Transcriptional repression of CD11b by overexpression of ZBP-89 in differentiating U937 cells and in Drosophila SL2 cells.

(A) Dose-dependent repression of CD11b by ZBP-89 in stable U937 cells in which CD11b-luciferase gene is incorporated in the genome. The various concentrations of pPacZBP-89 are indicated. Luciferase activity is normalized against β-galactosidase activity, and each histogram represents the mean ± the standard deviation of three independent experiments. In a typical experiment, the CD11b luciferase activity without ZBP-89 was 41,771 relative light units (RLU)(normalized to 100%), compared to 22 RLU in stable cell lines lacking the exogenous CD11b promoter.

(B) Histograms comparing the trans-repression activity of ZBP-89 on CD11b-wt and mutant CD11b-M4a gene expression. Each transfection was performed six times. Luciferase activity is normalized against β-galactosidase activity, and the standard deviation from the mean is presented. The fold above background (conferred by the negative control plasmid pATLuc) of CD11b-wt luciferase activity without ZBP-89 is 29.32± 2.36 (n=6) and this was considered as 100%.
(C) Derepression of CD11b promoter activity by blocking endogenous ZBP-89 binding. U937 cells were transfected with pALbWt or pALbM4a then treated for 16 hours with PMA prior to harvesting. The level of Wt luciferase reporter gene activity, normalized against β-galactosidase activity to correct transfection efficiency, is expressed relative to that conferred by the negative control plasmid pATLuc. It represented 143.85±8.5 (n=3) fold above background and equated to 100%.

(D) Transfection assays in Drosophila SL2 cells were performed using 1 µg of the reporter construct pALbWt, 0.2 µg of pPacSp1 expressing Sp1, 0.1 µg of PIE3LacZ expressing β-galactosidase, and an increasing amount of pPacZBP-89 (expressing ZBP-89) from 0 to 1 µg. The various concentrations of pPacZBP-89 are indicated. The final DNA concentration in all transfections was adjusted to 2.6 µg by adding the empty vector pPacO. Sp1-driven CD11b-wt promoter activity was calculated as a ratio of that of CD11b-wt promoter activity without Sp1. In the absence of ZBP-89 expression, the calculated level of Sp1-driven transcription (81.1±12.2 above background, n=3) was considered as 100%.

(E) The same experiment was performed as in (C) using pALbM4a instead of pALbWt (actual values of Sp-1-driven transcription were 91±10.68, n=3, above background).

Figure 5: Expression profiles of endogenous ZBP-89 protein and mRNA and CD11b mRNA during differentiation of U937.

(A) Morphology of U937 cells without (left panel) and with PMA-treatment for 72 hrs (right panel). The later cells form readily visible clusters (40x magnification).

(B) Real-time RT-PCR analysis of CD11b and ZBP-89 during U937 cell differentiation. The experiment was performed using total RNA isolated from U937 cells treated with PMA (100 ng/ml) for 0, 24, 48, and 72 h. The ratios, CD11b/GAPDH or ZBP-89/GAPDH in untreated U937 cells (0 h) were given a value of 1, and the ratios at 24, 48, and 72 h are displayed as fold above 1. (Inset) Thirty micrograms of nuclear extract from U937 cells treated with PMA (100 ng/ml) for 0 (lane 1), 24 (lane 2), 48 h (lane 3), and 72 h (lane 4) were resolved by 6 % SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and analyzed with an anti-ZBP-89 antibody followed by chemiluminescence. ZBP-89 protein is indicated with an arrow.
Figure 6: Inverse correlation between endogenous ZBP-89 protein and CD11b mRNA during differentiation of monocytes/macrophages.

(A) Morphology of human monocytes during differentiation and maturation into macrophages is shown at Day 1 (left panel) and Day 12 (right panel). Representative fields are shown using light microscopy (40x magnification).

(B) Real-time RT-PCR analysis of CD11b during monocyte/macrophage differentiation. Total RNA isolated from monocytes/macrophages differentiated in vitro for 1 or 12 days were reverse transcribed to make cDNA. Quantitative PCR was carried out using the TaqMan system and an ABI Prism 7700 instrument (see Materials and Methods). The copy number of CD11b is normalized to the copy number of GAPDH and expressed as CD11b/GAPDH. The CD11b/GAPDH ratio in monocytes at Day 1 was given a value of 1, and the ratio at Day 12 is shown as a relative value to 1. Each histogram represents the mean ± the standard deviation of three independent experiments.

(C) Western blot analysis showing ZBP-89 protein expression during differentiation of U937 and monocytes/macrophages. Five micrograms of nuclear extract from U937 cells treated with PMA for 0 h (lane 1) or 72 h (lane 2), and thirty micrograms of total protein lysate from monocytes/macrophages differentiated for 1 (lane 3) or 12 days (lane 4) were resolved by 6% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and detected with an anti-ZBP-89 antibody followed by chemiluminescence. ZBP-89 (arrow) migrates as a double band with apparent mobility of ~120kD, consistent with similar analyses by other investigators. 

M.W, molecular weight markers (Gibco-BRL).
Fig. 1

Fig. 2
Fig. 5

Fig. 6
The zinc finger transcription factor ZBP-89 is a repressor of the human β2 integrin CD11b gene

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