Calcineurin-mediated YB-1 Dephosphorylation Regulates CCL5 Expression during Monocyte Differentiation*

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**Background:** Transcription factor YB-1 constitutes a key regulator in immune cell homeostasis. It has been demonstrated to be involved in monocyte/macrophage differentiation. However, the underlying mechanisms are poorly understood.

**Results:** Protein phosphatase calcineurin (CN) regulates YB-1 activities on the CCL5 promoter during macrophage differentiation.

**Conclusion:** Dephosphorylation of YB-1 by CN is crucial to counteract the overwhelming pro-inflammatory propensities of YB-1.

**Significance:** Overshooting inflammation may be counteracted by dephosphorylation of YB-1.

Y-box (YB) protein-1 serves as a master regulator in gene transcription and mRNA translation. YB-1 itself is regulated at various levels, e.g. through post-translational modifications. In our previous work, we identified RANTES/CCL5 as a transcriptional target of YB-1. We previously demonstrated that YB-1 protein is transiently up-regulated during monocyte/macrophage differentiation evidenced in monocytic cells (THP-1 cells) that were differentiated using phorbol myristate acetate (PMA). Here we provide evidence that YB-1 phosphorylation, specifically at its serine residue 102 (Ser-102), increases early on in THP-1 cells following PMA treatment as well as in differentiated primary human monocytes. This process is mediated through the Akt signaling pathway. Ser-102-phosphorylated YB-1 displaces stronger binding affinity and trans-activating capacity at the CCL5 gene promoter. Notably, Ser-102-phosphorylated YB-1 disappears at later stages of the monocyte/macrophage differentiation process. We demonstrate that serine-threonine phosphatase calcineurin (CN) dephosphorylates YB-1 preventing it from binding to and trans-activating the CCL5 promoter. Co-immunoprecipitation assays prove a direct YB-1/CN interaction. Furthermore, analyses in kidney tissues from mice that were treated with the CN inhibitor cyclosporine A revealed an in vivo effect of CN on the YB-1 phosphorylation status. We conclude that YB-1 phosphorylation at Ser-102 is an important prerequisite for CCL5 promoter activation during macrophage differentiation. Our findings point to a critical role of YB-1 in the resolution of inflammatory processes which may largely be due to CN-mediated dephosphorylation.

Post-translational modifications of proteins increase the functional diversity of the proteome by covalent addition of functional groups. Phosphorylation and dephosphorylation are the most common protein modifications and crucially contribute to the fine-tuning of multiple biological processes that are often accompanied by intracellular translocation of the respective proteins (1, 2).

Calcineurin (CN),5 also known as protein phosphatase 2B (PP2B), constitutes a calcium-dependent serine-threonine (Ser/Thr) phosphatase. In response to increased intracellular calcium levels, CN dephosphorylates and thereby activates transcription factors including nuclear factor of activated T cells (NFAT). In a complex together with CN, NFAT shuttles into the nucleus and induces gene transcription, e.g. that of interleukin (IL)-2 (3, 4). This signal transduction pathway was initially characterized in T lymphocytes. In these cells, CN inhibitors (CNI), such as cyclosporine A (CsA) and tacrolimus (FK506), can efficiently block CN effects at various stages in the immune system. CN has evolved as a major target of immunosuppressant drugs and CNI s are an integral part of standard therapy regimens to prevent allograft rejection (5, 6). However, despite the beneficial effects on allograft survival, CNI s also exert nephrotoxic side effects contributing to acute or chronic allograft nephropathy (7). Recent findings from our group point...
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to profibrotic properties of Y-box protein-1 (YB-1) in CNI-challenged mesangial cells (MCS) (8).

YB-1 is a highly conserved protein that has been shown to associate with DNA elements encompassing inverted CAAT-box sequences (Y-boxes) as well as with RNA in the cytoplasm. By this, YB-1 is involved in the regulation of DNA transcription (9, 10), RNA splicing (11) and translational control of protein synthesis (8, 12). In vitro analysis in MCS revealed a severalfold induction of cellular YB-1 protein content upon CsA treatment that resulted in stabilization and generation of type 1 collagen (Col1A) mRNA (8). Whether CN can act directly on the YB-1 activity by dephosphorylation of the protein is unknown. Although there is growing knowledge of how YB-1 is phosphorylated (13, 14), little is known about its dephosphorylation processes. The Ser/Thr phosphatase PP2Cγ is involved in spliceosome assembly and has been shown to interact with YB-1 (15); however, a subsequent change in the phosphorylation state of YB-1 has not been investigated so far. In vivo and ex vivo data demonstrate that YB-1 is post-translationally phosphorylated at amino acid position 102 (serine 102 (Ser-102)) at the onset of lipopolysaccharide (LPS)-triggered inflammation (16). However, this modification is no longer detectable during the late phase of inflammation, pointing to a reversible phosphorylation of YB-1 (16).

We have previously demonstrated a pivotal regulatory role for YB-1 in CCL5/RANTES gene transcription by binding to its specific gene promoter in transplant rejection (17) and atherogenesis (18). Chemokines such as CCL5 permit monocytes to infiltrate the tissue and propagate a process denoted “differentiation” into macrophages (19, 20). YB-1 has the potential to contribute to profibrotic properties of Y-box protein-1 (YB-1) in CNI-challenged mesangial cells (MCS) (8).

For induction of YB-1 phosphorylation, rMCs (1 × 10^6) or HEK293T cells (2 × 10^6) were seeded in 75 cm^2 cell culture flasks and grown in serum-reduced media with 1% FCS 24 h prior to challenge. Cells were stimulated for 1 h with 100 ng/ml recombinant human epidermal growth factor (EGF) (ImmunoTools) or rat insulin-like growth factor (IGF) (Pros pec), respectively. To prevent PMA-induced phosphorylation of YB-1, THP-1 cells were preincubated either with 10 µM Ly294002 (Calbiochem) or 38 µM SL0101 (Calbiochem). To induce monocyte differentiation, 1 × 10^7 THP-1 cells were incubated with 100 nM PMA (Sigma-Aldrich) for the indicated periods.

**Plasmids**—Plasmids encoding for CCL5 promoter fused to the luciferase gene have been described previously (17). A full-length YB-1 expression plasmid (YB-1-pSG5) was kindly donated by J. Ting (Lineberger Comprehensive Center, University of North Carolina) (24). For immunofluorescence studies, an expression plasmid was used that encodes the full-length YB-1 protein with a C-terminal CFP-tag (pDREAM vector, Genscript). A pEYFP-N2 N-terminal protein fusion vector containing a CN-encoding DNA fragment was kindly donated by M. L. Dell’Acqua and W. A. Sather. Overexpression of CN in different cell lines was performed using a pEGFP-C3-CNAbeta expression plasmid (donated by Oliver Ritter, Ref. 23).

**Transient Transfections**—Transient transfections in HEK293T cells were performed using calcium phosphate precipitates as described previously (25). rMCs (2 × 10^6) were transfected with the lipid-based transfection reagent Fugene HD (Promega) according to the manufacturer’s instructions. Human THP-1 cells were transfected through electroporation. Briefly, 1 × 10^7 non-adherent cells were pelleted and resuspended in 1 ml of RPMI 1640 medium supplemented with 20% FCS. Next, 2.5 × 10^6 cells (250 µl) were added to electroporation cuvettes (0.4 cm gap, Bio-Rad) together with a total amount of 20 µg plasmid DNA, respectively. The mixture was incubated for 5 min on ice and THP-1 cells were electroporated at 250 V/1100 μF in a Gene Pulser II electroporation system (Bio-Rad). Cells were incubated another 5 min on ice prior to resuspension in 2 ml of RPMI 1640 medium supplemented with 20% FCS, transferred to 6-well tissue culture plates and incubated at 37 °C and 5% CO_2 for 6/24 h.

**Preparation of Whole Cell Lysates, Nuclear, and Cytoplasmic Cell Extracts and Immunoblotting**—For preparation of whole cell lysates, cells were harvested, washed once with ice-cold PBS, and lysed with 1 ml RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, protease inhibitors, phosphatase inhibitors (Roche Applied Science)). After 30-min incubation on ice, lysates were centrifuged (15 min, 4 °C, 14,000 rpm), and supernatants were collected and stored at −80 °C.

For preparation of nuclear and cytoplasmic cell extracts, 1 × 10^7 THP-1 or 2 × 10^6 HEK293T cells were grown in tissue culture dishes, washed with ice-cold PBS, pelleted and resuspended in 200 µl of hypotonic buffer A (10 mM HEPES-KOH 7.9, 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM DTT, 1 mM sodium orthovanadate, protease inhibitors (Roche Applied Science)). After 10 min of incubation on ice, samples were vortexed for

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human monocytes (THP-1) and rat mesangial cells (rMCs) were cultured in RPMI 1640, and human embryonic kidney cells (HEK293T) were grown in Dulbecco’s modified Eagle’s medium (DMEM, low glucose). Media and supplements (10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin) were purchased from Invitrogen. All cell lines were maintained in humidified air with 5% CO_2 content at 37 °C.

For induction of YB-1 phosphorylation, rMCs (1 × 10^6) or HEK293T cells (2 × 10^6) were seeded in 75 cm^2 cell culture flasks and grown in serum-reduced media with 1% FCS 24 h prior to challenge. Cells were stimulated for 1 h with 100 ng/ml recombinant human epidermal growth factor (EGF) (ImmunoTools) or rat insulin-like growth factor (IGF) (Pros pec), respectively. To prevent PMA-induced phosphorylation of YB-1, THP-1 cells were preincubated either with 10 µM Ly294002 (Calbiochem) or 38 µM SL0101 (Calbiochem). To induce monocyte differentiation, 1 × 10^7 THP-1 cells were incubated with 100 nM PMA (Sigma-Aldrich) for the indicated periods.

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For preparation of nuclear and cytoplasmic cell extracts, 1 × 10^7 THP-1 or 2 × 10^6 HEK293T cells were grown in tissue culture dishes, washed with ice-cold PBS, pelleted and resuspended in 200 µl of hypotonic buffer A (10 mM HEPES-KOH 7.9, 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM DTT, 1 mM sodium orthovanadate, protease inhibitors (Roche Applied Science)). After 10 min of incubation on ice, samples were vortexed for
10 s and the cell nuclei pelleted by centrifugation (5 min, 2,000 rpm, 4 °C). Supernatants (cytoplasmatic fractions) were harvested and frozen at −80 °C. Nuclear pellets were washed five times with buffer A and resuspended in 50 μl of buffer C (20 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 1 mM sodium orthovanadate, protease inhibitors). After 20 min of incubation on ice, the supernatants containing nuclear proteins were collected by spinning at 14,000 rpm (5 min, 4 °C).

Nuclear and cytoplasmic cell extracts of kidney tissue were prepared as follows: ∼50 μg of minced kidneys were lysed with 400 μl of buffer A, homogenized and sonicated (3 times, 10 s each cycle). The suspension was incubated for 10 min on ice and nuclei pelleted by centrifugation (5 min, 2,000 rpm, 4 °C). The cytoplasmic fraction was collected and nuclear protein extracts prepared as described above.

Protein concentrations were determined by the BCA protein assay (Interchim), using bovine serum albumin as a standard. Extracts were stored at −80 °C until 10–30 μg of lysates were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane and detected by Western blotting using antibodies against histone H3 (3H1), Cell Signaling. To ensure equal protein loading of whole cell lysates or cytoplasmatic extracts, blots were stripped and reprobed against histone H3 (monoclonal antibody against histone H3 (H1), Cell Signaling). For 10 ng of p-YB-1-specific antibody (Cell Signaling) or IgG as negative control (Millipore) overnight at 4 °C. A proportion (20%) of the samples was kept as “input” to represent the PCR amplification of the total sample. Immune complexes were precipitated with ChIP-grade Protein A/G Plus-agarose (Roche Scientific). DNA was prepared by incubation with proteinase K enzyme (Roche Applied Science) for 1 h at 45 °C, recovered using a QIAamp DNA Mini Kit protocol (Qiagen) and subjected to real-time PCR analysis.

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The real-time qPCR primer sequences used to amplify the regions within the CCL5 gene locus were as follows: 5’-CTC-TGAGGAGGACCCCTTCC-3’; 5’-TTCCTCTTTGACCAAGCACCC-3’. The amount of immunoprecipitated DNA was subtracted by the amplified DNA that was bound by the nonspecific IgG antibody and calculated relative to the respective input DNA. Further, real-time qPCR products were separated on 2% agarose gel stained with GelRed (Biotium).

Luciferase Assays—THP-1 cells were transfected by electroporation as described above including 10 ng of Renilla luciferase control plasmid per transfection. At 6/24 h after electroporation, the cells were harvested and lysed with 50 μl of passive lysis buffer. After incubation for 15 min on ice and centrifugation (5 min, 14,000 rpm, 4 °C) lysates were assayed for luciferase activity using the Dual-Luciferase® Assay System (Promega) following the manufacturer’s instructions on a Sirius lumino-meter (Berthold Detection Systems). Renilla luciferase activity was measured to control for transfection efficiency. Results were confirmed in at least three independent experiments and calculated as fold changes relative to luciferase activity measured with CCL5 promoter plasmid.

Electrophoretic Mobility Shift Analyses—Biotin 3’-end labeling of synthetic DNA probes corresponding to the antisense strands of the CCL5 promoter sequences was performed as described previously (17). Biotin-labeled DNA was incubated with nuclear cell extract for 20 min at room temperature, subjected to electrophoresis on native 6% polyacrylamide gels, and transferred to nylon membranes. The bands were visualized by streptavidin horseradish peroxidase conjugate and chemiluminescent substrate (Light-Shift chemiluminescent EMSA kit, Pierce).

For supershift analyses, the following antibodies were incubated with nuclear proteins 20 min prior to addition of probes: IgG as negative control (Millipore), polyclonal anti-calcineurin, polyclonal anti-Akt, and p-YB-1-specific antibody (all obtained from Cell Signaling). Supershifted complexes were indirectly deduced from decreased band intensities on the retardation gels, whereas bands should not change when control IgGs were added to the binding reactions. The binding reaction was per-
formed as described above, and samples were subjected to electrophoresis on 6% polyacrylamide gels.

Co-immunoprecipitation of CN Associated with YB-1—Prior to immunoprecipitation, polyclonal anti-calcineurin antibody (Cell Signaling) and nonspecific rabbit IgG (Santa Cruz Biotechnology) as negative control were covalently linked to protein A-Sepharose beads (50% suspension; Invitrogen), as described before (16). A total of 40 μl of protein A-Sepharose coupled with either polyclonal CN antibody or nonspecific IgG was incubated with 200 μg of total cell extracts or cytoplasmic (200 μg) and nuclear (100 μg) protein extracts from HEK293T cells, respectively, for 90 min at 4 °C in immunoprecipitation buffer (20 mM HEPES, 100 mM potassium chloride, 5 mM magnesium acetate, 1 mM DTT, 0.025% Triton X-100, and protease inhibitors). Sepharose beads were washed six times in immunoprecipitation buffer and one time in PBS buffer, and precipitated material was resuspended in 50 μl of Western blot sample buffer. Then 10 μl of samples were subjected to SDS-polyacrylamide gel electrophoresis.

Quantitative Real-time PCR—For quantitative real-time PCR (qRT-PCR), 1.25 × 10^7 THP-1 cells were transfected with either a CN-GFP or GFP control plasmid by means of electroporation as described above and cultured in RPMI 1640 medium supplemented with 20% FCS for 6 or 24 h, respectively. Total RNA was purified using a my-Budget RNA Mini Kit (Bio-Budget) according to the manufacturer’s protocol. First-strand cDNA was synthesized with Moloney-monkey leukemia virus reverse transcriptase (Invitrogen). qRT-PCR was carried out on the 7300 real-time PCR system (Applied Biosystems). TaqMan master mix and TaqMan primer sets were obtained for human CCL5 (Hs00174575_m1), human CN (Hs00917458_m1), and eukaryotic 18 S rRNA (Hs99999901_s1) as an internal control from Applied Biosystems. Results were calculated using the comparative deltaCT methodology. For ChIP analyses, realtime qPCR was performed using a SYBR Green PCR kit (Eugentec, Cologne, Germany).

Chronic CsA Nephropathy in Vivo—Animals were housed in cages with constant temperature and humidity and drinking water and food ad libitum. The local review board approved this animal experiment according to prevailing guidelines for scientific animal experimentation. One week prior to and during the experiments animals received a low salt diet with distilled water ad libitum. Female 16-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) with a weight of 20–24 g received daily subcutaneous injections of 30 mg/kg of CsA (Sandimmune®, Novartis) or identical volume of the vehicle (27% Cremophor® EL, 33% ethanol and 40% of a 0.9% NaCl solution) as control. After 5 weeks, the animals were sacrificed, and kidneys were removed and frozen on dry ice for protein extraction. Extraction of nuclear and cytoplasmic extracts from renal tissues was performed as described previously.

Isolation of Primary Human Monocytic Cells and FACS Analysis—Human peripheral blood mononuclear cells from pooled samples obtained from three healthy blood donors of the local blood bank were isolated by density gradient centrifugation by means ofuffy coats. CD14-positive monocytes were isolated by immunomagnetic separation (MACS; Miltenyi Biotec) according to the manufacturer’s instructions. Separated cells were analyzed fresh (day 0) or resuspended in RPMI 1640 containing 5% human serum to allow cells to adhere and differentiate for 24 h at 37 °C. Nuclear and cytoplasmic cell extracts were prepared as described above. Western blot analyses from these samples were run in three independent experimental approaches.

Effective differentiation process was assessed by surface expression of ICAM-1/CD54 as a macrophage marker molecule through FACS analysis. Briefly, 3 × 10^5 cells were washed with PBS supplemented with 0.5% BSA and 0.1% sodium azide and stained with a fluorescein-conjugated anti-CD54 antibody (R&D Systems). Specificity of staining was confirmed using the corresponding isotype control. Stained cells were washed and analyzed on a FACSCanto II (BD Biosciences). Measurements were quantified using FlowJo software (TreeStar, Ashland, OR).

Statistical Analysis—All values are expressed as means ± standard deviation (S.D.). Statistical significance was evaluated using the Student’s t test with significance accepted when p < 0.05. All experiments were performed at least in triplicates.

RESULTS

PMA-induced Monocyte Differentiation Results in Transient YB-1 Phosphorylation—Recently, we described the impact of YB-1 in the course of monocytic cell differentiation (17). To assess whether the posttranslational modification of YB-1 changes during this process, we analyzed the presence of YB-1 and (phosphorylated) p-YB-1 in cell lysates obtained at different time points during PMA-induced differentiation of monocytic THP-1 cells (26). In Western blot analysis, we used a polyclonal anti-YB-1 antibody that specifically detects YB-1 at its phosphorylated form (p-YB-1). Total YB-1 protein was still detectable in contrast to p-YB-1S102, total YB-1 protein was still detectable in contrast to p-YB-1S102, total YB-1 protein was still detectable in contrast to p-YB-1S102, total YB-1 protein was still detectable in contrast to p-YB-1S102, total YB-1 protein was still detectable in contrast to p-YB-1S102, total YB-1 protein was still detectable in contrast to p-YB-1S102, total YB-1 protein was still detectable in contrast to p-YB-1S102, total YB-1 protein was still detectable in contrast to p-YB-1S102, total YB-1 protein was still detectable in contrast to p-YB-1S102, total YB-1 protein was still detectable in contrast to p-YB-1S102. In Western blot analysis, we used a polyclonal anti-YB-1 antibody that specifically detects YB-1 at its phosphorylated serine residue 102 (p-YB-1S102), which is located within the highly conserved cold-shock domain. After h of PMA incubation, we observed enhanced expression of phosphorylated YB-1. Furthermore, an approximatively 30 kDa phosphorylated YB-1 fragment appeared at that time point in both the cytoplasm and the nucleus, which tapered off in the subsequent observation period until 48 h (Fig. 1A). Of note, in contrast to p-YB-1S102, total YB-1 protein was still detectable 48 h following PMA challenge (Fig. 1A, second row) pointing to a reversible process. Phosphorylation was prevented to a high degree by preincubation with the phosphoinositide 3-kinase inhibitor Ly294002 in the nuclear compartment (Fig. 1B, upper panel, right). In contrast, there was no effect of Ly294002 on cytoplasmic YB-1 (Fig. 1B, upper panel, left). To analyze subcellular localization of Akt following the differentiation process, we used cytoplasmic and nuclear protein extracts of untreated and PMA-challenged THP-1 cells and analyzed them for Akt protein content by Western blotting. Unchanged Akt protein amounts could be detected in the cytoplasm following PMA challenge, whereas nuclear Akt was enhanced (Fig. 1C). In contrast to Ly294002, pre-incubation with SL0101, an inhibitor of p90 ribosomal S6 kinase (RSK), effectively prevented phosphorylation of YB-1 in both compartments following PMA treatment (Fig. 1D).

To confirm phosphorylation and fragmentation of YB-1 following differentiation, primary human monocytes were isolated from healthy blood donors, pooled, and cytoplasmic and nuclear protein extracts thereof were analyzed by Western blot.
YB-1 and p-YB-1S102 levels were determined in freshly isolated cells and compared with cells that had been incubated for 24 h with human serum to induce differentiation toward a macrophage-like phenotype. Successful differentiation process was monitored by enhanced ICAM-1/CD54 expression (Fig. 1E). As observed in THP-1 cells, we detected increased levels of total YB-1 in both, cytoplasm and nuclei of differentiated primary human monocytes. Furthermore, we observed a phosphorylated, ~30 kDa YB-1 fragment in the nuclei of monocytes upon serum incubation. A representative immunoblot from three independent experiments is shown in Fig. 1F. Taken together, data from the differentiated primary human monocytes clearly accord with those obtained from the PMA/THP-1 differentiation model, underscoring the physiological relevance of our findings.

**Phosphorylation of YB-1 Leads to Enhanced Binding to the CCL5 Promoter**—Having demonstrated a YB-1 phosphorylation during monocyte differentiation, we next determined, whether Ser-102 phosphorylation directly influences YB-1-binding affinity to a well-known YB-1 target gene promoter, namely the CCL5 promoter. In previous studies, we were able to map a specific YB-1-binding motif in the very proximal promoter region (17). ChIP analyses in naive and PMA-activated THP-1 cells using antibodies against YB-1 and p-YB-1S102 con-
FIGURE 2. Phosphorylation of YB-1 at Ser-102 increases its binding affinity to CCL5 promoter region and CCL5 expression in monocytes (THP-1 cells). A, ChIP assays were performed in THP-1 cells incubated with PMA for 6 h using p-YB-1S102-specific antibody or unspecific IgG (negative control). The amount of included DNA was tested without preceding immunoprecipitation (input). B, protein-DNA binding from A was quantified and confirmed by qRT-PCR in three independent experiments. C, EMSA analyses were performed with the antisense Y-box region within the human CCL5 promoter including nuclear protein extracts from THP-1 cells previously incubated with PMA (100 nM, 6 h) or left untreated. Participation of p-YB-1S102 in the complex formation was confirmed by supershift analyses (*) using a p-YB-1S102-specific or nonspecific IgG antibody as control. D, CCL5 promoter activity was determined in THP-1 cells transfected with a plasmid harboring the proximal 1014 bp of the 5' regulatory sequence covalently coupled to luciferase reporter gene and with a plasmid encoding for empty vector, wt-YB-1, respectively. Prior to PMA stimulation (100 nM, 6 h), THP-1 cells were preincubated with Akt inhibitor Ly294002 (100 nM, 6 h) or vehicle for 2 h before CCL5 promoter activation. CCL5 mRNA (E) and secretion (F) was assessed by TaqMan and ELISA technology, respectively. Values were normalized to control transfection. Experiments were performed in at least three independent experiments, each performed in triplicate. Data are expressed as mean values ± S.D. G, EMSA analyses were performed with the antisense Y-box region within the human CCL5 promoter including nuclear protein extracts from human primary monocytes previously incubated with human serum for 24 h or left untreated. Participation of p-YB-1S102 in the complex formation was confirmed by supershift analyses (*) using a p-YB-1S102-specific or nonspecific IgG antibody as control.
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was observed with the control IgG antibody (Fig. 4D, lanes 1, 3, and 5).

In a next step, we assessed the cell compartment of YB-1-CN interaction using cytoplasmic and nuclear protein extracts of HEK293T cells. CN-co-immunoprecipitated endogenous YB-1 was nearly exclusively detected in nuclear protein extracts (Fig. 4E, lanes 6 and 8), and exceedingly in those cells that were pre-stimulated with IGF (Fig. 4E, lane 8). A minor amount of YB-1 that co-immunoprecipitated in the cytoplasmic compartment was solely detectable following extended exposure of the immunoblot (data not shown). No YB-1 protein was precipitated with nonspecific IgG (Fig. 4E, lanes 1, 3, 5, and 7). To prove whether incubation of HEK293T cells with IGF results not only in YB-1 phosphorylation but also in enhanced presence of YB-1 in the nucleus, we performed immunofluorescence of YB-1-CFP-transfected cells with and without IGF stimulation. Following IGF stimulation, an enhanced number of transfected cells displayed YB-1-CFP in the nuclear compart-

![Graphs and images related to the text]
Phosphorylation State of YB-1 Determines Its Function

In Vivo Inhibition of CN Results in Enhanced YB-1 Phosphorylation in the Kidney—To analyze CN-dependent effects on YB-1 in vivo, we examined YB-1 phosphorylation in the presence of the CN inhibitor CsA in BL/6 mice. Mice were injected with CsA (Sandimmune®) for 5 weeks following a one-week low-salt diet. The kidneys were obtained, and Western blot analyses of renal extracts from cytoplasmic and nuclear compartments were performed. In contrast to samples from control animals that received only the solvent, the content of p-YB-1S102 was strongly elevated in the nuclear compartment following CsA challenge (Fig. 5). There is increasing evidence that YB-1 has an important regulatory role in various inflammatory diseases, such as allergic asthma (31), transplant rejection (17), atherosclerosis (18), sepsis, and sterile inflammation (16). In previous studies, our group identified YB-1 as a cell-type-specific regulator of the chemokine CCL5 (17, 18), a key chemotactic factor for immune cell infiltration. From studies with gelatinase A (32) and GM-CSF (33) genes it is known that YB-1 may act as transcriptional activator and repressor of the same gene, depending on the cellular context. Along this line, on the one hand YB-1 activates CCL5 expression in monocytes, but upon differentiation of these cells to macrophages, YB-1 exhibits a trans-repressive effect on the CCL5 promoter. Thereby, YB-1 drives the early phase of inflammation and additionally contributes to its termination through the shutdown of CCL5 expression upon cell differentiation at later stages. The appearance of a high mobility complex in DNA binding studies indicates that partnering with other transcription factors/cofactors occurs and most likely mediates the repressive effect on gene transcription (17, 21). In our present study, we demonstrate that YB-1 directly interacts with phosphatase CN and that the binding of CN to YB-1 predominantly occurs in the nucleus, as only minor amounts of the CN-YB-1 complex were present in the cytoplasmic fractions. The phosphorylation of YB-1 is increased in response to IGF, and as a consequence thereof, YB-1 shuttles into the nucleus. Furthermore, these processes enhance the interaction of YB-1 with CN. In general, CN accomplishes multiple functions in cells through dephosphorylation of its substrates (NFAT, cdk-4, GABA) (34–36). A nuclear translocation of CN in a

DISCUSSION

In this study, we demonstrate in both, primary human monocytes and a monocytic cell line, that the differential regulation of CCL5 gene expression during monocyte/macrophage differentiation highly depends on the phosphorylation status of YB-1. Furthermore, we identified YB-1 as a novel partnering protein of phosphatase CN.

Expression, subcellular localization, and regulatory activities of YB-1 are tightly controlled by several mechanisms including phosphorylation processes that are accomplished by kinases such as Akt/PKB and p90 ribosomal S6 kinase (RSK) (14, 27). During the differentiation of human primary and cell line monocytic cells, p-YB-1S102 was detectable in both the cytoplasmic and the nuclear compartment. While RSK activity could be localized to both compartments, Akt/PKB-mediated phosphorylation predominantly occurred in the nucleus, as demonstrated in our studies by interventions with specific kinase inhibitors. In line with this, an enhanced presence of Akt/PKB in the nucleus occurred following PMA-triggered differentiation. It is well documented that Akt/PKB-mediated phosphorylation predominantly occurs in the nucleus, as only minor amounts of the phosphatase CN and that the binding of CN to YB-1 pre- dominantly occurs in the nucleus, as most likely mediates the repressive effect on gene transcription (17, 21). In our present study, we demonstrate that YB-1 directly interacts with phosphatase CN and that the binding of CN to YB-1 predominantly occurs in the nucleus, as only minor amounts of the CN-YB-1 complex were present in the cytoplasmic fractions. The phosphorylation of YB-1 is increased in response to IGF, and as a consequence thereof, YB-1 shuttles into the nucleus. Furthermore, these processes enhance the interaction of YB-1 with CN. In general, CN accomplishes multiple functions in cells through dephosphorylation of its substrates (NFAT, cdk-4, GABA) (34–36). A nuclear translocation of CN in a

FIGURE 3. CN-mediated dephosphorylation of p-YB-1S102 decreases CCL5 expression in monocytes (THP-1 cells). Rat mesangial (rMCs) (A) or THP-1 cells (B) were transfected with a CN-GFP expression plasmid or control vector, and cell lysates were analyzed for p-YB-1S102 and total YB-1 content by immunoblot analyses. Densitometric analyses were performed on p-YB-1S102 bands with normalization against values determined for GAPDH. Relative band intensities are depicted in bar diagrams below. C, ChIP assays were performed in THP-1 cells transfected with a CN-GFP expression plasmid or control vector. Four hours after transfection, cells were incubated with PMA for 1 h, and ChIP was performed using a p-YB-1S102-specific antibody or unspecific IgG (negative control) with oligos within the CCL5 promoter region. The amount of included DNA was tested without preceding immunoprecipitation (input). CCL5 promoter activity (D), CCL5 mRNA (E), and CCL5 protein secretion (F) were determined in THP-1 cells transfected with a CN-GFP expression plasmid or control vector. The cellular YB-1 content was manipulated by transfection of THP-1 cells with YB-1-psGS expression plasmid (D). Cells were harvested 6 or 24 h after transfection and CCL5 mRNA (E) and secretion (F) was measured by TaqMan and ELISA technology, respectively, and normalized to control transfection. G, overexpression of CN was confirmed by TaqMan analysis of THP-1 cells transfected with the CN-GFP expression vector. H, Western blot analyses of cytoplasmic (CE) and nuclear proteins (NE) of THP-1 cells in the course of PMA-induced monocyte differentiation. Compartamental localization of CN was assessed using a specific polyclonal antibody. Separation of cell compartments and equal protein loading were ensured by GAPDH and CREB levels. I, nuclear protein extracts from THP-1 cells prior to incubation for 24 or 48 h with PMA or left untreated were prepared and complex formation with the antisense element of the CCL5 promoter was assessed. A strong high-mobility nucleoprotein complex appeared after 48 h of PMA-induced differentiation, which was not detected in the presence of a CN-specific polyclonal antibody (arrowhead) but with a p-YB-1S102-specific or non-specific IgG-antibody as control. Experiments were performed in at least three independent experiments, each performed in triplicate. Data are expressed as mean values ± S.D. NE, nuclear extract.
complex with the transcription factor NFAT has already been shown, whereby CN continues to dephosphorylate NFAT, thereby ensuring the functional activities of this transcriptional regulator (22). In contrast to NFAT, however, CN-mediated dephosphorylation at Ser-102 leads to a deactivation of YB-1 with impact on binding to and regulation of the CCL5 promoter. Thus, transient overexpression of CN, co-localization and direct protein-protein interaction between CN and YB-1 result in a reduced amount of phosphorylated YB-1 and finally attenuated CCL5 promoter activity. In accordance with our data, it has been previously shown that the YB-1-binding capacities to the epidermal growth factor receptor (EGFR) promoter depend on the phosphorylation at Ser-102 (37). We demonstrate that a transient upregulation and phosphorylation of YB-1 occurs at the onset of the inflammatory response, whereas YB-1 dephosphorylation via CN constitutes a crucial mecha-
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