

AML1/Runx1 Recruits Calcineurin to Regulate Granulocyte Macrophage Colony-stimulating Factor by Ets1 Activation*

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Acute myeloid leukemia 1 (AML1), also denoted Runx1, is a transcription factor essential for hematopoiesis, and the AML1 gene is the most common target of chromosomal translocations in human leukemias. AML1 binds to sequences present in the regulatory regions of a number of hematopoiesis-specific genes, including certain cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) up-regulated after T cell receptor stimulation. Here we show that both subunits of the Ca²⁺/calmodulin-dependent protein phosphatase calcineurin (CN), which is activated upon T cell receptor stimulation, interact directly with the N-terminal runt homology domain-containing part of AML1. The regulatory CN subunit binds AML1 with a higher affinity and in addition also interacts with the isolated runt homology domain. The related Runx2 transcription factor, which is essential for bone formation, also interacts with CN. A constitutively active derivative of CN is shown to activate synergistically the GM-CSF promoter/enhancer together with AML1 or Runx2. We also provide evidence that relief of the negative effect of the AML1 sites is important for Ca²⁺ activation of the GM-CSF promoter/enhancer and that AML1 overexpression increases this Ca²⁺ activation. Both subunits of CN interact with AML1 in coimmunoprecipitation analyses, and confocal microscopy analysis of cells expressing fluorescence-tagged protein derivatives shows that CN can be recruited to the nucleus by AML1 *in vivo*. Mutant analysis of the GM-CSF promoter shows that the Ets1 binding site of the promoter is essential for the synergy between AML1 and CN in Jurkat T cells. Analysis of the effects of inhibitors of the protein kinase glycogen synthase kinase-3 β and *in vitro* phosphorylation/dephosphorylation analysis of Ets1 suggest that glycogen synthase kinase-3 β -phosphorylated Ets1 is a target of AML1-recruited CN phosphatase at the GM-CSF promoter.

The Runx family of transcription factors plays important roles in development, and the three Runx proteins are also tumor suppressors (1–4). Runx1, better known as acute myeloid leukemia 1 (AML1),¹ is essential for definitive hematopoiesis. Genetic ablation of AML1 leads to a complete lack of fetal

liver hematopoiesis, and AML1 is important at several levels of development of hematopoietic cells and regulation of the immune system (1, 2). The AML1 gene is the most common target for chromosomal translocations in human leukemia (5). In addition, point mutations in AML1 are found in acute human leukemias, and an inherited defect in one allele of AML1 leads to the disease familial platelet disorder with propensity to acute myeloid leukemia (3, 5). Runx2 is essential for osteoblast differentiation. Genetic ablation of Runx2 leads to a complete block of bone formation, and heterozygous Runx2 loss of function gives rise to the skeletal abnormalities disease cleidocranial dysplasia (3). Genetic ablation of Runx3 has shown that it is essential for antiproliferation and apoptosis of gastric epithelium, for differentiation of certain dorsal root ganglion nerve cells, and for establishment of mature CD8⁺ cytotoxic T lymphocytes (6–9). Runx3 expression is lost in 45–60% of gastric cancers (8).

The DNA binding domain of AML1, Runx2, and Runx3 is denoted the runt homology domain (RHD) (10) after the first identified member of this family of transcription factors, the *Drosophila* protein Runt. The Runx proteins interact with a protein called CBF β (11) or PEBP2 β (12). The free RHD exists in a conformation in which affinity for DNA is nonoptimal, and CBF β binding to the RHD triggers a switch in the RHD structure that stabilizes it in a conformation for sustained DNA binding (13). CBF β is essential for Runx protein function because a homozygous mutation of CBF β results in a complete lack of fetal liver hematopoiesis (1, 2) and severely defective bone formation (14, 15).

Runx/AML1 proteins bind to sites in the regulatory regions of a number of hematopoiesis-specific genes. These include genes encoding, for example, the subunits of the T cell receptor (TCR)-CD3 complex (16–18) and genes encoding certain cytokines up-regulated after T cell activation, such as granulocyte macrophage colony-stimulating factor (GM-CSF) (19). GM-CSF is a multipotent cytokine involved in hematopoiesis and many functions of hematopoietic cells. It plays a major role in the physiological response to infection and in inflammatory responses. GM-CSF and related cytokines can increase the antimicrobial activities of monocytes, neutrophils, and macrophages (20). Many cell types including T helper cells, mantle zone B lymphocytes, mast cells, macrophages, fibroblasts, endothelial cells, and epithelial cells can produce GM-CSF in response to different immune activating and inflammatory stimuli (21, 22). T cells that respond to TCR activation are a major source of GM-CSF. TCR activation initiates a bipartite signal transduction cascade that involves both activation of a kinase cascade and an increase in intracellular Ca²⁺ that can be mimicked by treatment

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¹ The abbreviations used are: AML1, acute myeloid leukemia 1; BSA, bovine serum albumin; CN, calcineurin; CsA, cyclosporin A; EGFP, enhanced green fluorescence protein; GFP, green fluorescence protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; GSK, glycogen synthase kinase; hCMV, human cytomegalovirus; Ni-NTA, nickel-

el-nitrilotriacetic acid; PMA, phorbol 12-myristate 13-acetate; RHD, runt homology domain; RSV, Rous sarcoma virus; TCR, T cell receptor; CBF, core binding factor; PEBP, polyoma enhancer binding protein; PBS, phosphate-buffered saline.

with a Ca^{2+} ionophore such as ionomycin (23). The promoter and the enhancer of the GM-CSF gene are both involved in the GM-CSF response to TCR activation. A number of conserved binding sites for transcription factors in the promoter are important for its activity. These include sites for Ets, NF-AT, and AP-1 transcription factors located ~40 bp upstream from the transcription start. A binding site for Runx/AML1 proteins is located nearby on the upstream side; and further upstream, ~70–90 bp from the start site, are binding sites for SP-1 and NF- κ B (see Fig. 1A). Ca^{2+} stimulation of GM-CSF transcription is mediated by the calmodulin-dependent phosphatase calcineurin (CN); and the NF-AT, AP-1, and NF- κ B transcription factors have been implicated in this activation (for review, see Ref. 24; see also Ref. 25).

In this study we show that CN interacts directly with AML1 and Runx2 in their N-terminal RHD-containing part. We show that relief of the negative effect of the AML1 sites is of importance for Ca^{2+} activation of the GM-CSF promoter/enhancer and that AML1 overexpression increases Ca^{2+} activation. We show also that constitutively active CN displays synergistic activation of the GM-CSF promoter together with AML1 or Runx2 and that CN can be recruited to the nucleus by AML1 *in vivo*. Mutant analysis of the GM-CSF promoter, analysis of effects of inhibitors of the protein kinase glycogen synthase kinase (GSK)-3 β , and *in vitro* phosphorylation/dephosphorylation analysis of Ets1 suggest that GSK-3 β -phosphorylated Ets1 is a target of AML1-recruited CN phosphatase at the GM-CSF promoter.

EXPERIMENTAL PROCEDURES

Expression and Reporter Plasmids—The mouse Runx1 expression plasmids pBJ9AML1b and pBJ9AML1a and the mouse Runx2 expression plasmid pBJ9PEBP2 α A1 have been described previously (26). The constitutively active murine CN A subunit Δ CN was PCR amplified from the CN α -4 cDNA (27) with *Pfu* polymerase (Stratagene) using the upstream primer 5'-CCGCTCGAGCCATATGTCCGAGCCCAAGGC-G-3' and the downstream primer 5'-GCGGATCCCTATTCCTCCGG-GCTGCG-3', which introduces a stop codon instead of amino acid 395. Δ CN was cloned with XhoI(SalI)/BamHI into the pBJ9 Ω vector. The Δ CN_{151N} mutant was made using standard PCR techniques. The Ets1 expression plasmid has been described previously (26).

The expression plasmid for green fluorescent AML1 was constructed by amplifying the AML1b cDNA from the pBJ9AML1b plasmid (26) with *PfuTurbo* using the primers 5'-CCCAAGCTTATGCGTATCCCGTAGATGC-3' and 5'-AAAGGTACCAGGTAGGGCCGCCACACGGCCT-3' (HindIII and KpnI sites underlined) and cloning of the PCR product into the pEGFP-N plasmid (Clontech) with HindIII and KpnI. For construction of plasmids expressing red fluorescent A and B subunits of CN and the C-terminally truncated CN A subunit, Δ CN, the cDNAs were amplified with *PfuTurbo* using the primers 5'-CCGCTCGAGATGTCGAGCCCAAGGCATTG-3' and 5'-CGCGGATCCCGCTGGATATTGCTGCTATTACTG-3 for CNA, 5'-CCGCTCGAGATGGGAAGTGAGGCGAGTTAC-3' and 5'-CGCGGATCCCGCACATCCACCACCATT-3' for CNB, and 5'-CCGCTCGAGATGTCGAGCCCAAGGCATTG-3' and 5'-CGGGATCCCGTTCCTCCGGGCTGC-3' for Δ CN (XhoI and BamHI sites underlined), respectively. The PCR products were cloned into the DsRed plasmid (Clontech) with XhoI and BamHI.

To construct the expression plasmid for FLAG-tagged AML1, the AML1b cDNA bordered by XhoI and XbaI sites was amplified with *PfuTurbo* replacing the N-terminal 25 codons of AML1b with an NdeI site. The PCR product was cloned with XhoI/XbaI creating the pBJ9AML1b Δ 25 plasmid. The FlagAML1 plasmid expressing AML1 with two FLAG tags was constructed from this plasmid by inserting the annealed oligonucleotides 5'-TCGAGATGGATTACAAGGATGACGACGATAAGATTACAAGGATGACGACGATAAGCA-3' and 3'-CTACCTAATGTTCCTACTGCTGCTATTCTAATGTTCCCTACTGCTGCTATTTCGTA-T-5' (start codon underlined and FLAG sequences in bold) between the XhoI and NdeI sites.

The luciferase reporter plasmid containing a 716-bp segment of the enhancer and a 0.6-kb segment of the promoter of the human GM-CSF gene has been described previously (28). The AML1 site mutations from 5'-TTTGTGGTCA-3' to 5'-TTTGTAAATCA-3' at nucleotides -59 to -50 in the promoter and from 5'-ATCGTGGTCC-3' to 5'-ATCGTAATCC-3' at nucleotides 285–294 in the enhancer were created by PCR with the

PfuTurbo polymerase. The deleted human GM-CSF promoter (bp -125 to +30) was constructed by PCR amplification from the GM-CSF promoter and enhancer reporter plasmid by *PfuTurbo* polymerase using the primers 5'-GAAGATCTCCGTTCCCATGTGTGGCTG-3' and 5'-CCCAAGCTTTAGCCTTCTCTCTGTGTAGCTGGGCTCACTGGC-3' (BglII and HindIII sites underlined). The deleted promoter was cloned with BglII and HindIII into the pGL2-Basic reporter plasmid. Mutations of Ets1/NF-AT and AP-1 sites in the reporter plasmid were created by PCR. The primers at the mutation sites contained the two strands of the mutant sequences; mAP-1, 5'-GAGCTCTAAATACACAGAGGAAATGCTATCTGTTGACCACAAAATGCCAGGGAG-3'; mNF-AT, 5'-CTCAGCTGGCAAAAAGAGCTCTTAAATACACAGAGGAAGTGATTAATGGTG-3'; mEts1, 5'-CTCAGCGAAAAGAGCTCTTAAATACACAGAGGAAAAGATTAATGGTG-3'; and mEts1/mNF-AT, 5'-CTCAGCTGGCAAAAAGAGCTCTTAAATACACAGAAAGATTAATGGTG-3' (mutations underlined). The nucleotide sequences of all DNA segments constructed by PCR were confirmed by DNA sequencing.

The derivatives of the pGL2-Basic reporter plasmid containing the germ line I α 1 promoter and the TCR β gene enhancer have been described previously (26, 29).

Cell Lines and Transient Transfections with GM-CSF Reporter Plasmids—The human cell lines DG75, an Epstein-Barr virus-negative Burkitt's lymphoma, Jurkat, a T cell line, and K562, an early erythroleukemia, were cultured as described previously (29, 30). The murine fibroblast cell line NIH3T3 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Transient transfections of DG75, Jurkat, and K562 cells were performed as described previously (30), using 2 μ g of hCMV- β -galactosidase plasmid (reference plasmid for normalization), 4 μ g of reporter plasmid, and 5 μ g of each expression plasmid indicated. Where necessary, the corresponding empty expression vector was added to a total of 10 μ g of expression plasmids. 10^7 cells were electroporated followed by incubation in 10 ml of medium. The cells were harvested 18–20 h after electroporation.

Expression of Runx Proteins and CN in *Escherichia coli* and Purification of Proteins—The expression plasmids for Δ AML1 (amino acids 1–185 of Runx1) and Δ Runx2 (amino acids 1–228 of Runx2 (PEBP2 α A)) have been described previously (26). Truncated genes encoding AML1_{RHD} (amino acids 51–185) and Runx2_{RHD} (amino acids 94–228) were constructed by PCR using *Pfu* polymerase. AML1 was N-terminally deleted by employing the primer 5'-ACATGCATGCATGGTG-GAGGTACTAGC-3', and Runx2 was N-terminally deleted by employing the primer 5'-ACATGCATGCATGGTGAGATCATCGC-3'. AML1_{RHD} and Runx2_{RHD} were cloned into pET21a+His as described previously for Δ AML1 and Δ Runx2 (Δ α A) (26).

Δ CN and Δ CN_{151N} were PCR amplified from their respective pBJ9 Ω vector employing 5'-GCGGATCCTTCCTCCGGGCTGCG-3' as the downstream primer, and CNB was PCR amplified from a cDNA of the β 1 isoform of the B subunit of CN (31) with the primers 5'-CCGCTCGAGCCATATGGGAAGTGAGGCGAG-3' and 5'-GCGGATCCACATCCACCACCATTT-3'. The PCR products were inserted with NdeI/BamHI into pET-20aCThrHis, a pET-20a derivative with a thrombin site between the C terminus of inserted proteins and the His tag (a kind gift from Dr. S. Hermann).

Proteins were expressed in the *E. coli* strain BL21(DE3)pLysS according to the manufacturer's instructions. After harvesting, cells were lysed by freeze-thawing followed by sonication and centrifugation. Supernatants were mixed with Ni-NTA-agarose (Qiagen) in binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole) at 4 °C for 1 h. After washing, the proteins were eluted by increasing the imidazole concentration to 100 mM. AML1_{RHD} protein with amino acids 47–185 of AML1 was obtained from Δ AML1 after cleavage with thrombin for several days. The AML1_{RHD} was purified by Ni-NTA-agarose chromatography.

Protein Binding Assays and Electrophoretic Mobility Shift Assays— Δ AML1, Δ Runx2, AML1_{RHD} (amino acids 47–185) and bovine serum albumin (BSA) were immobilized on CNBr-activated Sepharose 4B (Amersham Biosciences) according to the procedure suggested by the manufacturer. 5–7 μ g of protein was mixed with 30 μ g of Sepharose 4B containing immobilized protein in 150 μ l of binding buffer (25 mM HEPES, pH 8.0, 10% glycerol, 100 mM NaCl, 1 mM EDTA, 0.05% Triton X-100). After incubation for 2 h at 4 °C, the column was washed three times with binding buffer, and protein was eluted with 1 M NaCl in binding buffer, unless otherwise stated. Column fractions were concentrated by acetone precipitation, separated by SDS-PAGE, and visualized by silver staining. Bovine brain CN was purchased from Upstate Biotechnology.

Electrophoretic mobility shift assays were performed essentially as described previously (32) using complementary oligonucleotides optimized for AML1 binding (33).

Immunoprecipitation Assays—8 μ g of each plasmid was transfected

to 10^7 K562 or DG75 cells by electroporation or to a 35-mm dish of 70–80% confluent NIH3T3 cells by the polyethyleneimine method (Sigma) according to the manufacturer's instructions. The transfected cells were harvested after 20 h of culture by centrifugation at 1,200 rpm for 10 min. The cell pellets were washed once with PBS and dissolved in 300 μ l of radioimmune precipitation assay buffer (1 \times phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with freshly added protease inhibitor mixture (Roche Diagnostics GmbH). The cells were sonicated for 10 s and incubated for 10 additional min on ice, and the cell extracts were cleared by centrifugation at 18,000 rpm for 5 min. The cleared cell extracts were transferred to tubes with 20 μ l of radioimmune precipitation assay buffer equilibrated anti-FLAG antibody M2-coupled agarose beads (Sigma) that were incubated with shaking at 4 $^{\circ}$ C for 3 h. The beads were collected by centrifugation at $3,000 \times g$ for 5 min and washed three times with 500 μ l of radioimmune precipitation assay buffer. 30 μ l of SDS sample buffer was added to the beads, and after 5 min of boiling, the samples were subjected to 12% SDS-PAGE. Western blotting was performed using the WesternBreeze Kit (Invitrogen) according to the manufacturer's instructions. The Western blottings were with a monoclonal antibody against DsRed from Clontech and a rabbit antibody against CNB from Calbiochem.

Confocal Microscopy— 10^7 K562 cells were transfected with 5 μ g of expression plasmid DsRed-CNA, DsRed-CNB, DsRed-ACN, pEGFP-AML1b, or pEGFP vector separately or in combination as indicated. 18 h post-transfection, the cells were harvested by centrifugation and washed twice with PBS. The cells were permeabilized and fixed with freshly made 0.2% Triton X-100 in 3% paraformaldehyde in PBS, pH 7.5, for 5 min at room temperature and then further fixed in 3% paraformaldehyde in PBS, pH 7.5, for 10 min at room temperature. The fixed cells were washed twice with PBS, and the cells were stained by 1 ng/ml DAPI in PBS for 5 min at room temperature. The cells were washed twice with PBS and finally dissolved in 100 μ l of Long Antifade mounting solution (Molecular Probes). 10 μ l of the cell suspension was spread onto glass slides. The localization of the green fluorescent AML1 and the red fluorescent CN subunits was visualized by confocal laser scanning microscopy using a Leica SP2 confocal microscope. Each optical section was acquired sequentially using the 488 and 546 nm laser lines to excite EGFP and DsRed, respectively. Data presented in the same figure were registered with the same laser and detector settings. Coupled images of the DAPI stain were acquired after excitation at 785 nm with a multiphoton laser (Tsunami, Specha Physics).

Phosphorylation of Ets1 by GSK-3 β and Dephosphorylation by CN in Vitro—*E. coli*-produced and purified His-tagged wild type, mutant, or C-terminally truncated human Ets1 (10 μ g) was phosphorylated with 10 units of GSK-3 β (New England Biolabs) at 30 $^{\circ}$ C for 1.5 h in 25 μ l of 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol, 200 μ M [γ -³²P]ATP.

GSK-3 β -phosphorylated Ets1 was purified from GSK-3 β by the addition of 20 μ l of Ni-NTA-agarose beads in PBS (final volume 200 μ l) followed by incubation with shaking at 4 $^{\circ}$ C for 1 h and centrifugation at 4,000 rpm for 10 s. The beads were washed three times with cold PBS and three times with phosphatase assay buffer (50 mM Tris-HCl, pH 7.2, 0.1 mM CaCl₂). The beads were resuspended in 50 μ l of 2 \times phosphatase assay buffer. Half the suspension was incubated with 200 milliunits of CN (Upstate Biotechnology), and half was mock incubated with 125 ng of BSA for 1.5 h at 37 $^{\circ}$ C in presence of 1 mM NiCl₂ and 25 μ g/ml calmodulin (Upstate Biotechnology) in a final volume of 50 μ l. After incubation, the Ni-NTA-agarose beads were pelleted as above and washed three times with PBS and three times with 50 mM NaH₂PO₄, 0.5 M NaCl, 30 mM imidazole, pH 8.0. Finally, the Ets1 proteins were eluted in 25 μ l of 50 mM NaH₂PO₄, 0.5 M NaCl, 250 mM imidazole, pH 8.0. An equal volume of 2 \times SSC was added to the eluates followed by incubation at 70 $^{\circ}$ C for 5 min and separation by 10% SDS-PAGE. Gels were dried and autoradiographed overnight.

RESULTS

Synergistic Activation of GM-CSF Transcription by AML1 and Constitutively Active CN—Stimulation of GM-CSF transcription by Ca²⁺ is mediated by CN, and the transcription factors NF-AT, AP-1, and NF- κ B have been implicated in this activation (24, 25). To analyze whether AML1 could also have a role in the activation of GM-CSF by CN, we used a luciferase reporter plasmid under the control of the human GM-CSF promoter and enhancer (Fig. 1A) in transient transfection experiments. The regulatory region of GM-CSF contains an

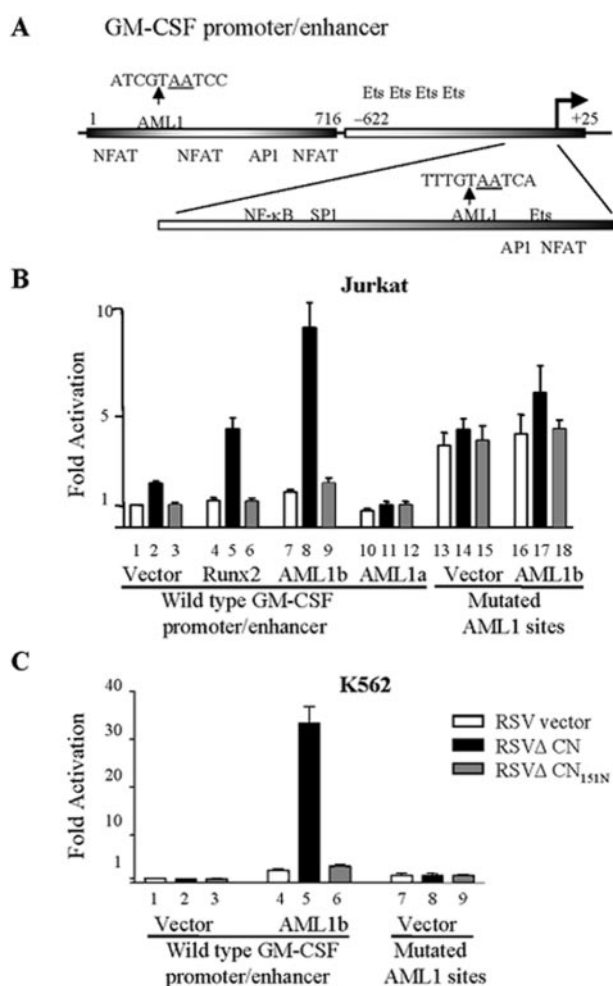


FIG. 1. Activation of the GM-CSF promoter/enhancer by co-transfected AML1 and constitutively active CN. A, schematic drawing of the human GM-CSF promoter/enhancer of the reporter plasmid. Identified transcription factor binding sites (24, 25, 57–59) and the AML1 site mutations are indicated. The mutations were from 5'-TTTGTGGTCA-3' to 5'-TTTGTAAATCA-3' at nucleotides -59 to -50 in the promoter and from 5'-ATCGTGGTCC-3' to 5'-ATCGTAAATCC-3' at nucleotides 285–294 in the enhancer. B and C, synergistic activation of the GM-CSF promoter/enhancer by cotransfected AML1 and constitutively active calcineurin (Δ CN) in Jurkat (B) and K562 (C) cells. 5 μ g of RSV-based AML1b, AML1a, Runx2, Δ CN, or Δ CN_{151N} expression plasmid was added where indicated, and empty RSV vector was added where necessary to achieve a total of 10 μ g of RSV expression vector in each transfection. Bars represent average luciferase activity expressed from the reporter plasmid in 5–15 independent transfections \pm S.E., using β -galactosidase expression from an hCMV- β -galactosidase plasmid for normalization.

AML1 site in the promoter (19) and a putative AML1 site at nucleotides 285–294 in the enhancer (Fig. 1A). The part of the GM-CSF promoter containing the AML1 site has previously been shown to act as a negative element for GM-CSF transcription in several cell types, including the Jurkat T cell line (34). In accordance with these results, mutation of both AML1 sites gave a 4.2 ± 0.4 -fold increase in transcription from the GM-CSF promoter/enhancer in Jurkat cells (Fig. 1B). Expression of Δ CN, a C-terminally truncated and thereby Ca²⁺/calmodulin-independent constitutively active A subunit of CN, activated the wild type GM-CSF reporter \sim 2-fold in Jurkat, whereas it had no significant effect on the AML1 site-mutated reporter (Fig. 1B, cf. bars 1, 2, 13, and 14). Thus, the constitutively active CN subunit displayed AML1 site-dependent activation of the GM-CSF promoter/enhancer.

To analyze whether the AML1 content of Jurkat cells was

limiting for the effect of AML1 or Δ CN, we cotransfected an expression plasmid for mouse AML1b (26), the longest murine splice form of AML1 (35). This overexpression of AML1b had only a marginal effect on transcription from the GM-CSF promoter/enhancer (Fig. 1B, bar 7). In contrast, coexpression of AML1b and Δ CN resulted in 9.0 ± 1.1 -fold activation (Fig. 1B, bar 8). This synergy was dependent on the AML1 sites because even upon AML1 overexpression the effect of Δ CN was hardly significant on the AML1 site-mutated reporter (Fig. 1B). Thus, coexpression of AML1b and Δ CN made the AML1 sites positive instead of negative for the activity of the GM-CSF promoter/enhancer.

AML1 was not the only member of the Runx transcription factor family whose activity was improved by CN. Expression of mouse Runx2 also resulted in synergy with Δ CN, albeit to a lower level than for AML1b (Fig. 1B).

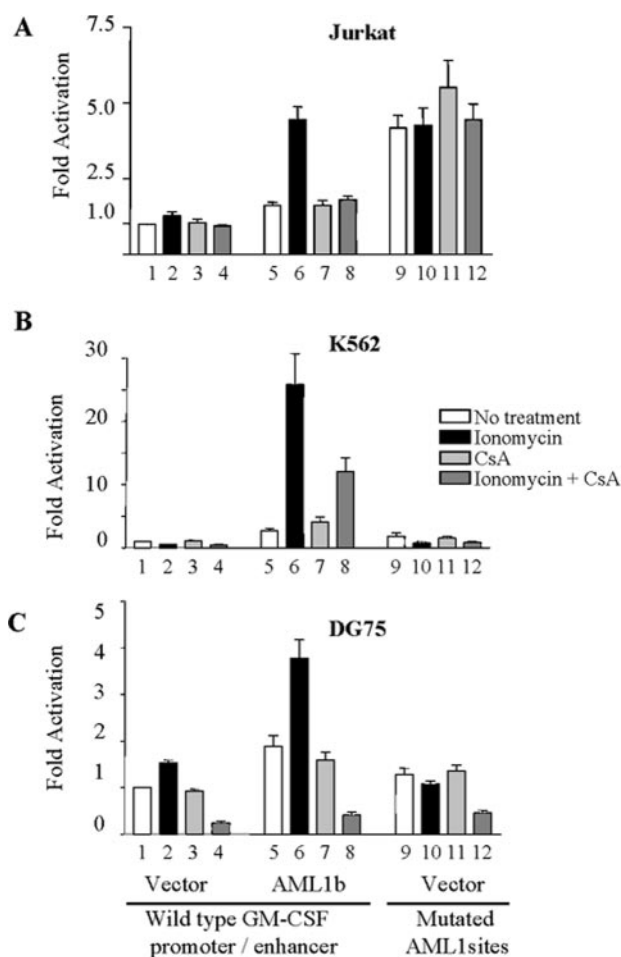
The synergistic activation required the transactivating domains in the C-terminal part of AML1 because AML1a, a shorter AML1 splice variant containing only 73 amino acids C-terminal to the RHD, showed no synergy with Δ CN (Fig. 1B). Furthermore, AML1a blocked the activation by the Δ CN expression vector, indicating that it inhibited the synergy of Δ CN with endogenous AML1 or a related factor(s).

To determine the importance of the catalytic activity of Δ CN, we constructed a derivative, Δ CN_{151N}, containing a His¹⁵¹ \rightarrow Asn substitution in the active site. Mutations of this critical histidine have been shown to result in the loss of several orders of magnitude in the activity of CN and related phosphatases (36–38). Δ CN_{151N} retained only a very small part of the strong synergistic effect of Δ CN (Fig. 1B), showing that the phosphatase activity of CN is important for its effect on AML1 activation. The synergism between Δ CN and AML1 proteins was also blocked by treatment with the CN inhibitor cyclosporin A (CsA; data not shown), further supporting that CN requires phosphatase activity to achieve this synergistic effect.

The effects of Δ CN and AML1b were also studied in the early erythroleukemia cell line K562. This cell line was selected because, in contrast to B and T cell lines, it shows low expression of Runx proteins (39–41). AML1b overexpression increased the activity of the GM-CSF promoter/enhancer and turned the AML1 sites from being negative to being positive for the activity of the reporter. Importantly, Δ CN had no significant effect on transcription of the GM-CSF reporter in the absence of cotransfected AML1b expression plasmid in this cell line (Fig. 1C), further supporting that the activation by constitutively active CN was dependent on synergy with AML1 or another Runx protein. Moreover, cotransfection of AML1b and Δ CN expression plasmids gave 33.3 ± 3.6 -fold activation in K562. Catalytically active CN was required for the synergy also in K562 cells because Δ CN_{151N} had little effect.

These results show that a constitutively active catalytic CN subunit, Δ CN, can activate the GM-CSF promoter/enhancer in synergy with AML1 and Runx2. Furthermore, overexpression of the regulatory B subunit of CN also activated the GM-CSF promoter/enhancer in synergy with AML1 (data not shown).

Calcineurin-dependent Ca²⁺ Activation of the GM-CSF Promoter/Enhancer by AML1—To analyze whether the GM-CSF promoter/enhancer can also be activated through AML1 by a Ca²⁺ signal, we studied the effects of AML1 and mutation of the AML1 binding sites on the Ca²⁺ activation. The expression of GM-CSF or a reporter driven by the GM-CSF promoter/enhancer is strongly up-regulated upon T cell activation or mimicking of T cell activation by treatment with phorbol ester plus Ca²⁺ ionophore (42, 43). Interestingly, the negative effect of the AML1 sites of the promoter/enhancer in Jurkat cells (Fig. 1B) was to a large extent relieved upon mimicking T cell acti-



stimulation of transcription of the GM-CSF promoter/enhancer independent of PMA costimulation.

The transfection analysis in K562 cells showed that ionomycin did not induce the GM-CSF reporter in this cell line but instead resulted in a negative effect that was not mediated by CN because it was not blocked by the CN inhibitor CsA (Fig. 2B). The negative CN-independent Ca^{2+} effect was also observed when the AML1 sites were mutated, suggesting that this was not the result of AML1. A negative AML1 site-independent and CN-independent Ca^{2+} effect was also present in DG75 cells (Fig. 2C). Our recent analysis of the CN-independent Ca^{2+} inhibition of GM-CSF has shown that this inhibition is caused by Ca^{2+} /calmodulin-dependent kinase II phosphorylation of serines in the autoinhibitory domain for DNA binding of the transcription factor Ets1 (28).

To analyze whether the AML1 content of the cells was limiting for the AML1 site-dependent Ca^{2+} stimulation, we co-transfected the expression plasmid for AML1b. Overexpression of AML1b increased transcription from the GM-CSF promoter/enhancer in all three cell lines, but the increases were relatively small in the absence of a Ca^{2+} signal (Fig. 2). However, they were dramatically potentiated by an increase in intracellular Ca^{2+} . To address whether CN was involved in the Ca^{2+} stimulations, we analyzed whether the CN inhibitor CsA could block them. The Ca^{2+} inductions in Jurkat and DG75 cells and the Ca^{2+} inductions when AML1b was overexpressed in all three cell lines were CsA-sensitive (Fig. 2), showing that CN is important for the positive AML1-dependent calcium effect in all of these cell lines.

We conclude that in addition to the previously described CN-dependent Ca^{2+} stimulation of GM-CSF transcription through NF-AT, AP-1, and NF- κ B and the CN-independent Ca^{2+} inhibition of GM-CSF through Ets1 phosphorylation, there is also a CN-dependent Ca^{2+} stimulation through synergy with AML1. The levels of both the CN-dependent Ca^{2+} stimulation in synergy with AML1 and the CN-independent Ca^{2+} inhibition showed a big variation between the cell lines (Fig. 2). For example, AML1b overexpression in the presence of ionomycin gave a dramatic 55-fold increase in transcription in K562 (*cf. bars 2 and 6* in Fig. 2B), showing that the synergy between AML1 and CN can be very strong.

Direct CN Interaction with AML1—To search for a mechanism for the synergy of CN with AML1 and Runx2, we analyzed whether CN could interact directly with these proteins. Unfortunately, full-length AML1 and Runx2 are highly protease-sensitive and unstable (32, 44) and have not been possible to express heterologously (Ref. 10 and data not shown). Therefore, we expressed mouse AML1 and Runx2 derivatives with C-terminal truncations just after the Runt domain (amino acids 1–185 of AML1 and 1–228 of Runx2), denoted Δ AML1 and Δ Runx2, respectively, in *E. coli*, purified the proteins, and prepared affinity columns with them (see “Experimental Procedures”). Bovine brain CN bound to the Δ Runx2 and Δ AML1 affinity columns (Fig. 3A, lanes 3 and 4, respectively), whereas CN did not bind to a control BSA affinity column (lane 2). The specific binding occurred in the absence of calmodulin, and it was independent of Ca^{2+} because it occurred even in the presence of EDTA (Fig. 3A). Thus, the interaction with AML1 and Runx2 was not the process in the synergy of CN with these proteins that was dependent on Ca^{2+} and on the enzymatic activity of CN.

To exclude the possibility that the purified brain CN contained an impurity mediating the interaction, both a C-terminally truncated catalytic subunit, Δ CN, and the regulatory Ca^{2+} binding CNB subunit (Fig. 3A) were expressed in *E. coli*, and the proteins were purified. Both Δ CN and CNB specifically

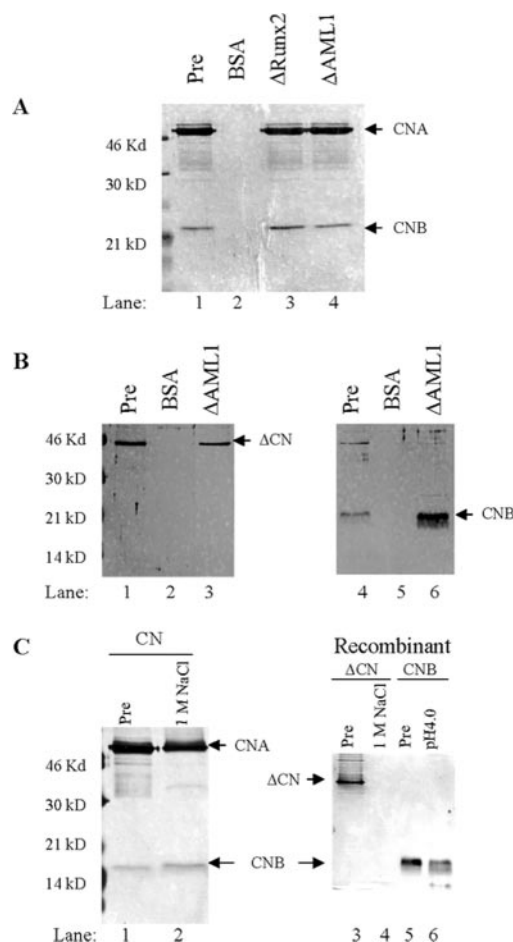


FIG. 3. Specific binding of CN to C-terminally deleted Runx2 (Δ Runx2), AML1 (Δ AML1), and the runt homology domain of AML1 (Δ AML1_{RHD}). A, binding of the catalytic subunit (CNA) and regulatory subunit (CNB) of bovine brain CN to Δ Runx2 and Δ AML1 columns but not to a BSA column. Preloaded CN (25% of loading) (lane 1) and the 1 M NaCl eluates from BSA (lane 2), Δ Runx2 (lane 3), and Δ AML1 (lane 4) columns were separated by SDS-PAGE and visualized by silver staining. B, binding of *E. coli*-produced and Ni-NTA-agarose-purified CN subunits Δ CN (lanes 1–3) and CNB (lanes 4–6) to Δ AML1. Preloaded calcineurin subunit (20% of loading) was added in lanes 1 and 4; lanes 2 and 5 contain the eluates from the BSA column, and lanes 3 and 6 contain the eluates from the Δ AML1 column. Elutions were with 1 M NaCl for Δ CN and with 100 mM sodium acetate, pH 4.0, 0.5 M NaCl for CNB. C, analysis of binding of bovine brain CN (lanes 1 and 2) and *E. coli*-produced and Ni-NTA-agarose-purified Δ CN (lanes 3 and 4) and CNB (lanes 5 and 6) to AML1_{RHD} (amino acids 47–185). Lanes 1, 3, and 5 contain preloaded proteins (10% of loading), lanes 2 and 4 contain 1 M NaCl eluates, and lane 6 contains 100 mM sodium acetate, pH 4.0, 0.5 M NaCl eluate from the AML1_{RHD} column separated by SDS-PAGE and visualized by silver staining. The protein-binding buffer was 25 mM HEPES, pH 8.0, 10% glycerol, 100 mM NaCl, 1 mM EDTA, 0.05% Triton X-100.

bound to the Δ AML1 affinity column (Fig. 3B, lanes 3 and 6), whereas neither of the proteins displayed any binding to a control BSA column (lanes 2 and 5). Only a few percent of the Δ CN bound at these conditions (lane 3), whereas the binding of the CNB subunit was very strong because almost all CNB bound, and in this case 1 M NaCl gave only a partial elution (data not shown), and low pH elution (pH 4.0) was used instead (lane 6).

An affinity column with the isolated Runt domain of AML1 (amino acids 47–185) bound bovine brain CN (Fig. 3C, lane 2) and *E. coli*-produced CNB (lane 6), whereas Δ CN did not bind to the isolated Runt domain (lane 4), showing that the 46 N-terminal amino acids of AML1 are necessary for its weak interaction with Δ CN. Furthermore, a lower proportion of the A

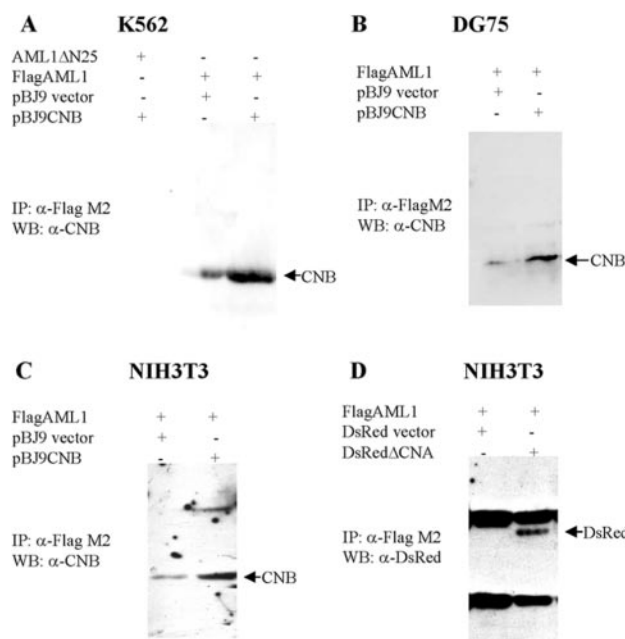


FIG. 4. Coimmunoprecipitation of calcineurin with AML1. A–C, the indicated cell lines were transiently transfected with pBJ9 plasmid derivatives expressing the B subunit of calcineurin (pBJ9CNB), AML1 with the N-terminal 25 amino acids replaced with two FLAG sequences (FlagAML1), the corresponding AML1 lacking the FLAG (AML1AN25) and/or the pBJ9 expression vector control plasmid as indicated. Equal amounts of whole cell extracts were subjected to immunoprecipitation (IP) with the monoclonal anti-FLAG antibody M2 (see “Experimental Procedures”). Coimmunoprecipitated CNB was detected by Western blot (WB) analysis with anti-CN antibody. D, NIH3T3 cells were transiently transfected with a DsRed vector plasmid derivative expressing the DsRed-fused constitutively active A subunit of calcineurin (DsRed Δ CNA), FlagAML1, and/or the DsRed expression vector control plasmid as indicated. Immunoprecipitations were from whole cell extracts and with the monoclonal anti-FLAG antibody M2. Coimmunoprecipitated DsRed Δ CNA was detected by Western blot analysis with anti-DsRed antibody.

subunit than the B subunit of CN was eluted from the Runt domain column (*cf. lanes 1 and 2*). These results indicate that the interaction of the A subunit of CN with the Runt domain was at least partially indirect via the B subunit.

We conclude that CN can interact with the N-terminal Runt domain-containing part of AML1 and Runx2. At least in the case of AML1, the interaction is direct, and the interaction with the CNB subunit is very strong. The CNB subunit can also bind directly to the isolated Runt domain.

To analyze further whether AML1 could interact with CN, we performed coimmunoprecipitation experiments (Fig. 4). Because the interaction between purified AML1 and CN subunits could occur in the absence of Ca^{2+} (Fig. 3), we performed the coimmunoprecipitations without Ca^{2+} stimulation of the cells and with the chelator EDTA present during cell extract preparations (see “Experimental Procedures”). Commercially available anti-AML1 antibody did not perform well in coimmunoprecipitations (data not shown). Therefore, the coimmunoprecipitations were performed with FLAG-tagged AML1. For this purpose, a derivative of the AML1b expression plasmid that expressed AML1 with the N-terminal 25 amino acids replaced with a 17-amino acid sequence encoding two copies of the FLAG sequence was constructed (see “Experimental Procedures”). AML1 was found to interact efficiently with the regulatory B subunit of CN in the coimmunoprecipitation experiments in all cell lines tested, K562, DG75, and NIH3T3 (Fig. 4, A–C). The CNB band obtained was dependent on immunoprecipitation with AML1 because very little of the band was detected when AML1 lacking the two FLAGS was trans-

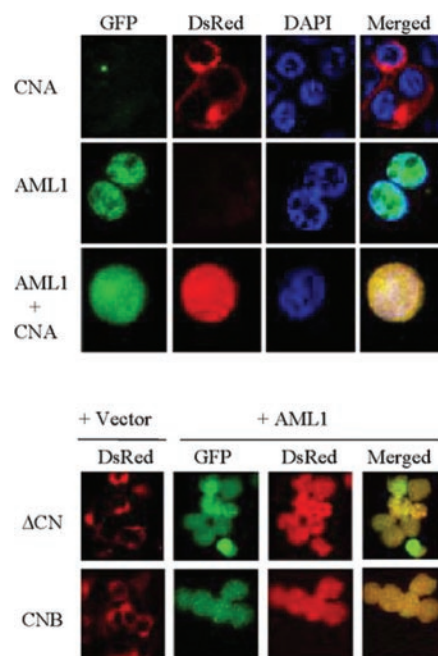


FIG. 5. Calcineurin recruitment to the nucleus by AML1. Confocal laser scanning microscopy of K562 cells transfected singly or in combination with expression vectors for GFP-fused AML1b and DsRed-fused CN derivatives. *Upper panel*, transfection of GFP-fused AML1b, DsRed-fused full-length CNA subunit, or cotransfection of both constructs. The cell nuclei were fluorescence stained blue with DAPI; and the GFP, DsRed, and DAPI fluorescence stainings were merged to the right. *Lower panel*, transfection of the DsRed-fused CNB subunit or constitutively active calcineurin A (Δ CN) together with GFP-fused AML1b expression plasmid or the vector control as indicated. The GFP and DsRed fluorescence stainings were merged to the right.

fected (Fig. 4A). Immunoprecipitation of CNB with FlagAML1 was obtained in the absence of CNB overexpression in all cell lines tested, and overexpression of CNB increased the coimmunoprecipitation of CNB in all of the cell lines (Fig. 4, A–C).

To analyze the interaction between the A subunit of CN and AML1, a constitutively active CN A subunit was expressed as a fusion protein with the red fluorescent protein DsRed, and anti-DsRed antibodies were used. In line with the weaker interaction of the A subunit than the B subunit with AML1 (Fig. 3), much weaker interaction with the A subunit was also seen in coimmunoprecipitations. However, coimmunoprecipitation of overexpressed A subunit of CN with AML1 was over background in some of the tested cell lines (Fig. 4D and data not shown). Ionomycin stimulation of the cells and the presence of Ca^{2+} instead of EDTA during extract preparation and immunoprecipitations did not further increase the coimmunoprecipitation of CNA (data not shown).

CN Recruitment to the Nucleus by AML1—The direct physical interaction observed between AML1 and CN *in vitro* and the coimmunoprecipitations prompted us to analyze whether the proteins could colocalize *in vivo* and whether they could influence the localization of each other. To address these questions, the localization of AML1 and CN was analyzed by confocal fluorescence microscopy in K562 cells. AML1b was expressed as an N-terminal fusion protein by coupling the cDNA with a DNA sequence coding for the green fluorescence protein EGFP, and the CN A and B subunits and the C-terminally truncated A subunit Δ CNA were N-terminally fused by coupling the cDNA with a DNA sequence coding for DsRed (see “Experimental Procedures”). As expected, when green fluorescent AML1b was expressed, the AML1 was found in the nucleus because the green fluorescence overlapped with the blue nuclear DAPI staining (Fig. 5A). Conversely, when red fluores-

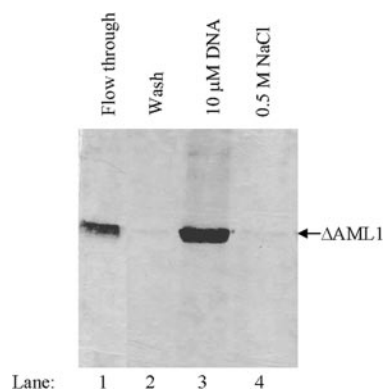


FIG. 6. DNA elution of *E. coli*-produced Δ AML1 from a CNB column. The flow-through (lane 1), the second wash (lane 2), the eluate with 10 μ M AML1 site containing DNA segment 5'-GGCCCCACCAGC-CAGACCACAGCCAGAC-3' (lane 3), and the subsequent eluate with 0.5 M NaCl (lane 4) were separated by SDS-PAGE and visualized by silver staining.

cent CNA, CNB, or Δ CNA was expressed, the red fluorescent CN was detected almost exclusively in the cytoplasm (Fig. 5, upper and lower panels). This is in agreement with previous reports that CN is a cytoplasmic protein in the absence of a Ca^{2+} signal and NF-AT, which can mediate nuclear entry of CN in the presence of a Ca^{2+} signal (45). Interestingly, the intracellular localization of both AML1 and CN was changed when AML1 and a CN subunit were coexpressed. The CN subunit was not only in the cytoplasm but was also present at about the same level in the nuclear compartment, and conversely the AML1 was not only in the nucleus but was also present at about the same level in the cytoplasmic compartment (Fig. 5, upper and lower panels). Thus, both AML1 and CN were localized in both the nucleus and the cytoplasm when AML1b and a CN subunit were coexpressed. These results show that AML1 can recruit both A and B subunits of CN to the nucleus where gene activation takes place. The recruitment occurred in the absence of any treatment increasing the intracellular Ca^{2+} concentration, suggesting that the CN recruitment to the nucleus was not dependent on Ca^{2+} loading of calmodulin or CN. The low level of intranuclear CN in the absence of EGFP-AML1b expression is not surprising considering the low level of expression of endogenous Runx proteins in K562 cells (40, 41) and the large transcriptional effect when AML1b overexpression is combined with ionomycin treatment or Δ CN coexpression in these cells (Figs. 1C and 2B).

DNA Binding Inhibits CN Interaction with AML1—Because AML1 could recruit CN to the nucleus and the DNA binding Runt domain could bind to CN, it raises the question of whether one interaction of the Runt domain excludes the other or whether the domain can bind simultaneously to both. The addition of CN neither inhibited the DNA binding of Δ AML1 or the isolated Runt domain in electrophoretic mobility shift assay analysis, nor did it supershift the protein-DNA complex (data not shown). Thus, no sign of CN inhibition of the DNA binding of the Runt domain or simultaneous DNA and CN binding was obtained. Instead, we found that Δ AML1 could be eluted efficiently from a CNB column by addition of a DNA segment containing an AML1 site (Fig. 6, lane 3). The elution was efficient because no further Δ AML1 could be eluted by a subsequent wash with 0.5 M NaCl (lane 4). Thus, CNB binding to Δ AML1 is blocked by a DNA segment that can bind to the Runt domain.

AML1-recruited CN Can Activate GM-CSF Expression through the Ets1 Site—The synergy between AML1 and CN was not generally found on AML1-controlled promoters. No effect of coexpression of Δ CN was seen on the AML1 activation of the germ line $I\alpha 1$ promoter, and only a small Δ CN effect was

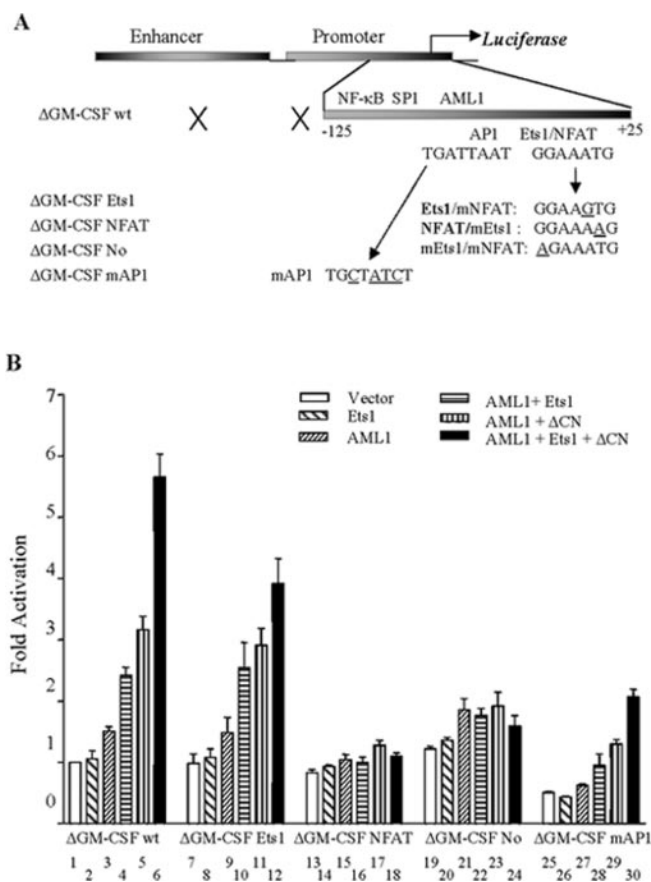


FIG. 7. The Ets1 site of the GM-CSF promoter is essential for transcriptional activation by AML1-recruited CN. A, mutations introduced in a luciferase reporter plasmid without GM-CSF enhancer and with the GM-CSF promoter sequences upstream of -125 deleted. The positions of the overlapping binding site for Ets1 and NF-AT and the sites for AP-1, AML1, SP-1, and NF- κ B (24, 25, 57–59) are indicated, and the mutations introduced are shown. *wt*, wild type. B, analysis of the synergy in transfected Jurkat cells between AML1 and the constitutively active Δ CN in transactivation of the deleted GM-CSF promoter. 5 μ g of AML1b, Δ CN, and/or Ets1 expression plasmid was added where necessary, and the empty vector was added where necessary to achieve a total of 15 μ g of expression vector in each transfection. The reporter plasmid contained either the GM-CSF promoter deleted to -125 or this promoter with mutations of the indicated transcription factor binding sites. In Δ GM-CSF Ets1, the Ets1 site is intact, and the NF-AT site is mutated; in Δ GM-CSF NFAT, the NF-AT site is intact, and the Ets1 site is mutated; in Δ GM-CSF No, both the Ets1 and NF-AT sites are mutated. Bars represent average luciferase activity expressed from the reporter plasmid in at least three independent transfections \pm S.E., using β -galactosidase expression from an hCMV- β -galactosidase plasmid for normalization.

obtained on the AML1 activation of the TCR β gene enhancer (data not shown). The differential effect of CN on AML1 activation of different promoters suggests that the target of the CN phosphatase is a component of differential importance in the various promoters/enhancers. However, the high number of transcription factor binding sites at the GM-CSF promoter/enhancer hampers identification of a transcription factor that is important for the differential synergy of AML1 and CN. To reduce the complexity, we analyzed whether AML1 and CN could still synergize in transactivation when the reporter contained no GM-CSF enhancer and the GM-CSF promoter had the sequences upstream of position -125 deleted. This deleted promoter contains one overlapping binding site for Ets1 and NF-AT and one site each for AP-1 and AML1 near this overlapping site, and it contains SP-1 and NF- κ B sites further upstream (Fig. 7A). Analysis in Jurkat cells showed that most of the synergy between AML1 and Δ CN on the GM-CSF pro-

motor/enhancer remained in this deleted GM-CSF promoter. The coexpression of AML1 and Δ CN resulted in a more than 3-fold increase in transcription, whereas AML1b alone had only a 1.5-fold effect (Fig. 7B, cf. bars 1, 3, and 5).

To analyze the role of the AP-1 transcription factor, which binds next to AML1, the AP-1 site was mutated (Fig. 7A). This mutation decreased the activity of the deleted promoter, but coexpression of AML1b and Δ CN still gave a much higher transcriptional activation compared with AML1b alone (Fig. 7B, bars 25, 27, and 29). Thus, the AP-1 site is not essential for the AML1/CN synergy. To analyze the role of Ets1 and NF-AT, which bind next to the AP-1 and AML1 sites, the overlapping Ets1/NF-AT site was mutated to become only an Ets1 site, only an NF-AT site, or without both the Ets1 and the NF-AT binding (Fig. 7A). The mutations (see "Experimental Procedures") of critical nucleotides for the interactions were designed based on the structural information on how Ets1 (46, 47) and NF-AT (48) interact with their specific DNA recognition sequence. Mutation of the site to become only an Ets1 site did not decrease the synergistic effect of combined overexpression of AML1 and Δ CN (Fig. 7B, cf. bars 7, 9, and 11). In contrast, most of the synergistic effect of coexpression of AML1 and Δ CN was lost both for the mutant promoter where the overlapping site was made into only an NF-AT site and for the mutant promoter with both the Ets1 and the NF-AT binding lost (Fig. 7B, cf. bars 13, 15, and 17 and bars 19, 21, and 23). This strongly favors that Ets1 is a transcription factor whose binding is important for the differential synergy of AML1 and CN at the GM-CSF promoter. We therefore overexpressed Ets1 either alone, together with AML1, or together with AML1 plus Δ CN. Ets1 had little effect when overexpressed alone, but Ets1 had a synergistic effect on the transcription from the reporters having a functional Ets1 binding site both when cotransfected with AML1 and with AML1 plus Δ CN (Fig. 7B, bars 2, 4, 6, 8, 10, 12, 26, 28, and 30). As expected, the synergistic effect of Ets1 together with AML1 or AML1 plus Δ CN was lost in the two GM-CSF promoter mutants without Ets1 binding site (cf. bars 14, 16, and 18 and bars 20, 22, and 24). Thus, the results favor that Ets1, but not NF-AT or AP-1, is essential for the synergistic activity of AML1 and CN on the GM-CSF promoter in Jurkat cells.

AML1-recruited CN Can Activate GM-CSF Expression through Counteraction of GSK-3—The protein kinase GSK-3 has a broad range of substrates. GSK-3 has, for example, been identified as a major kinase adding the phosphorylations on the transcription factor NF-ATc1 that are substrates of the CN phosphatase in the Ca^{2+} -dependent activation of NF-ATc1 (45, 49). We therefore asked whether GSK-3 was also relevant in the phosphorylation(s) that is reversed by CN in the activation of GM-CSF in synergy with AML1. Lithium ion and valproate are two different inhibitors of GSK-3 (50, 51). We analyzed the effects of these GSK-3 inhibitors on the transcriptional activity of the GM-CSF promoter/enhancer. Treatment with either inhibitor increased the activity of the GM-CSF promoter/enhancer 2–4-fold in DG75 cells both in absence and presence of overexpressed Ets1 and/or AML1 (Fig. 8). The similar effects of the two different GSK-3 inhibitors favor that their effects indeed are through their common inhibitory effect on GSK-3. Importantly, these inhibitors had no positive effect on the increased level of GM-CSF promoter/enhancer transcription when Δ CN was overexpressed together with AML1 or AML1 plus Ets1 (Fig. 8). This indicates that the GSK-3 inhibitors lithium and valproate inhibit the same phosphorylation(s) in the GM-CSF promoter/enhancer activation as that/those reversed by the CN phosphatase.

Dephosphorylation of GSK-3-phosphorylated Ets1 by CN—Our

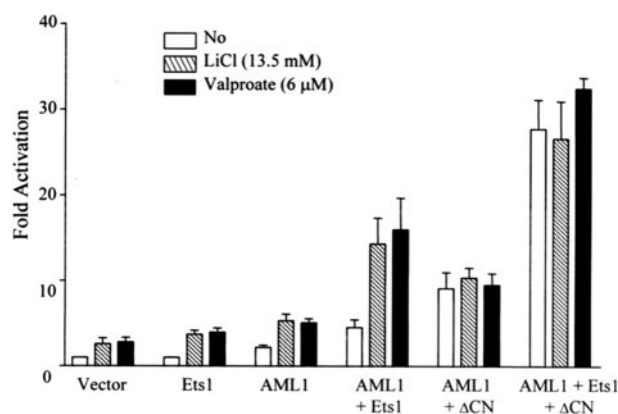


FIG. 8. Activation of GM-CSF expression by AML1-recruited CN through counteraction of GSK-3. The effects of the two GSK-3 inhibitors, lithium ion (13.5 mM LiCl) and valproate (0.9 μg/ml), on the transcriptional activity of the luciferase reporter with the GM-CSF promoter/enhancer without and with overexpression of AML1, Ets1, and/or the constitutively active Δ CN were analyzed. Empty vector was added where necessary to achieve a total of 15 μg of expression vector in each transfection. Either GSK-3 inhibitor increased the activity of the GM-CSF promoter/enhancer at all analyzed conditions except when the reporter was activated by Δ CN overexpression. Bars represent the average ratio of luciferase to β -galactosidase activity from three independent transfections \pm S.D., using β -galactosidase expression from an hCMV- β -galactosidase plasmid for normalization.

identification of Ets1 binding as important for the CN-AML1 synergy at the GM-CSF promoter together with the finding that GSK-3 inhibitors have the same effect as Δ CN overexpression on the GM-CSF promoter/enhancer *in vivo* prompted us to analyze whether GSK-3 could phosphorylate Ets1. Ets1 was indeed phosphorylated efficiently by *E. coli*-produced GSK-3 β (Fig. 9, A and B). His-tagged Ets1 m3, with the four Ca^{2+} /calmodulin-dependent kinase II target serines in the autoinhibitory domain mutated to alanines (Fig. 9F), was also phosphorylated efficiently with GSK-3 β *in vitro* (Fig. 9, A and B). To analyze whether the GSK-3 β -phosphorylated Ets1 could be targeted by CN, *in vitro* CN dephosphorylation analyses were performed. GSK-3 β -phosphorylated Ets1 could be partially dephosphorylated by treatment with CN (Fig. 9E), suggesting that Ets1 contains both GSK-3 β phosphorylation sites that can be targeted by CN and sites that cannot. To localize further the CN-sensitive GSK-3 β phosphorylation site(s), a C-terminally deleted Ets1 containing the N-terminal 250 amino acids of the protein was prepared. This deleted Ets1, Δ C, was phosphorylated efficiently by GSK-3 β (Fig. 9, C and D). Interestingly, the Ets1 Δ C mutant was also dephosphorylated efficiently by CN (Fig. 9E). Thus, Ets1, which is important for the GM-CSF promoter activation by AML1-recruited CN, contains a GSK-3 β phosphorylation site(s) in the N-terminal 250 amino acids that can be dephosphorylated efficiently by CN activation.

DISCUSSION

In this report we have shown that the two subunits of CN interact directly with the N-terminal RHD-containing part of AML1 and Runx2 and that constitutively active CN together with AML1 or Runx2 synergistically activates the GM-CSF promoter/enhancer. Efficient synergy requires both the catalytic activity of CN and the C-terminally located transactivating parts of AML1. We have also shown that relief of the negative effect of the AML1 sites is important for Ca^{2+} activation of the GM-CSF promoter/enhancer and that AML1b overexpression increases this Ca^{2+} activation.

CN has previously been shown to interact physically with members of the NF-AT family of transcription factors (37, 52). The interaction between CN and NF-AT is believed to occur in

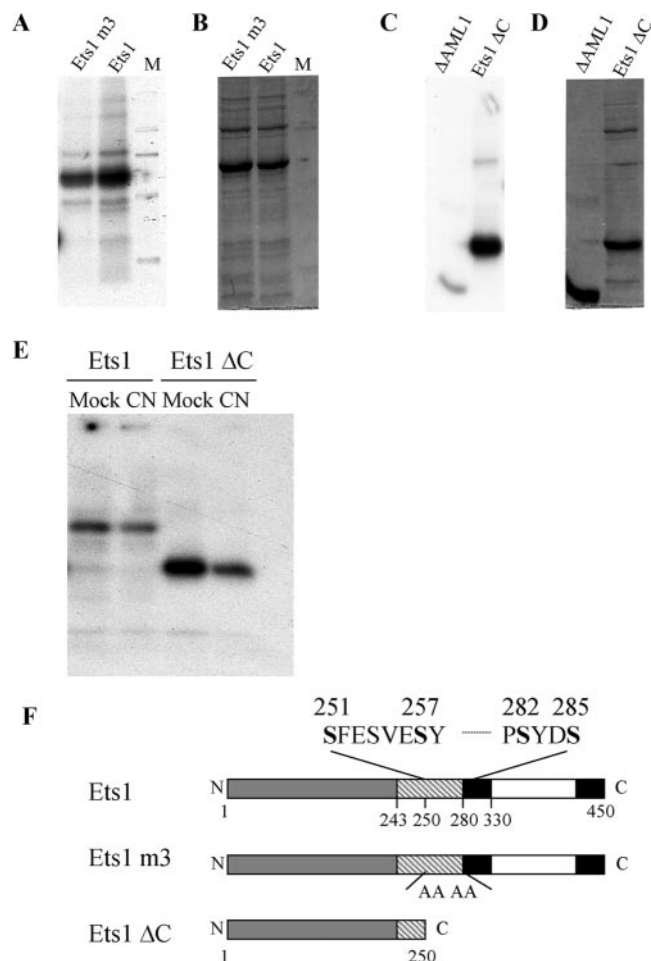


FIG. 9. Phosphorylation of Ets1 by GSK-3 β and dephosphorylation by calcineurin. *A–D*, phosphorylation of Ets1 wild type, Ets1 m3, and Ets1 Δ C mutants by GSK-3 β . Equal amounts of the N-terminal half of AML1 (Δ AML1) (26) were incubated with the GSK-3 β as a negative control. *M* indicates lanes with marker proteins. *A* and *C*, autoradiography of the proteins phosphorylated with [γ - 32 P]ATP. *B* and *D*, Coomassie Blue staining of the proteins. *E*, dephosphorylation of GSK-3 β -phosphorylated Ets1 wild type and Ets1 Δ C by calcineurin (CN) (see “Experimental Procedures”) with the mock treated proteins as controls. *F*, schematic drawing of the structures of the Ets1 wild type, Ets1 m3, and Ets1 Δ C proteins. The Ets domain of Ets1 is *nonfilled*, and the domains regulating DNA binding of the Ets domain are *solid* or *hatched*.

the cytoplasm and lead to a dephosphorylation of NF-AT and nuclear translocation of a CN·NF-AT complex. Both the dephosphorylation and the nuclear translocation are dependent on CN activity because it can be blocked by CsA or FK506 (37). However, the physical interaction between NF-AT and CN is independent of the phosphorylation status of NF-AT (49, 52).

In analogy with NF-AT, the AML1 synergy with CN is blocked by CsA, supporting that the phosphatase activity of CN is required for relief of the negative effect of the AML1 sites and activation of GM-CSF. However, the physical interaction between AML1 and the A and B subunits of CN *in vitro* is independent of both Ca $^{2+}$ and calmodulin, and it does not require CN activity. Likewise, AML1 interacted with the CN subunits in coimmunoprecipitation experiments performed without Ca $^{2+}$ stimulation of the cells and with the chelator EDTA present during cell extract preparations (Fig. 4). Confocal microscopy analysis of cells expressing fluorescence-tagged protein derivatives showed that CN can be recruited to the nucleus by overexpression of AML1 *in vivo*. This suggests that AML1 recruits CN to the nucleus analogous to the case of NF-AT. The alternative, that AML1 overexpression recruits

CN to the nucleus and to the control region of the GM-CSF gene indirectly through a neighboring protein at the GM-CSF promoter/enhancer instead of through the AML1 interaction, cannot be excluded, but it appears less likely. The most likely candidate protein for such an alternative hypothesis would be NF-AT because of its known binding both to the GM-CSF promoter (24, 25, 53) and to CN (37, 52) and the known important role of NF-AT as a CN target (45, 49). However, the AML1-CN synergy remained with a reporter plasmid having a core GM-CSF promoter with the NF-AT site point mutated (Fig. 7), further arguing against this hypothesis. Thus, our data favor that the physical interaction between AML1 and CN is a necessary prerequisite for the efficient synergy seen *in vivo*.

Is the mechanism of the CN synergy with AML1 the same as for the synergy with NF-AT? This is highly unlikely because AML1, as reported previously (54), is a nuclear protein even in the absence of any calcium signal or CN activation (Fig. 5). The activity of the Ca $^{2+}$ /calmodulin-dependent phosphatase was important for the AML1-CN synergy because most of the synergy was lost in an inactive mutant, Δ CN $_{151N}$, replacing a critical histidine in the active site. The results therefore support the notion that the physical interaction of CN with AML1 results in a dephosphorylation improving a functional interaction or cooperation of AML1 *in vivo*. What could be improved by the dephosphorylation? It could be the binding of AML1 to DNA and/or to CBF β /PEBP2 β that improves the DNA binding. Because most of the CN effect *in vivo* is dependent on its catalytic activity, a CN effect on the DNA or CBF β binding would imply the presence *in vivo* of a phosphorylation that is inhibitory for that binding. However, we have no evidence for such an inhibitory phosphorylation, although we can at present not exclude its existence. Furthermore, a positive effect of CN on the DNA binding of AML1 would not be expected to be promoter-specific, and also the positive effect of CBF β on the DNA binding of AML1 has not been found to be promoter-specific, whereas the CN-AML1 synergy found here shows promoter specificity. No CN effect was seen on AML1 activation of the germ line I α 1 promoter, and only a small CN effect was obtained on AML1 activation of the TCR β gene enhancer (data not shown). The differential CN effect on AML1 activation of different promoters therefore supports the notion that dephosphorylation by CN improves the collaboration of AML1 with a component specific for activation of the GM-CSF promoter.

AML1 has to collaborate with a number of other proteins involved in the transcriptional activation process at the GM-CSF promoter/enhancer. One of these is the Ets1 transcription factor because the GM-CSF promoter contains several Ets1 binding sites. Ets1 is a member of the Ets family, which plays an important role in regulation of critical genes in cell proliferation, development, differentiation, apoptosis, angiogenesis, and transformation. Ets1 is highly expressed in the B and T lymphocyte lineages and is important for their homeostasis, differentiation, and activation (55, 56). Ets1 can transactivate the GM-CSF promoter in Jurkat T cells stimulated with a Ca $^{2+}$ ionophore and phorbol ester (57), and it can also up-regulate GM-CSF expression in mast cells (58). Ets1 stimulates the GM-CSF promoter in a synergistic relationship with NF- κ B and AP-1 (24, 59). In this report we have shown that a functional Ets1 binding site is essential for the synergy between AML1 and CN on the GM-CSF promoter in Jurkat T cells. Ets1 has many phosphorylation sites that include a mitogen-activated protein kinase phosphorylation site and several Ca $^{2+}$ /calmodulin-dependent kinase II phosphorylation sites as well as sites where the phosphorylating kinase and the role of the phosphorylation have not been determined (60). Here we report that inhibitors of the protein kinase GSK-3 β gave the same

increase in GM-CSF transcription as did expression of constitutively active CN (Fig. 8). Furthermore, Ets1 could be phosphorylated by GSK-3 β *in vitro*, and this phosphorylation could be dephosphorylated by CN (Fig. 9). The results suggest that GSK-3 β -phosphorylated Ets1 is a target of AML1-recruited CN phosphatase at the GM-CSF promoter. The identification of GSK-3 as a key kinase in this regulation is in line with previous reports that GSK-3 is a key kinase counteracting CN in T lymphocyte activation through NF-AT (45, 49). Furthermore, GSK-3 has been shown to be a negative regulator of lymphocyte activation (61), and costimulation of T cells by CD28 or by endothelial cells has been shown to activate NF-AT through inactivation of GSK-3 β (62, 63).

Ets1 is known to be able to interact physically with Runx proteins (57, 64, 65), and Ets1 and Runx proteins reciprocally relieve autoinhibition of the DNA binding of the other protein (65–67). However, the presence of both Ets and AML1/Runx protein binding sites at a promoter does not appear to be a sufficient prerequisite for the synergy with CN at the promoter because AML1 and Ets proteins function in synergy also at the I α 1 promoter (26) where no synergy between CN and AML1 was found (data not shown). It is possible that the main difference between the GM-CSF promoter and the I α 1 promoter causing the differential synergy with CN is the presence of additional nucleotides between the Runx and Ets sites in the GM-CSF promoter. Whereas the Runx and Ets binding sequences are directly next to each other at the I α 1 promoter (26), enabling direct contact between Ets1 and AML1/Runx2, additional nucleotides at the GM-CSF promoter constitute a binding site for AP-1, and together, the AP-1 and Ets binding sites constitute the important CLE0 element (24, 59). Our results may indicate that CN activity is not needed at a promoter where Ets1 functions in direct physical interaction and reciprocal relief of autoinhibition with a Runx protein, whereas when AML1 cooperates with nearby AP-1, then Ets1 is dependent on activation through CN.

In addition to NF-AT, also another eukaryotic transcription factor, Elk1, has previously been reported to be a CN target (68, 69). Interestingly, Elk1 is like Ets1 belonging to the Ets family of transcription factors. However, dephosphorylation by CN has an opposite effect on Elk1 compared with the activating effects on NF-AT and Ets1 because Elk1 is activated by phosphorylation through different mitogen-activated protein kinase cascades, and activation of CN leads to a direct dephosphorylation of these phosphorylations of Elk1 and thereby inhibition of its activity (69).

Ca²⁺ signaling has previously been reported to regulate positively transcription from the GM-CSF promoter/enhancer through CN activation of the transcription factors NF-AT, NF- κ B, and AP-1 (24, 25) and negatively through Ca²⁺/calmodulin-dependent kinase II phosphorylation of serines in the autoinhibitory domain for DNA binding of Ets1 (28). In the present study, we have shown that Ca²⁺ signaling can regulate GM-CSF transcription also through dephosphorylation that activates Ets1 by AML1/Runx protein-recruited CN. It is interesting that the Ca²⁺ second messenger can elicit many different effects on transcription of the same gene. The TCR or B cell receptor activation-induced Ca²⁺ signaling shows both frequency and amplitude modulation. In naive B cells, foreign antigen triggers a large biphasic Ca²⁺ response, whereas the basal Ca²⁺ level is increased, and self-antigen stimulates low Ca²⁺ oscillations in tolerant B cells (70). Correspondingly, the basal Ca²⁺ concentration is elevated in T cells induced to anergy, and when T cell anergy is induced by altered peptide ligands the Ca²⁺ responses are of comparatively low amplitude (71–74). Furthermore, TCR-stimulated T cells show Ca²⁺ os-

cillations with a period of ~1.5 min (71–74). Moreover, immature B cells have a higher amplitude Ca²⁺ response to antigen than mature B cells, and α -hemolysin of uropathogenic *E. coli* induces Ca²⁺ oscillations with a period of ~12 min in renal epithelial cells (75, 76). Therefore, because differential Ca²⁺ signaling plays a key role in the distinct responses to self and nonself, it is not surprising that Ca²⁺ can regulate transcription of a cytokine in many ways. It is notable in this context that each of the three CN-activated transcription factors previously reported to participate in GM-CSF activation, NF-AT, AP-1, and NF- κ B (24, 25), displays a different pattern of amplitude and frequency modulation in response to Ca²⁺ signaling (77, 78).

Taken together, we show that AML1 and Runx2 are targets of Ca²⁺ regulation of GM-CSF through CN. The CN activation with AML1 and Runx2 is mediated through a novel mechanism, and we show data suggesting that it involves dephosphorylation of Ets1 at the GM-CSF promoter/enhancer at least in Jurkat T cells. The findings provide a starting point for studies to elucidate the role that Ca²⁺ activation through CN might play in other regulatory systems controlled by Runx proteins.

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