Binding of Two Nuclear Complexes to a Novel Regulatory Element within the Human S100A9 Promoter Drives the S100A9 Gene Expression*

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S100A9, also referred to as MRPI4, is a calcium-binding protein whose expression is tightly regulated during differentiation of myeloid cells. The present study was performed to study the cell type- and differentiation-specific transcriptional regulation of the S100A9 gene. Analysis of the S100A9 promoter in MonoMac-6 cells revealed evidence for a novel regulatory region from position −400 to approximately −374 bp, termed myeloid-related protein regulatory element (MRE). MRE deletion resulted in a 5.2-fold reduction of promoter activity. By electrophoretic mobility shift analysis two nuclear complexes binding to this region were identified and referred to as MRE-binding complex A (MbcA) and MRE-binding complex B (MbcB). By mutagenesis the MRE-binding motif could be narrowed to a 12-bp region. The relevance of MRE is deduced from the observations that the formation of either MRE-binding complex A or MRE-binding complex B strongly correlated with S100A9 gene expression in a cell type-specific, activation- and differentiation-dependent manner. Moreover, DNA affinity chromatography and Western blot studies indicate that a Kruppel-related zinc finger protein and the transcriptional intermediary factor 1β (TIF1β) are involved in an MRE-binding complex, thereby regulating the S100A9 gene expression.

Mononuclear phagocytes play a pivotal role in host defense to pathogens, wound healing, angiogenesis, and various types of chronic inflammation, e.g. granulomatous reaction, fibrosis, and arteriosclerosis. They originate from hematopoietic precursor cells in the bone marrow, and their lineage differentiation is coordinated by the closely regulated expression of cytokines, colony-stimulating factors, receptors, and transcription factors (for review, see Refs. 1–5). To study lineage differentiation several cell surface antigens, adhesion molecules (e.g. CD11b, CD18, CD64), and primary granule proteins (e.g. myeloperoxidase and neutrophil elastase) have been used as myeloid stage-specific markers. Additional interesting markers of myeloid differentiation represent the two myeloid-related proteins MRP8 (S100A8) and MRP14 (S100A9). Studies using human leukemia models of myelomonocytic development indicate that S100A8 and S100A9 expression is restricted to a specific stage of myeloid differentiation. Moreover, their expression is also regulated in mature blood cells because they are expressed in circulating neutrophils and monocytes but not in mature tissue macrophages (6–7).

S100A8 and S100A9 belong to the S100 family of calcium-binding proteins (for review, see Refs. 8 and 9). Although the exact functions of both proteins remain unknown, they form heteromeric complexes and bind polyunsaturated fatty acids in a calcium-dependent manner (10–13), indicating that the complex may be involved in the intra- and transcellular arachidonic acid metabolism. They are used as marker antigens for activated or recruited phagocytes because the first cells that migrate to inflammatory lesions express S100A8 and S100A9 (7). These findings together with the presence of enhanced S100A8/S100A9 levels in sera from patients suffering from a number of inflammatory disorders (14–16) have led to the assumption that S100A8 and S100A9 affect leukocyte trafficking and display a propagating role in inflammatory responses.

The molecular mechanisms underlying the cell type-specific expression of S100A9 are unknown. The human S100A9 gene has been cloned and sequenced, and an upstream 1-kb fragment of its promoter was shown to drive the gene expression in myeloid cells (6). A number of distinct regulatory regions upstream of the transcription initiation site have been demonstrated to either activate or repress promoter activity in a differentiation and tissue/cell-specific manner. For example, two still unidentified factors were found to bind to the upstream regions of S100A9 gene during differentiation of HL-60 cells into monocyte-like cells; one adjacent to the TATA box and another in the region between −400 and −150 (17). Another study revealed a CCAAT/enhancer-binding protein (C/EBP)1-binding motif located at position −81 upstream of the S100A9 gene. Both C/EBPα and -β bind to this motif in a myeloid/macrophage differentiation-dependent manner (18). C/EBP was shown to be sufficient alone to drive S100A9 expression in

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1 The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift analysis; KRAB, Kruppel-associated box; MRE, MRPI regulatory element; Mbc, MRE-binding complex; MRP, myeloid-related protein; TMβ, transcriptionsal intermediary factor 1β; RTTC, fluoresecin isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; TK, thymidine kinase; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PMA, phorbol 12-myristate 13-acetate.
otherwise negative cells. C/EBP up-regulation is antagonized by myb, a transcription factor active in differentiated myeloid/monocytic cells (19). The presence of distinct epithelial and myeloid-specific regulatory regions upstream of the transcription initiation site has been demonstrated by detailed deletion analysis (20). Besides the very specific action of particular upstream DNA elements, the S100A9 gene contains a potent enhancer, which is harbored within positions 153–361 of its first intron (21). The functional relevance of this enhancer in S100A9 expression is supported by its conservation in human and murine S100A9 genes at almost identical positions.

The present study was performed to identify regulatory elements within the human S100A9 promoter that drive the cell type-specific and differentiation-dependent protein expression. We found a novel 27-bp region referred to as MRE regulatory element (MRE), which exhibits these characteristics. The functional relevance of this DNA regulatory region is shown by (i) the binding of two nuclear factors to the MRE by electrophoretic mobility shift analysis, and (ii) the finding that the formation of the nuclear protein complexes closely correlates with the myeloid-specific expression of the S100A9 gene. Further extensive investigations provide strong evidence that a complex of a Kruppel-related zinc finger protein and the transcriptional intermediary factor 1β (TIF1β) are involved in the regulation of the myeloid-specific S100A9 gene expression.

**EXPERIMENTAL PROCEDURES**

Antibodies—Fluorescein isothiocyanate (FITC)-conjugated anti-human CD38 (clone T16), phycoerythrin (PE)-conjugated anti-human CD33 (clone D3HL60.251), and FITC-conjugated anti-human CD15 (clone 80H5) were purchased from Beckman Coulter (Unterschleisheim, Germany). Allophycocyanin (APC)-conjugated anti-human CD34 (clone 5G12), PE-conjugated anti-human CD11b (clone D12), and APC-conjugated anti-human CD14 (clone MoP9) were from BD Pharmingen. Biotin-conjugated anti-human S100A9 (clone S22.5) was from Dianova (Hamburg, Germany). Mouse monoclonal C/EBPβ antibody (H7) was from Santa Cruz Biotechnology, and the anti-chicken C/EBPα and C/EBPβ antibodies were kind gifts of Dr. Karl-Heinz Kemppnaeur (Institut of Biochemistry, University of Muenster, Muenster, Germany). The mouse monoclonal anti-TIF1β antibody was a kind gift of Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire Illkirch, Cedex, France).

Four-color Flow Cytometry—To investigate S100A9 gene expression during normal hematopoiesis, bone marrow samples obtained to rule out systemic malignant disease (n = 4), to exclude bone marrow involvement in patients with non-Hodgkin lymphoma or sarcoma (n = 3), or to confirm continuous remission following chemotherapy for acute leukemia (n = 11) were analyzed by four-color flow cytometry. To analyze S100A9 expression at different stages of myeloid differentiation we used the antibody combinations of anti-CD38-FITC/anti-CD33-PE/anti-S100A9/anti-CD34-APC (immature cells) and anti-CD15-FITC/anti-S100A9/anti-CD34-APC (mature myeloid cells).

Approximately 3–5 × 10⁶ non-separated bone marrow cells were first incubated for 15 min at room temperature with saturating amounts of the antibodies binding to myeloid differentiation markers on the cell surface. Subsequently, the cell membranes were treated with the Fix & Perm cell permeabilization kit (An-der-Grub, Kaumberg, Austria) according to the manufacturer’s protocol to measure expression of intracellular S100A9. The cells were then incubated with biotinylated anti-human S100A9 antibody for 15 min at room temperature followed by incubation with streptavidin-PerCP. The cells were washed twice with phosphate buffered saline. Data acquisition and analysis was performed on a 2-laser (488 and 633 nm) FACSCalibur (BD Pharmingen) using CellQuest and Paint-A-Gate-Pro software (BD Pharmingen). For time delay calibration, APC beads (Calibrite APC, BD Pharmingen) were applied according to the manufacturer’s instructions. Isotype controls were included in all analyses.

Cell Culture—The human monocytic cell line MonoMac-6 (DSM ACC 124) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin/streptomycin, and 1 mmol/liter glutamine, 1% non-essential amino acids, 1 mmol/liter pyruvate, and 9 μg/ml bovine insulin (Sigma). The human histiocyte lymphoma cell line U937 (ACC 5) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The human B-lymphocytic cell line Raji, the fibroblast
kinase promoter (CLONTECH) was inserted into the pSP72 vector (Promega) to generate pTKC (21). Additionally, the region from −400 to −378 bp of the S100A9-promoter was cloned 5′ to the pTKC construct (MRE-pTKC).

Transfections and CAT Assay—Transfection was performed as described by Melkonyan et al. (23) with minor modifications.

Nuclear Extraction and Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared essentially as described (24). For the EMSA reaction, a double-stranded oligonucleotide encompassing nucleotides −400 to −357 of the S100A9 promoter was used. The sense oligonucleotide CAGACCATCCTTGAGACTAAAGGAAGGGGAAGATCCTGGCAGTCCATG and its antisense strand CATGGCGATCTCCGTCTGCCCTTTGTTGGACTAAAAGGAAGGGGCACTGCCATG were annealed and end-labeled by thyroxine polynucleotide kinase and [γ-32P]ATP (Hartmann Analytic, Braunschweig, Germany). EMSAs were performed with nuclear extracts as follows: nuclear protein (50 μg) was mixed with 3 μg of sheared genomic salmon sperm DNA and 100,000 cpm of the labeled probe (−1 ng) in EMSA buffer (20 mmol/liter Hepes, pH 7.5, 1 mmol/liter MgCl2, 75 mmol/liter KCl, 1 mmol/liter dithiothreitol, 0.018% (v/v) Nonidet P-40) in a total volume of 36 μl. In the competition

FIG. 2. CAT reporter gene analysis of the human S100A9 promoter. Deletion constructs of the S100A9 promoter 5′ flanking region extending from position −1000 to −153 bp fused to the CAT reporter gene were generated and transfected in MonoMac-6 cells as described under “Experimental Procedures.” A schematic representation of each CAT reporter construct is shown on the left. The expression level (rel. CAT activity) of the basic promoter construct pHH1.0T was set as 100%. The values are the mean ± S.E. from at least twelve independent transfections.

FIG. 3. CAT reporter gene analysis of the pH763TΔ-400(−356) promoter construct. The promoter construct pH763TΔ-400(−356) was generated as described under “Experimental Procedures” by deletion from the pH763T construct. A schematic representation of some other CAT reporter deletion construct is shown on the left. The expression level (rel. CAT activity) of the promoter construct pH763T was set as 100%. Results represent a minimum of three separate experiments, each done in duplicate.
experiments, a 100-fold molar excess of unlabelled competitor oligonucleotide was added to the mixture prior to the addition of nuclear protein extracts. This mixture was allowed to incubate for 60 min at 4 °C. Samples were mixed with 12 µl of sample buffer (50% (w/v) sucrose, 0.5 × Tris borate EDTA buffer (where 1 × Tris borate EDTA buffer is 90 mmol/liter Tris borate, 2 mmol/liter EDTA, pH 8.0) and then run on a non-denaturing 5% polyacrylamide gel in 0.25 × Tris borate EDTA buffer. The gels were dried and exposed to Kodak XAR-5 x-ray film.

**DNA Affinity Chromatography**—The biotinylated MRE oligonucleotides were synthesized by Applied Biosystems Oligo Factory (Weiterstadt, Germany). For DNA affinity purification, 0.5–2 mg of nuclear protein was incubated with 400 pmol double-stranded, biotinylated MRE oligonucleotide coupled to 500-µg magnetic beads via streptavidin (Dynal, Hamburg, Germany) and 100 µg of sheared genomic salmon sperm DNA in 1000 µl of EMSA-buffer. After rotating the samples for 1 h at 4 °C, the proteins bound to MRE beads were eluted with EMSA-buffer supplemented with 0, 100, 250, and 1,000 mM NaCl. Aliquots of the different fractions were analyzed by EMSA and subjected to SDS-PAGE followed by Western blot analysis using standard protocols.

**Northern Blotting**—Total cellular RNA was extracted from cells by the SDS-citric acid method of Dreier et al. (25). The Northern blot analysis was performed using standard protocols.

**Mass Spectrometry**—After DNA affinity chromatography the 1 mM NaCl eluate displaying DNA-binding activity was subjected to SDS-PAGE, and the proteins were visualized by silver staining. The proteins were excised from the gel according to Shevchenko et al. (26) and Zhang et al. (27). Briefly, the gel slices were shrunk in acetonitrile, dried, and re-swollen in 20 µl of 50 mM NH₄HCO₃ containing 400 ng of trypsin. Excess trypsin solution was removed, sufficient buffer was added to cover the gel slices, and digestion was carried out at 37 °C overnight. Digestion was stopped by adding 5–10 µl of 100% acetic acid, and the supernatant was removed. The gel slices were extracted twice with 70 µl of acetonitrile, and the supernatants were pooled and lyophilized. The lyophilized peptides were dissolved in 7 µl of 0.1% aqueous trifluoroacetic acid followed by hydrophobic chromatography using ZipTips (Millipore, Bedford, MA). The peptides were eluted with 7 µl of 70% acetonitrile. Samples of 10 µg of α-cyano were washed with acetonitrile and dissolved in 1 ml of 50/50 (v/v) acetonitrile/ethanol containing 1% of 0.1% aqueous trifluoroacetic acid. Then, 0.7 µl of this matrix preparation was spotted onto the target followed by the same volume of sample. Both solutions were directly mixed on the target. MALDI-mass spectrometry was carried out using a TofSpec-2E instrument (Micromass, Manchester, UK). Digests were run in positive ion reflection mode using a matrix suppression of 500. Masses were externally calibrated and internally corrected using either the lock mass option of the

**RESULTS AND DISCUSSION**

**Expression of S100A9 in Normal Bone Marrow**—The expression of S100A9 appears to be restricted to a specific stage of myeloid differentiation because the protein is expressed in circulating neutrophils and monocytes but not in mature tissue macrophages. In peripheral blood monocytes its expression is down-regulated during maturation to macrophages (6, 7). In
the past, several investigators had used various human promyelocytic leukemia cell lines, such as HL-60, U937, and Mono-Mac-6 cells, as cellular models to study differentiation-dependent S100A9 gene expression (6, 18, 28). However, less is known about S100A9 gene expression during the differentiation of human hematopoietic cells. Therefore, we investigated S100A9 expression during normal hematopoiesis using four-color flow cytometry of human bone marrow. Immature cells were distinguished from more mature myeloid cells by staining for either CD38, CD33, and CD34 (immature cells) or CD15, CD11b, and CD14 (mature myeloid cells) that reflect different stages of myeloid differentiation (29, 30).

Primitive hematopoietic cells express the progenitor antigen CD34 but lack expression of CD38 or any lineage-specific markers. With differentiation toward the myeloid lineage, immature progenitor cells acquire expression of the myeloid antigen CD33 followed by down-regulation of CD34. As shown in Fig. 1, primitive, uncommitted CD34−CD38−CD33− (data not shown) and early myeloid CD34+CD33+ progenitor cells lack expression of S100A9 (Fig. 1, green events, upper right dot blot). During neutrophil maturation, S100A9 is only detectable in cells that already express CD15, but S100A9 expression slightly precedes and then correlates with the up-regulation of CD11b (Fig. 1, blue cells, lower left and right dot blots). Mature CD15+CD16−CD33− neutrophils are also S100A9-positive (data not shown). Other lineages, including CD19+ B-cells, CD3 T-cells, and glycophorin A-positive erythroid cells, did not express S100A9 (data not shown). The acquisition of CD15 (Lewis x antigen) marks the transition from myeloblasts to promyelocytes and the up-regulation of the αM-integrin CD11b differentiation of promyelocytes to myelocytes (29). Hence, the expression of S100A9 is first initiated during maturation of promyelocytes toward myelocytes and then maintained up to the level of mature neutrophils.

Within the monocytic pathway, up-regulation of CD11b precedes acquisition of intermediate expression of CD15. The up-regulation of S100A9 correlates with the expression of CD11b (Fig. 1, red events, lower left dot blot) and CD14 (data not shown).

In contrast to neutrophil development, in monocytes S100A9 expression precedes up-regulation of CD15 (Fig. 1, red events, lower right dot blot). Thus, during both neutrophil and monocyte development the up-regulation of S100A9 correlates with the acquisition of CD11b. However, due to the differential expression of CD15 in both cell lineages, S100A9 expression precedes acquisition of CD15 in the monocytic pathway and succeeds CD15 expression during neutrophil maturation. This flow cytometric characterization shows for the first time that S100A9 expression is tightly regulated in a differentiation- and lineagespecific manner within the hematopoietic system. Note that our study clearly indicates that the S100A9 protein also represents a myeloid stage-specific marker.

**Identification of a Novel Regulatory Element within the S100A9 Promoter**—Due to the differentiation- and lineage-dependent expression of S100A9, we investigated the transcriptional regulation of the human S100A9 gene. MonoMac-6 cells were transfected with various S100A9 promoter constructs containing CAT as a reporter gene. The promoter constructs used in this functional analysis were a 1-kb fragment from position −1,000 extending to the transcriptional start site and various deletions of this fragment generated by exonuclease digestions as described under “Experimental Procedures.” This 1-kb proximal region has been found to be sufficient to drive lineage-specific expression of the S100A9 gene (6, 21). Therefore, the mean transcriptional activities of the deletion constructs were normalized to the mean activity of the 1-kb fragment. As shown in Fig. 2, regions of both positive and negative regulation were present within the 1-kb fragment. The construct pHH153T was sufficient to drive S100A9 gene expression in MonoMac-6 cells, indicating that this region might represent a minimal promoter. The construct pHH763T had a 1.7-fold increase in CAT activity compared with the basic promoter construct pHH1.0T. Subsequent deletions to position −400 bp (pHH533T, pHH520T, pHH432T, and pHH400T) only slightly affected the relative transcriptional activity. However, deletion of the region from −400 to −374 bp (promoter construct pHH374T) prevented significant CAT activity.
resulted in a remarkable reduction of the relative transcriptional activity compared with pHH763T (5.2-fold).

To verify the relevance of this 27-bp region for the transcriptional regulation of the S100A9 gene, we generated a promoter construct of pHH763T in which the 27-bp region was deleted. Fortunately, the S100A9 promoter adjacent to the region from −400 to −374 bp contained upstream and downstream recognition sites of SacI and NcoI. The pHH763T construct was digested with these two endonucleases as described under “Experimental Procedures,” and the resulting promoter construct pHH763TΔ-400(−356) was transiently transfected into MonoMac-6 cells. The mean activity of the deletion construct was normalized to the mean activity of the pHH763T construct. The pHH763TΔ-400(−356) construct exhibited a relative transcriptional activity similar to the pHH374T construct (Fig. 3), indicating that the 27-bp region may represent a strong positive regulatory element. We therefore termed this region MRP (myeloid-related protein) regulatory element (MRE).

Two Nuclear Factors Bind to MRE—Further functional analysis of the MRE was performed by cloning the MRE adjacent to the heterologous herpes simplex thymidine kinase promoter (pTKC). The promoter constructs MRE-pTKC as well as pTKC were transiently transfected into either MonoMac-6 or U937 cells (Fig. 4). The relative transcriptional activity of MRE-pTKC was slightly increased in MonoMac-6 compared with pTKC, indicating that MRE does not function as a context-independent enhancer element.

In the human monocytic cell line U937 the MRE-pTKC construct resulted in a strong decrease (7.2-fold) of the relative transcriptional activity compared with pTKC (Fig. 3B). To estimate this result we performed Northern blot analysis of U937 cells and found that U937 cells did not express S100A9 (data not shown). This result is in accordance with another study (18). Therefore, we suggested that MRE behaves as a strong negative regulatory element in S100A9-negative cells.

Next, EMSA was performed to analyze the nuclear proteins binding to the region from −400 to −357 bp, thereby using nuclear extracts prepared from MonoMac-6 and U937 cells. As shown in Fig. 5, in both nuclear extracts DNA-protein complexes were formed. The binding of proteins to MRE was specific, as an excess of non-labeled MRE oligonucleotide efficiently competed with the labeled probe in complex formation, whereas an unrelated double-stranded oligonucleotide probe did not compete. However, the resulting DNA-protein complexes in the nuclear extracts of either MonoMac-6 or U937 cells showed different electrophoretic mobilities. The complex exhibiting slower electrophoretic mobility is referred to as MRE-binding complex A (MbcA) and the other complex as MbcB, respectively.

For control, we performed analogous EMSA analysis with nuclear extracts of human blood monocytes (S100A9-positive) and human blood lymphocytes (S100A9-negative). The results were very similar for MonoMac-6 cells and the human blood monocytes, and DNA-protein complexes with similar electrophoretic mobilities were detected. Incubation of the probe with nuclear extracts from human blood lymphocytes resulted in the formation of a complex with a similar electrophoretic mobility to the complex formed in U937 cells (data not shown). Therefore, we assume a correlation between S100A9 protein expression and binding of the different protein complexes to the MRE driving the gene expression within these cells. Based on our findings, the complex MbcB presumably drives S100A9 gene expression, whereas MbcA might have a negative regulatory role.

Characterization of the Protein-binding Sequence in MRE—To characterize the binding site of the nuclear proteins, first various double-stranded DNA probes with overlapping and flanking sequences to the region from −400 to −357 bp were synthesized and used as cold competitors in EMSA analysis of nuclear extracts of monocytes. The results are summarized in Fig. 6. The competition analysis of MRE binding revealed that most likely the subregion from −400 to −379 bp upstream of the transcriptional start side contained the MRE. Similar results were obtained for MbcA with the nuclear extracts prepared from lymphocytes (data not shown).

We then used various mutant oligonucleotides as cold competitors (Fig. 6). The formation of MbcB was specifically competed by two oligonucleotides exhibiting mutations within either the −400 to −392 or the −376 to −368 region, whereas the double-stranded oligonucleotide exhibiting mutations within the −388 to −379 region did not compete for MbcB. Therefore, we concluded that the 12-bp element (5′-GTTGGACTAAAA-3′) was involved in the binding of both nuclear factors.

MRE Complex Formation Is Cell-specific and Activation- and Differentiation-dependent—Next, we performed EMSAs with nuclear extracts from various human cell lines to determine the cellular distribution and lineage specificity. As shown in Fig. 7, the formation of MbcB was largely restricted to myeloid cell lines as it was only present in MonoMac-6 cells and monocytes. The formation of MbcA was observed in the nuclear extracts of the B-lymphoid cell line Raji and in non-hematopoietic cells, such as HeLa epithelial carcinoma cell line, L132 fibroblasts, HaCaT cells, and keratinocytes. These data confirm our hypothesis that the formation of either MbcA or MbcB correlates with S100A9 expression.

Our finding that the formation of both complexes was observed in the nuclear extract of 7-day-cultured monocytes is of interest because S100A9 gene expression is down-regulated during maturation of human blood monocytes to macrophages (6, 7). Therefore, we investigated the time course of MRE complex formation during monocyte differentiation. Nuclear extracts from monocytes cultured for different time periods were subjected to EMSA analysis. As seen in Fig. 8, the formation of complex MbcB was decreased during cultivation, whereas the
formation of complex MbcA was induced at day 3 during the differentiation of monocytes to macrophage-like cells. In control experiments, we confirmed the down-regulation of S100A9 mRNA transcripts by Northern blot analysis (data not shown).

Short-term stimulation of monocytes by either the calcium ionophore A23187 or the phorbolester PMA also resulted in down-regulation of S100A9 mRNA expression (31, 32). Therefore, nuclear extracts of human blood monocytes stimulated by either A23187 or PMA were subjected to EMSA analysis. As shown in Fig. 9, the formation of MbcB was observed in the nuclear extract prepared from non-stimulated monocytes, whereas MbcA was found in the nuclear extracts of both calcium ionophore- and phorbolester-stimulated monocytes. For control, the nuclear extract of lymphocytes was also subjected to EMSA analysis. Through Northern blot analysis we confirmed that short-term incubation with these agents indeed resulted in down-regulation of S100A9 mRNA transcripts by Northern blot analysis (data not shown). The changes in EMSA banding patterns observed upon cell stimulation were not due to de novo protein synthesis as confirmed by the simultaneous addition of actinomycin D (Fig. 9).

Identification of Proteins Participating in Complex Formation—To identify the proteins participating in the complex formation we performed DNA affinity chromatography. Nuclear extracts of lymphocytes were subjected to affinity purification employing MRE oligonucleotides as affinity matrix. Nuclear proteins bound to MRE were eluted within a stepwise gradient with 0, 100, 250, and 1,000 mM NaCl and then analyzed by EMSA. Exclusively, the proteins of the 1M NaCl eluate displayed DNA-binding activity, indicating that they specifically interacted with the probe (Fig. 10A). This fraction was then subjected to SDS-PAGE, and the proteins were visualized by silver staining (Fig. 10B). The most prominent protein band with an apparent molecular mass of 45 kDa, which was exclusively detected in the 1 mM NaCl eluate, was excised from the gel, digested with trypsin, and subsequently analyzed by MALDI-TOF mass spectrometry. The analysis of the obtained spectra by using Swissprot and NCBI databases indicated that the 45-kDa protein resembled the human Kruppel-related zinc finger protein ZNF184 (GenBank™ accession no. GI:1769490).

Unfortunately, we did not succeed in analyzing the amino acid sequence of some peptides by electrospray ionization-mass spectrometry analysis. Therefore, the exact identity of the zinc finger protein remains unknown. Additional evidence for the Kruppel-related zinc finger protein in the nuclear complex formation was given by a detailed computer search using the TRANSFAC Matrix Table (34). This search indicated that MRE contains the putative binding site for Kruppel-related zinc finger proteins (data not shown). Interestingly, this binding site is identical to the motif identified by the competition studies (Fig. 6).

ZNF184 is a classical (C2H2) zinc finger with 19 highly conserved zinc finger motifs and a Kruppel-associated box (KRAB) domain at the C terminus of the protein. Kruppel-like zinc finger proteins, named after the Drosophila segmentation gene Kruppel (35), form one of the largest families of transcription factors with a broad expression pattern. For example, Abrink et al. (36) isolated 42 different cDNA clones for Kruppel-related zinc finger proteins that were expressed in the human monoblast cell line U937. The Kruppel-like zinc finger proteins can be divided into several subfamilies based on the number of zinc finger motifs, sequence homology between the zinc-fingers, and the presence of specific repressor and activation domains (37–40). The differential expression of several KRAB domain-containing zinc finger proteins during myeloid differentiation suggests that they could be important in this developmental process.

The KRAB domain, originally identified as a 75-amino acid sequence in numerous Kruppel-type zinc finger proteins, is a potent DNA-binding transcriptional repressor region that is believed to function through interaction with the TIF1β (37, 41–43). TIF1β has been identified as an early response gene for the differentiation of HL-60 cells to either macrophages or granulocytes, for the differentiation of U937 cells to macrophages, and for the differentiation of polymorphonuclear leu-

![Fig. 8. Formation of MRE complexes during monocyte differentiation. EMSAs using radiolabeled MRE oligonucleotides were performed with nuclear extracts from human blood monocytes that were cultured for different time periods as indicated. For further details see "Experimental Procedures."](Image 94x507 to 252x729)

![Fig. 9. Effect of phorbolester and calcium ionophore on MRE complex formation. Human blood monocytes were prepared and cultured overnight. Subsequently, cells were stimulated for 1 h with either 100 nmol/liter PMA or 4 μmol/liter A23187 in the presence and absence of actinomycin D (25 μg/ml). Cells were then harvested, and nuclear extracts were prepared and subjected to EMSA analysis using the radiolabeled MRE oligonucleotide extending from −400 to −357 bp.](Image 349x481 to 513x728)
ocytes to macrophages (44). It belongs to the immediate-early (IE) genes (for review, see Ref. 33).

To further characterize the DNA-binding protein complex we performed Western blots with the different fractions derived from the DNA affinity chromatography. We found that a protein with an apparently molecular mass of 110 kDa was immunodetectable with an antibody directed against TIF1/H9252 in the 1 M NaCl eluate (Fig. 11A). Because TIF1/H9252 alone cannot bind DNA, this result clearly indicates that the nuclear complex binding to MRE most likely consisted of TIF1/H9252 and its DNA-binding interaction partner Kruppel-like zinc finger protein. TIF1/H9252 has also been shown to bind to and act as a cofactor for C/EBP/H9252 (45, 46). However, both competition with an ideal C/EBP oligonucleotide as well as super shift experiments with antisera to several C/EBP isoforms excluded the possibility that a C/EBP isoform represented the DNA-binding factor (data not shown).

To investigate whether TIF1β was involved in S100A9 gene regulation we extended the Western blot analysis with nuclear extracts of various human cell lines. As shown in Fig. 11, B and C, TIF1β was expressed in nuclear extracts of lymphocytes, the B-lymphoid cell lines Raji and Malme, and in non-hematopoietic cells, such as HeLa epithelial carcinoma cell line, L132 fibroblasts, and HaCaT cells. TIF1β was also immunodetectable in S100A9-negative myeloid cell lines such as HL-60 and U937. TIF1β was not present in freshly isolated human blood monocytes. However, its expression was up-regulated at day 3–5 during differentiation to macrophages. Furthermore, TPA-stimulated monocytes were positive for TIF1β. Thus, the expression pattern of TIF1β closely correlates with both the formation of MbcA and S100A9 expression. Therefore, we suggest from our data that TIF1β is one subunit of the negative regulatory nuclear complex MbcA.

The S100A9 expression is restricted to a specific stage of myeloid differentiation. In the present study we give evidence that a nuclear complex consisting of a Kruppel-related zinc finger protein and TIF1/H9252 is involved in S100A9 gene expression in a cell type-specific, activation-, and differentiation-dependent manner. Members of the Kruppel-related zinc finger protein family have been shown to play a pivotal role in myeloid differentiation and development. The importance of TIF1/H9252 is highlighted by the fact that TIF1β is essential for early embryogenesis (48). Although the physiological functions of KRAB domain-containing zinc finger proteins are unknown at present, they appear to play an important role in myeloid differentiation and development. The importance of TIF1β is highlighted by the fact that TIF1β is essential for early embryogenesis (48). Although the physiological functions of KRAB domain-containing zinc finger proteins are unknown at present, they appear to play an important role in regulating expression of specific genes during cell differentiation and development. KRAB domain-containing zinc finger proteins show a temporally and spatially regulated expression pattern (Ref. 49 and references therein). The KRAB domain-containing zinc finger proteins ZNF43 and ZNF91 exhibit expression that is mainly restricted to lymphoid cells, suggesting roles as transcriptional regulators specific for lymphoid cell differentiation (50, 51). Others, such as HPF4, HTF10, and HTF34, are down-regulated during myeloid differentiation (44). In addition, a
number of KRAB zinc finger proteins are candidate genes for human diseases based on their chromosomal locations (52, 53). Our finding that one function of the Kruppel-related zinc finger protein/TIF1β complex may be to regulate the expression of the myeloid-specific S100A9 gene represents the first link between these nuclear factors and the S100 protein family. This study gives first insight into the molecular mechanism of S100A9 gene regulation. Nevertheless, we are aware that the exact elucidation of the DNA/protein complexes will require further intensive investigations.

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