The Hematopoietic Transcription Factor AML1 (RUNX1) Is Negatively Regulated by the Cell Cycle Protein Cyclin D3

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The family of cyclin D proteins plays a crucial role in the early events of the mammalian cell cycle. Recent studies have revealed the involvement of AML1 transactivation activity in promoting cell cycle progression through the induction of cyclin D proteins. This information in combination with our previous observation that a region in AML1 between amino acids 213 and 289 is important for its function led us to investigate prospective proteins associating with this region. We identified cyclin D3 by a yeast two-hybrid screen and detected AML1 interaction with the cyclin D family by both in vitro pull-down and in vivo coimmunoprecipitation assays. Furthermore, we demonstrate that cyclin D3 negatively regulates the transactivation activity of AML1 in a dose-dependent manner by competing with CBF β for AML1 association, leading to a decreased binding affinity of AML1 for its target DNA sequence. AML1 and its fusion protein AML1-ETO have been shown to shorten and prolong the mammalian cell cycle, respectively. In addition, AML1 promotes myeloid cell differentiation. Thus, our observations suggest that the direct association of cyclin D3 with AML1 functions as a putative feedback mechanism to regulate cell cycle progression and differentiation.

AML1, also known as RUNX1, CBFA2, or PEBP2 α B, has an important role in hematopoiesis and leukemogenesis (45). Its involvement in the development of blood cells is exemplified by its regulation of various myeloid and lymphoid promoters and enhancers (26). Its crucial importance was recognized in AML1^{-/-} mice, which display no definitive hematopoiesis (35, 51). In addition, AML1 is commonly found in chromosomal translocations in both myeloid and lymphoid leukemias (40). Furthermore, AML1 was shown to regulate the cell cycle by shortening the G₁/S phase in hematopoietic cells through the binding and induction of cyclin D promoters (4, 46). Subsequently, this function of AML1 was shown to require its C-terminal transactivation domains (3). In addition, AML1 is known to be involved in the differentiation of hematopoietic cells (48) and in promoting senescence in a p53-dependent fashion in primary mouse fibroblasts (54). Thus, AML1 seems to have a dual role in promoting cell cycle progression and differentiation, which could be dependent on the presence of different factors that interact with it during each stage of the development of a cell.

The regulation of the cell cycle is controlled by a combination of cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitors, which together with the tumor suppressor retinoblastoma (Rb) are involved in the tight control of the cell cycle machinery. Cyclin D proteins function as holo-enzymes when

complexed with Cdk4 and Cdk6, which promote the phosphorylation of Rb. The hypophosphorylated Rb proteins (Rb, p107, and p130) are known to inhibit the function of the E2F proteins, which promote the transcription of factors essential for DNA synthesis (41). Thus, phosphorylation by the cyclin D-Cdk complexes relieves inhibition by Rb, promoting the entry of cells into S phase. More-recent observations have implicated the cyclin D proteins as being not only cell cycle regulators but also transcription regulators. This is exemplified by the association of cyclin D proteins with the transcription factor DMP1 (13), which inhibits transactivation by DMP1 (17).

Cyclin D proteins have also been characterized as oncogenes (1) due to observations of amplification (19) and overexpression in a variety of tumors (7, 15, 28) or by in vitro overexpression studies (5, 9). Furthermore, cyclin D3 is specifically associated with t(6;14) in patients with B-cell malignancies (44). Thus, cyclin D proteins are involved in the tumorigenesis of various human malignancies.

AML1 is known to regulate promoters of various myeloid genes, such as macrophage colony-stimulating factor (CSF) receptor, granulocyte-macrophage CSF (GM-CSF), interleukin 3, neutrophil elastase, and myeloperoxidase and promoters/enhancers of lymphoid genes, such as the B-lymphoid kinase (BLK) promoter and enhancers of T-cell receptor α and immunoglobulin α (Ig α) (reviewed in references 2, 31, and 49). Our previous studies identified a region of AML1 between amino acids (aa) 268 and 289 that plays a critical role in regulating AML1 activity (36). To understand the molecular mechanism of AML1 function in activating gene expression, we performed yeast two-hybrid studies to identify proteins that associate with a region encompassing aa 213 to 289 of AML1 using a cDNA library prepared from the hematopoietic cell line EML (50). We determined that the cell cycle regulator

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cyclin D3 bound directly to AML1. We further showed that all three cyclin D proteins associated with AML1 and that the Runt homology domain of AML1 is also involved in the interaction with cyclin D. Interestingly, cyclin D3 worked as a negative regulator of AML1 in transactivation studies; cyclin D3 competed with core binding factor β (CBF β) for binding to AML1 and diminished AML1 affinity for DNA. AML1 is known to directly regulate cyclin D gene expression (4, 46). These observations suggest a mechanism of feedback regulation of AML1 transactivation by the G₁ cyclin-cyclin D proteins themselves and suggest a timely balanced regulation of cell division and differentiation by AML1 and cyclin D.

MATERIALS AND METHODS

Reagents and plasmids. The p(Mono)₁TK81-luc and pBLK-luc reporter constructs pCMV5-AML1, pCMV-CBF β , and pCMV-C/EBP α were described previously (25, 36, 56). The pCMV-RL and pNull-RL constructs express the *Renilla* luciferase protein under the cytomegalovirus (CMV) promoter and no promoter, respectively (Promega). pcDNA6-HA-AML1 was made by cloning the NotI-ApaI fragment from pBS-HA-AML1-ETO and the ApaI-XbaI fragment from pCMV5-AML1 into NotI-XbaI-digested pcDNA6/V5-HisB (Clontech), which expresses full-length AML1. Hemagglutinin (HA)-tagged mutant AML1 expression construct pcDNA6-HA-AML1(1-289) was generated by NotI-ApaI digestion of pcDNA6-HA-AML1 and ApaI-XbaI digestion of pCMV5-AML1(1-289) and by the ligation of NotI-ApaI and ApaI-XbaI fragments into NotI-XbaI-digested pcDNA6/V5-HisB. HA-tagged mutant AML1 expression construct pcDNA6-HA-AML1(1-213) was generated by EcoRI-HindIII digestion of pcDNA6-HA-AML1 and HindIII-BamHI digestion of pCMV5-AML1(1-213) and by the ligation of the EcoRI-HindIII and HindIII-BamHI DNA fragments into EcoRI-BamHI-digested pcDNA6/V5-HisB. AML1 mutant numbering is based on the AML1B numbering system. pCMV5-AML1(1-381), pCMV5-AML1(1-289), and pCMV5-AML1(1-213) were kindly provided by Scott Hiebert. pRcCMV-cyclin D3-HA, pRcCMV-cyclin D2-HA, and pRcCMV-cyclin D1-HA were kindly provided by Mark Ewen, and pRSV-cyclin D3 was provided by Charles Sherr. The glutathione *S*-transferase (GST)-AML1 fusion protein expression constructs were generated by PCR of various AML1 fragments and subcloning them into either pEBG (33) for mammalian expression or pGEX-2T GST-expressing vectors (Amersham-Pharmacia) for bacterial expression. pEBG-Runt and pEBG-AML1(315-395) were made by PCR using the primer sets 5' CGCGATCCGGCGAGCTGGTGC-3' and CCGATGCGG CCGCgaattcTGCCGATGCTCTCGAG and 5'-CGGGATCCCTGCAGAACTTT CCAGT-3' and CCGATGCGGCGCGgaattcTACGGGCGCTCCCTGCGCT, respectively, digested with BamHI and NotI, and cloned into pEBG-BamHI/NotI. pEBG-213-395 was constructed with the primer set 5' CGCAGATCTCAGACC AAGCCCGGGAG and the same 3' oligonucleotide used for pEBG-315-395, cut with BglII-NotI, and cloned into pEBG-BamHI-NotI. pGEX-Runt was made with the primer set 5' CGCGATCCGGCGAGCTGGTGC and CCGATGCGGCG CgaattcTGCCGATGCTCTCGAG and cloned into pGEX-2T-BamHI/EcoRI. pGEX-AML1(88-381) was constructed by cutting pGEX-2T-Runt with HindIII and EcoRI and ligating the HindIII-EcoRI fragment from pCMV5-AML1(1-381) into pGEX-Runt-HindIII/EcoRI. Boldface and underlining denote the endonuclease restriction sites present in the primers. p3XFlag-CBF β was constructed by PCR using the following primers for the PCR: 5'-GACAAGCTTCCGCGCGTCGTG CCGAC-3' and 5'-GGGTCTAGACTAGGTCTTGTGTCTTCTTCCAGT TACTGCC-3'. Following digestion with HindIII and XbaI, the PCR fragment was ligated into the HindIII and XbaI sites of p3XFlagCMV (Sigma).

Antibodies used were mouse anti-HA (Babco), mouse anti-Flag (Sigma), rabbit anti-GST (Molecular Probes), rabbit anti-cyclin D3, rabbit anti-C/EBP α , rabbit anti-cyclin D1, rabbit anti-PU.1 (Santa Cruz), rabbit IgG total, mouse anti-FLAG M2-horseradish peroxidase (HRP), mouse anti-HA-HRP (Sigma), and rabbit anti-AML1 (active motif) or rabbit anti-AML1, kindly provided by Paul Erickson. Secondary antibodies for Western detection were donkey anti-rabbit IgG HRP linked (Amersham Biosciences) and sheep anti-mouse IgG HRP linked (Amersham Biosciences). Trichostatin A (TSA) was purchased from Sigma.

The monkey kidney cell line CV-1 and the human fetal kidney cell line 293T were cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (FBS) (Sigma), 2 mM L-glutamine, and 1% Pen-Strep (Invitrogen). Human hematopoietic K562 and Jurkat cells and the murine

myeloid progenitor line 416B were cultured in RPMI 1640 (Invitrogen) with 10% FBS, 2 mM L-glutamine, and 1% Pen-Strep.

GST pull-down assays. GST fusion proteins, pGEX-AML1(88-381), pGEX-Rb(379-970), pGEX-Runt, pGEX-CBF β , and pGEX were made as described previously using *Escherichia coli* BL21 (36). The amount of GST fusion protein unbound or bound to the glutathione agarose beads (Amersham Pharmacia Biotech AB) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining using bovine serum albumin (BSA) as a standard. Cyclin D3 was in vitro translated from pRcCMV-cyclin D3-HA with the reticulocyte in vitro transcription-translation system as described by the manufacturer (Promega), and its quantity was determined by SDS-PAGE-radiography or overexpressed in 293T cells. HA-AML1 was overexpressed in 293T cells. The binding was performed at 4°C with 2 μ g of GST, GST-AML1(88-381), or GST-Rb and 2 μ g of cyclin D3 in 300 μ l binding buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], and 1 μ g/ml of pepstatin, chymostatin, leupeptin, and BSA) or using 1 μ g and 5 μ g of each GST fusion protein with 200 μ g of protein for 293T cells expressing cyclin D3-HA in buffer A (30 mM HEPES, pH 7.8, 60 mM KCl, 30 mM NaCl, 1 mM EDTA, 200 mM sucrose, 1 mM PMSF, and 2.5 μ g/ml of leupeptin, antipain, and pepstatin). The beads were then washed four times with the binding buffer or buffer A, boiled with 20 μ l Laemmli buffer, and loaded on a 12% SDS-PAGE gel. The gel was fixed for 30 min with 10% acetic acid-10% methanol-water, followed by incubation in Amplify (Amersham Biosciences) for 20 min. The gel was then dried and exposed to film overnight. In the case of cyclin D3-HA, or the cyclin D3 and HA-AML1 expressed in 293T cells, Western blot analysis was conducted with mouse anti-HA (1:1,000 dilution) and the appropriate secondary antibody. Mammalian pull-down assays were performed by transfecting into 293T cells 1 μ g of pEBG, pEBG-Runt, pEBG-AML1(213-395), or pEBG-AML1(315-395) with 1 μ g of pRcCMV-cyclin D3-HA, using the calcium phosphate precipitation method. The precipitate was removed 8 h after the transfection, and the cultured cells were harvested at 20 h after the transfection. Cells were washed with PBS and then lysed in 400 μ l buffer A. The lysate was passed through a 25-gauge needle six times, and then 20 μ l of 20% Triton X-100 was added. The protein content was measured by the Bradford assay (Bio-Rad), and 100 μ g of each protein was subjected to a pull-down assay with 20 μ l of glutathione agarose beads at 4°C for 2 hours. The beads were washed with a washing buffer (25 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 0.5% Triton X-100) three times and boiled in 20 μ l of Laemmli buffer. Following SDS-PAGE, the protein was transferred to a polyvinylidene difluoride membrane (NEN) and Western blot analysis was conducted with mouse anti-HA (1:1,000 dilution) and the appropriate secondary antibody. The signal was detected by chemiluminescence (NEN). The same blot was stripped and reprobed with rabbit anti-glutathione (1:1,000 dilution) and the appropriate secondary antibody. The competitive pull-down assay for the analysis of GST-CBF β -bound AML1 in the presence of increasing amounts of cyclin D3 was conducted with overexpressed HA-AML1 and cyclin D3 in 293T cells lysed into buffer A. One hundred twenty μ g of HA-AML1-containing lysate was spiked with 120 μ g cyclin D3 lysate up to 600 μ g and compensated for with 293T cell lysate to a final concentration of 720 μ g protein per tube with the 293T empty vector-transfected lysate and brought to a 500- μ l final volume with buffer A. Ten μ l of each resulting mix was used as the input control. The lysates were precleared with 10 μ l of GST beads for 1 h. The supernatant was incubated with 1 μ g of GST-CBF β protein for an hour, and 10 μ l of GST-beads was added and incubated overnight at 4°C with rotation. The samples were subsequently washed four times with wash buffer and boiled in 10 μ l of 2 $\times$ Laemmli buffer. Following SDS-PAGE and Western blotting, the analysis was performed for HA-AML1, cyclin D3, and GST fusion proteins with mouse anti-HA, rabbit anti-cyclin D3, and rabbit anti-glutathione (1:1,000 dilution) using the appropriate secondary antibody and chemiluminescence for detection.

Immunoprecipitation. 293T cells were transfected with various protein expression constructs as indicated in the figure legends using Polyfect (QIAGEN). Following culture, the cells were washed once with PBS and harvested in buffer A, as described above. One hundred micrograms of protein lysate was precleared with either mouse IgG or rabbit serum and 30 μ l protein A-Sepharose for 45 min at 4°C. The supernatant was then incubated with either mouse anti-HA or rabbit anti-cyclin D3 for 1 hour or overnight at 4°C. Thirty μ l protein A-Sepharose was added to the above-described mixture and incubated at 4°C for another hour. The beads were washed three times with washing buffer and boiled in 20 μ l Laemmli buffer. Proteins in the supernatant were separated on a 12% SDS-PAGE gel. Following transfer as described above, the blots were probed with either rabbit anti-cyclin D3, rabbit anti-C/EBP α , mouse anti-Flag, or mouse anti-HA or with either rabbit anti-AML1 or mouse anti-HA and appropriate secondary antibodies and detected by chemiluminescence.

FIG. 1. In vitro association of AML1 with cyclin D proteins. A. Cyclin D3 protein was produced by an in vitro transcription/translation system and used for in vitro pull-down assays with bacterially produced GST, GST-Rb, and GST-AML1(88-381) immobilized on glutathione-agarose beads. B. Two hundred micrograms of protein from 293T cells transfected with pRc-CMV-cyclin D3-HA was incubated with 1 or 5 μ g of bacterially produced GST, GST-Rb, and GST-AML1(88-381) immobilized on glutathione-agarose beads and subjected to Western blot analysis with anti-HA antibody.

In vivo immunoprecipitation. Jurkat cells or K562 cells (4×10^7) with or without stimulation by 100 nM phorbol myristate acetate (PMA) for 3 days were lysed in 1 ml of PBS-0.5% Triton X-100 containing 1 mM PMSF, 2 mM Benzamidine, and 2.5 μ g/ml of leupeptin, antipain, and pepstatin. Following sonication, the sample was centrifuged at $13,000 \times g$ for 10 min and the supernatant collected. Protein concentration was determined by the Bradford assay (Bio-Rad) according to the manufacturer's instructions. One milligram of protein was precleared with 2 μ g of rabbit IgG plus 30 μ l 50% slurry of protein A-Sepharose for 1 h at 4°C. The sample was then centrifuged at $6,000 \times g$ for 30 seconds and the supernatant collected. The beads were washed with the lysis buffer three times and boiled in 20 μ l SDS sample loading buffer. Five μ l of rabbit anti-AML1 (provided by P. Erickson, University of Colorado) was added to the supernatant and incubated overnight at 4°C with rotation. Then 30 μ l of a 50% slurry of protein A-Sepharose was added and further incubated for 1 hour at 4°C. The beads were sedimented, washed three times with the lysis buffer, and boiled as described above. The proteins were resolved on an SDS-PAGE gel, transferred to a PVDF membrane, immunoblotted with 1:1,000-diluted anti-cyclin D3 (Santa Cruz) and then with 1:5,000 goat anti-rabbit-HRP, and developed by chemiluminescence (NEN).

Transactivation studies. CV-1 cells were transfected by the calcium phosphate precipitation method as described previously (36), using 6 μ g of the reporter p(Mono)₄TK81-luc and 600 ng of pCMV-CBF β , with the addition of 30 ng of pCMV-Renilla or 500 ng of pNull-Renilla and 100 ng of pEGFP-N3. pCMV5-AML1 was added at 600 ng. Multiple samples of 600 ng of pRc-CMV-cyclin D3-HA, pRc-CMV-cyclin D2-HA, or pRc-CMV-cyclin D1-HA were included as described in the figure legends. The amount of total DNA was adjusted to 10.5 μ g with the pCMV5 vector. Following 40 h of culture, the cells were washed with PBS and collected into $1 \times$ passive lysis buffer (Promega) and assayed for dual luciferase activity with the Monolight 3010 apparatus (Pharmingen) as described by the manufacturer. K562 cells (2.5×10^6) in plain RPMI 1640 were transfected by electroporation (Bio-Rad Gene Pulser II) with a total of 25 μ g of DNA at 260 V and 950 μ F in 0.4-cm cuvettes (Invitrogen), using 10 μ g of reporter construct with 2 μ g pCMV-CBF β , with the addition of 30 ng of pCMV-Renilla or 500 ng of pNull-Renilla and 100 ng of pEGFP-N3. Two μ g of pCMV5-AML1 with multiple samples of 2 μ g of pRc-CMV-cyclin D3-HA was

added with or without 2 μ g of pCMV-C/EBP α . The transfected cells were cultured in 5 ml of RPMI 1640 with 10% FBS for 16 to 20 h and then washed with PBS and assayed for dual luciferase activity as described above.

Gel shift assays and chromatin immunoprecipitation. GST fusion and in vitro transcription and translation (Promega) of cyclin D3-HA or the empty vector were performed as described above. The GST fusion proteins were eluted with 10 mM Tris, pH 9.5, 1 mM EDTA, and 10 mM glutathione (Sigma); dialyzed into 10 mM Tris, pH 7.6, 0.1 mM EDTA; and concentrated to 25% of the original volume (Amicon). The samples were brought to a final concentration of 10 mM Tris, pH 7.6, 0.1 mM EDTA, and 37.5% glycerol. Gel shift assays were performed with annealed double-stranded oligonucleotides of the c-fms promoter AML1 DNA binding site as described previously (36). Sixty ng of GST-Runt was incubated with 40 ng of GST-CBF β in the presence of 1, 3, or 5 μ l of cyclin D3-HA reticulocyte lysate, compensated to a 5- μ l final volume with empty vector reticulocyte lysate, and brought to a final volume of 20 μ l containing 10 mM HEPES, pH 7.8, 30 mM KCl, 5 mM MgCl₂, 25% glycerol, 0.5 mM dithiothreitol, 0.2 mM PMSF, 50 μ M EDTA, and 100 ng/ μ l poly(dI-dC) (Amersham). Chromatin immunoprecipitations were performed using 2×10^7 Jurkat and K562 cells that had been cultured at 2×10^5 cells/ml overnight. The cells were fixed with 1% formaldehyde for 30 min at 37°C in the culture media, resuspended in 5 ml PBS-0.125 M glycine, and centrifuged for 5 min. Following a wash in PBS, they were lysed in SDS lysis buffer (0.5% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0) at 1×10^7 cells/ml. Following sonication to shear the DNA to ~ 700 bp, 1 ml of cells was diluted with 4 ml of PBS containing 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, and 2 mM Benzamidine (immunoprecipitation buffer). Fifty μ l of a 50% slurry of protein G in PBS, pretreated with 10 μ g herring sperm DNA and 5 μ g of BSA for 2 h and washed, was added to 1-ml aliquots of the diluted sample and incubated at 4°C for 2 h with rocking. The samples were centrifuged at $6,000 \times g$, and the supernatant was transferred. One μ g of rabbit IgG, rabbit anti-AML1, or rabbit anti-cyclin D3 was added to the samples and incubated overnight at 4°C with rocking. Then 30 μ l of the protein G 50% slurry was added and the samples rocked at 4°C for 2 h. The beads were washed six times with radioimmunoprecipitation assay buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate) and then once with LiCl washing buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.0), and once with $1 \times$ TE (10 mM Tris, pH 8.0, 1 mM EDTA) with rotation (5 min each wash) at room temperature. DNA elution and reversal of cross-linking were done as described by Upstate Cell Signaling Solutions (Chicago). The precipitated DNA was dissolved in 25 μ l of water. Thirty-five cycles of PCR were performed with 3 μ l of DNA and the primers 5'-CCCCTG TTCACACTCACAGGAGAAACC-3' (MIP1F) and 5'-CCTCTTTATAGG CAGCCCTGGCGGAT-3' (MIP1R). Input sample was prepared from 20 μ l of the original cell lysis preparation, and 5% of the sample was used for the PCR.

Analysis of then cyclin D3 effect on PU.1 expression. Electroporations of 416B cells were done at 270 V and 960 μ F with a Bio-Rad Gene Pulser using 2×10^7 cells in 0.5 ml plain RPMI 1640. The cells were cultured in 25 ml of RPMI medium containing 10% FBS, 2 mM L-glutamine, and 1% Pen-Strep for 16 h. Fifteen ml of cells was harvested for RNA preparation with RNA-Bee as described by the manufacturer (Tel-Test Inc.). Five ml of cells was harvested for protein extraction in PBS-0.5% Triton X-100 containing 1 mM PMSF, 2 mM Benzamidine, and 1 mM EDTA. The Northern blot analysis was performed as previously described (38). Real-time PCR was performed with 1 μ l of cDNA, which was prepared from 1 μ g of an RNA sample using reverse transcription with random primers (Invitrogen), with the mouse PU.1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primer sets being used for 40 cycles in an iCycler (Bio-Rad) with Platinum SYBR green qPCR supermix UDG (Invitrogen).

RESULTS

Association of AML1 with cyclin D3. AML1 is known to associate with various factors, including transcription factors, coactivators, and corepressors, to regulate the promoters and enhancers of various genes (26, 31). Our previous studies indicate that a region of AML1 between aa 268 and 289 is important for the control of AML1 activity (36), which led us to hypothesize that another factor(s) may associate with AML1 in this region. Therefore, we employed a yeast two-hybrid approach using aa 213 to 289 of AML1 to investigate this possibility. From our screening with the EML library, we retrieved seven clones. Two of the seven clones harbored the

FIG. 2. Cyclin D interacts with AML1 in vivo. A. Schematic depiction of HA-tagged AML1 proteins and GST-AML1 fusion proteins used in the experiments. runt, Runt homology domain; AD, activation domain. B. HA-tagged AML1 proteins were expressed in the presence of cyclin D3

FIG. 3. Cyclin D proteins are associated with AML1 in vivo. A. 293T cells were transfected with AML1 and cyclin D expression constructs as indicated in the figure. Cell lysates were immunoprecipitated (IP) with anti-HA antibodies (α-HA). The immunoblot was sequentially probed with the anti-AML1 antibodies (top panel) and anti-HA antibodies (bottom panel). Lane 1, AML1-positive control (10 μg of protein lysate from 293T cells expressing AML1). B. K562 cells were treated with or without 100 nM PMA for 3 days. Western blot analysis for endogenous AML1 and cyclin D3 expression. C. Western blot analysis of immunoprecipitates of AML1 from K562 and Jurkat cells for endogenous cyclin D3.

sequence of the cyclin D3 cDNA. To confirm the association between AML1 and cyclin D3, we performed an in vitro pull-down assay, using bacterially expressed GST-AML1(88-381) and in vitro-translated cyclin D3. As shown in Fig. 1A, GST-AML1(88-381) pulls down in vitro-translated cyclin D3 like the

GST-Rb control (11) but contrary to GST alone. In addition we repeated the in vitro binding by using increasing amounts of GST, GST-Rb, and GST-AML1(88-381) with 293T cell extracts containing HA-tagged cyclin D3 (Fig. 1B). Again GST-Rb pulls down cyclin D3 (Fig. 1B, lanes 3 and 4) as does

by cotransfection in 293T cells. One hundred micrograms of protein lysate was subjected to immunoprecipitation (IP) and Western blotting (WB) with the indicated antibodies. IgH, immunoglobulin heavy chain. C. Analysis of domains of AML1 associating with cyclin D3. GST alone and GST-AML1 fusion proteins, GST-Runt, GST-AML1(213-395), and GST-AML1(315-395), were coexpressed with cyclin D3-HA in 293T cells and subjected to glutathione-agarose bead pull-down assays. The blot was sequentially probed with antibodies against the HA tag to demonstrate the association of AML1 with cyclin D3 and probed with antibodies against GST to demonstrate the expression of the fusion proteins.

FIG. 4. Effect of AML1 on cyclin D3 kinase activity. A. 293T cells were transfected with 1 μ g of pRc-CMV-cyclin D3-HA and 0, 1, or 3 μ g of pCMV5-AML1. Two-hundred-microgram samples of protein extracts were subjected to anti-HA immunoprecipitation. The immunoprecipitates were used in a kinase assay with 1 μ g GST-Rb and [γ - 32 P]ATP. Twenty-microgram samples of transfected cell protein extracts were analyzed by Western blotting to examine the expression of AML1 and cyclin D3. B. HCT116 cells were transfected with 1 μ g of each indicated expression construct. Western blot analysis for phosphor-Rb Ser780, total Rb, AML1, and cyclin D3 expression following 24 h of culture and serum starvation. C. As described for panel B, but cells were cultured for 48 h with 10% serum.

GST-AML1(88-381), while GST alone does not. Thus, AML1 associates with cyclin D3 in both these assays.

Coimmunoprecipitation of AML1 and cyclin D. To verify the association of cyclin D3 with full-length AML1, we cotransfected HA-tagged AML1 (Fig. 2A) and untagged cyclin D3 expression constructs in 293T cells. Cell lysates were prepared 48 h after the transfection and used in the coimmunoprecipitation assays.

This assay shows that in a cyclin D3 immunoprecipitate, AML1 protein was clearly detected. In the reciprocal coimmunoprecipitation assay, antibodies against the HA tag pulled down both HA-tagged AML1 and cyclin D3 (Fig. 2B). These results demonstrate that the full-length AML1 protein associates with cyclin D3.

In addition to performing studies of the full-length AML1

protein, we performed additional studies to localize the region of AML1 critical for its interaction with cyclin D3. Two AML1 C-terminal deletion constructs—AML1(1-289) and AML1(1-213)—were used in this study. AML1(1-289) does and AML1(1-213) does not include the region of AML1 that was used in the original yeast two-hybrid assays (aa 213 to aa 289). Interestingly, we observed the association of both AML1(1-289) and AML1(1-213) with cyclin D3 (Fig. 2B). This result indicates that more than one region of AML1 can interact with cyclin D3. This prompted us to investigate via an *in vivo* pull-down assay whether the Runt homology domain of AML1 was involved in the interaction with cyclin D3. For this purpose, three GST-AML1 fusion protein mammalian expression constructs were generated (Fig. 2A): a GST-AML1 Runt homology domain fusion protein, a GST-AML1 fusion protein containing aa 213 to 395, which includes the AML1 peptide used to pull out cyclin D3 in the yeast two-hybrid assay, and a GST-AML1 fusion protein containing aa 315 to 395. To analyze the association of cyclin D3 with different GST-AML1 fusion proteins, 293T cells were cotransfected with a tagged cyclin D3 and the different GST-AML1 constructs. Cell lysates harvested from the transfectants were used to perform GST pull-down assays. GST alone and the GST-AML1(315-395) fusion protein were not able to pull-down cyclin D3; however, both the GST-AML1 Runt homology domain and the GST-AML1(213-395) fusion protein showed clear association with cyclin D3 (Fig. 2C). Together, these results suggested that two domains of AML1 (the Runt homology domain and aa 213 to aa 289) associated with cyclin D3 independently.

We further investigated whether AML1 can associate with all three members of the cyclin D family of proteins by coimmunoprecipitation assays using protein lysates prepared from 293T cells transfected with expression constructs for AML1 and either cyclin D1, cyclin D2, or cyclin D3. As depicted in Fig. 3A, full-length AML1 associates with all cyclin D members, while a control was not able to coimmunoprecipitate AML1. This observation shows that there was no difference between these cyclin D proteins in their interaction with AML1. We thus demonstrate that the cell cycle-related cyclin D proteins associate with AML1, suggesting a possible mechanism of control for the regulation of cell cycle progression.

To test endogenous association of these proteins, we subjected K562 cells to growth arrest/megakaryocyte induction by a phorbol ester (PMA) treatment (37, 53), which induces both AML1 and cyclin D3 (Fig. 3B), and immunoprecipitated for AML1. Following SDS-PAGE of the immunoprecipitate and Western analysis, we observed the association of cyclin D3 with AML1 in these cells before and after PMA induction (Fig. 3C). In addition, we detected the *in vivo* association of AML1 and cyclin D3 in the T-cell line Jurkat (Fig. 3C). Judging from the amount of protein immunoprecipitated in both cases, we deduce that a small portion of the AML1 pool is associated with cyclin D3. Thus, AML1 and cyclin D3 associate in both myeloid and lymphoid cell types.

Cyclin D inhibits AML1 transactivation activity. We hypothesized that the interaction between AML1 and cyclin D may have a dual role in that AML1 affects cyclin D kinase activity and cyclin D affects AML1 transactivation. We first investigated whether AML1 affects cyclin D kinase activity. 293T cells were transfected with cyclin D3 in the presence or absence of

AML1. The cell lysates were immunoprecipitated for the tagged cyclin D3 and used in *in vitro* kinase assays with GST-Rb as the substrate (32). These experiments showed that cyclin D3 *in vitro* kinase activity was not perturbed in the presence of AML1 (Fig. 4A). We further examined if the expression of cyclin D3 with or without AML1 affects the endogenous phosphorylation status of Rb at Ser780, a target phosphorylation site of cyclin D/Cdk4 (21). We transfected the HCT116 colon cancer cell line with cyclin D3 or cyclin D3 and AML1 and looked at the phosphorylation status of Ser780 of Rb following serum starvation for 24 h. The results showed the enhanced Rb phosphorylation at Ser780 with cyclin D3 expression (Fig. 4B). However, AML1 did not further increase Rb phosphorylation (Fig. 4B, lane 4). Under non-serum starvation conditions (Fig. 4C), we could see a very slight induction by cyclin D3 alone that was not affected by the presence of AML1. AML1 alone was able under serum starvation conditions to increase phosphor-Rb but not significantly under normal culture conditions (Fig. 4B and C). In addition, an *in vitro* kinase assay for cyclin D3-associated p58^{PITSLRE} kinase (57) with histone H1 as a substrate showed that AML1 did not disrupt this cyclin D3-associated kinase activity (data not shown). These results indicate that AML1 does not significantly interfere with cyclin D3-associated kinase activities under these assay conditions.

To analyze whether cyclin D affects AML1 transcription factor function, we performed transactivation experiments. A luciferase reporter construct containing a tetramer of the M-CSF receptor C/EBP-AML1 binding sites upstream of the basal thymidine kinase promoter, p(Mono)₄TK81-luc (56), and increasing amounts of cyclin D3 in the presence of AML1 and its heterodimer partner CBF β were transfected into CV-1 cells. As shown in Fig. 5A, cyclin D3 inhibits the transactivation activity of AML1 in a dose-dependent fashion up to ~70% while not affecting the basal level of promoter activity in the absence of AML1. Subsequently, we analyzed if this inhibition occurs in the myeloid cell line K562. As depicted in Fig. 5B, cyclin D3 strongly inhibited AML1 transactivation in K562 by approximately 80%. Overall, the inhibitory effect of cyclin D3 was higher in K562 cells than in CV-1 cells.

We have previously reported that the B-cell-specific tyrosine kinase (BLK) promoter contains adjacent AML1 and B-cell-specific activator protein (BSAP) DNA binding sequences and that these transcription factors synergize in activating this promoter (25). We therefore analyzed whether inhibition by cyclin D3 affects this natural promoter and show that AML1 transactivation of the BLK promoter was inhibited by cyclin D3 (Fig. 5C).

As all cyclin D family members interact with AML1, we studied whether this inhibitory effect by cyclin D3 can also be observed with the other two cyclin D proteins. Transactivation experiments similar to those described above in the presence of different cyclin D family members were conducted. Both cyclin D3 and cyclin D1 were more potent in inhibiting AML1 transcriptional activity than the other family member (Fig. 5D). Although the cyclin D2 level of expression was lower than those of cyclin D3 and cyclin D1 (Fig. 5D), it nevertheless suggests that the individual members of cyclin D family have different potentials to inhibit AML1 function.

To examine further the biological effect of cyclin D3 on AML1 target gene expression, we expressed cyclin D3 in 416B

FIG. 5. Inhibitory effect of cyclin D3 on AML1 transactivation. A. CV-1 cells were transfected with 6 μ g of the luciferase reporter p(Mono)₄TK81-luc, 600 ng of pCMV5-CBF β , and 30 ng of pRL-CMV in every transfection. Six hundred ng of pCMV5-AML1 was used with an increasing amount of pRcCMV-cyclin D3-HA from 0.6 to 2.4 μ g (depicted by + to 4+). The total DNA content was adjusted with the pRc-CMV empty vector to 10.5 μ g. The promoter activity is presented as a percentage of induction relative to the transfection containing p(Mono)₄TK81-luc, pCMV5-AML1, and pCMV5-CBF β . Transfection efficiency was normalized according to cotransfected pRL-CMV *Renilla* luciferase activity. B. K562 cells were transfected with 10 μ g of p(Mono)₄TK81-luc, 2 μ g of pCMV5-CBF β , and 0.5 μ g of pNull-*Renilla* in every transfection. Two μ g

FIG. 6. Effect of exogenous cyclin D3 on endogenous PU.1 gene expression. 416B cells were transfected by electroporation with 40 μ g of pRe-CMV or pRe-CMV-cyclin D3-HA and harvested for RNA and protein 16 h posttransfection. A. Western blot analysis of the exogenously expressed cyclin D3-HA. B. Northern blot analysis of PU.1 expression in 10 μ g of total RNA, and ethidium bromide-stained gel showing 28S and 18S rRNAs as loading controls. C. Real-time PCR depicting the level of PU.1 expressed as corrected for GAPDH expression in each sample, with a standard deviation from results of four separate reactions from two sets of experiments.

cells and analyzed the expression of a reported AML1 target gene, PU.1 (14). Cells were transfected with either the plasmid vector or a cyclin D3 expression construct. Western blot analysis showed that the overexpressed cyclin D3 did not alter the level of endogenous AML1 significantly (Fig. 6A). The effect

of cyclin D3 overexpression on PU.1 expression was first analyzed by Northern blotting (Fig. 6B). A noticeable decrease of PU.1 mRNA in the presence of overexpressed cyclin D3 was observed using 28S and 18S rRNA as loading controls. Furthermore, real-time quantitative reverse transcription PCR confirmed that the level of PU.1 was reduced to 25% upon cyclin D3 expression (Fig. 6C). These data indicate that cyclin D3 negatively regulates endogenous AML1 target gene expression.

Cyclin D inhibition of AML1 does not involve TSA-sensitive HDAC activity. Cyclin D1 has been shown to inhibit the transcriptional activity of the thyroid hormone receptor by recruitment of histone deacetylase 3 (HDAC3) (27). AML1 is known to associate with various repressor proteins, which attract HDAC activity to the complex and thereby promote the inhibition of AML1 targets (18, 24). To address whether cyclin D requires HDACs for the inhibition of AML1, we performed luciferase reporter assays with K562 cells in the presence of various concentrations of the HDAC inhibitor TSA. With increasing concentrations of TSA, an overall increase in transactivation was observed (Fig. 7A). However, the inhibitory effect of cyclin D was not reversed even at the highest concentration of TSA (Fig. 7B). From these experiments we conclude that the inhibition of AML1 by cyclin D3 does not involve TSA-sensitive HDACs.

C/EBP α decreases the inhibitory effect of cyclin D on AML1. We have shown above that two regions of AML1 are involved in the association with cyclin D3. Because the AML1 Runt homology domain associates with various factors that cooperate with AML1 in regulating gene transcription, we inquired if such a factor was able to reverse the inhibitory effect of cyclin D3. For this we used the transcription factor C/EBP α , which we have previously shown to associate and synergize with AML1 (36). The p(Mono)₄TK81-luc reporter construct contains the C/EBP DNA binding sites 5' to the AML1 binding sites (56). We cotransfected the cyclin D3 expression construct in the presence and absence of C/EBP α . Again we observed an inhibition of AML1 in the absence of C/EBP α . In the presence of C/EBP α , the inhibitory effect of cyclin D3 was still evident (Fig. 8A). However, the level of inhibition was diminished when we compared the 65% decrease in the absence and 21% decrease in the presence of C/EBP α (Fig. 8B). C/EBP α itself was not significantly affected by cyclin D3 (Fig. 8A and B). This suggests that C/EBP α and cyclin D3 may potentially coregulate AML1 activity by competition. To address this hypothesis of competition between AML1-associated factors in the regulation of transcription, we analyzed the interaction of AML1 and cyclin D3 in the presence of increasing amount of C/EBP α .

of pCMV5-AML1 was added in the absence or presence of 2 or 8 μ g of pReCMV-cyclin D3-HA. The total DNA content was adjusted with herring sperm DNA to 25 μ g. All the other parameters were the same as described for panel A. The averages and standard deviations were generated from results of duplicates of two independent experiments (four sets of data). C. Cyclin D3 inhibits AML1 activation of the B-cell-specific promoter BLK. K562 cells were transfected with 10 μ g of pBLK-luc and 2 μ g of pCMV5-CBF β . Two micrograms of AML1 with or without 4 μ g of cyclin D3 expression constructs was added. Values were calculated as described above. The results represent the averages with standard deviations from two independent duplicate experiments and were normalized to *Renilla* luciferase expression. D. Three members of cyclin D proteins inhibit AML1 activity. CV-1 cells were transfected as described for panel A with the variation of the addition of pReCMV-cyclin D1-HA and pReCMV-cyclin D2-HA. The transfection results depicted are normalized to the amount of *Renilla* luciferase expressed from pRL-CMV. The 100% induction is set for AML1 transactivation in the absence of cyclin D. The results shown are average results of duplicates of two independent experiments (four sets of results) with standard deviations. Western blot analysis for the expression of the cyclin D family members in one of the experiments is shown with EGFP as the loading control.

FIG. 7. HDAC activity is not required for cyclin D3 inhibition of AML1. K562 cells were transfected with 10 μ g of p(Mono)₄TK81-luc, 2 μ g of pCMV5-CBF β with or without 2 μ g of pCMV5-AML1, and/or 4 μ g of pRcCMV-cyclin D3-HA. The cells were cultured for 6 h, and then TSA was added at the indicated concentrations and cultured for an additional 16 h. A. Stimulatory effect of TSA on basal reporter activity. Depiction of the control vector p(Mono)₄TK81-luc with pCMV5-CBF β after treatment of cells for 16 h with the indicated concentrations of TSA. The averages and standard deviations were generated from the results of duplicates of two independent experiments. B. Each set of bars represents a concentration of TSA where AML1 only was set at 100% and the level of induction in that set was calculated according to the luciferase activity of AML1 transfection alone. The average and standard deviation were generated from results of duplicates of two independent experiments.

Both cyclin D3 and C/EBP α were detected in an AML1 immunoprecipitate (Fig. 8C). However, increasing amounts of C/EBP α did not displace cyclin D3 from AML1, suggesting that C/EBP α did not block the negative effect of cyclin D3 on AML1 transcription via competition for binding.

Cyclin D negatively regulates AML1 by competing with CBF β . Since the AML1 heterodimer partner CBF β can enhance AML1 transactivation, we next examined the possible competition between cyclin D3 and CBF β in their association with AML1. AML1 and cyclin D3 expression constructs were transfected into 293T cells with increasing amounts of a CBF β expression construct. Increasing CBF β expression reduced the association of cyclin D3 with AML1 in a coimmunoprecipitation assay (Fig. 9A). In addition, increased expression of cyclin D3 reduced CBF β association with AML1 (Fig. 9A). These

results indicate that CBF β and cyclin D3 compete with each other in binding to AML1 and suggest that the inhibitory effect of cyclin D3 could rely on such a mechanism. We thus looked at this phenomenon by performing a gel shift analysis using GST-fusion proteins of the Runt DNA binding domain of AML1 in the presence or absence of GST-CBF β and in the presence or absence of increasing amounts of in vitro-transcribed/translated cyclin D3-HA. These experiments demonstrated that cyclin D3 is able to perturb the DNA binding affinity of the Runt DNA binding domain of AML1 in the presence or absence of CBF β in a dose-dependent manner (Fig. 9B). To assess this further, we performed a pull-down assay with GST-CBF β and HA-AML1, with increasing quantities of cyclin D3 added during the binding. As shown in Fig. 9C, cyclin D3 is able to sequester HA-AML1 away from

FIG. 9. Cyclin D3 competes with CBF β for AML1 association and disrupts AML1 DNA binding. A. Competitive association between cyclin D3 and CBF β with AML1. 293T cells were cotransfected with 5 μ g of pRSV-cyclin D3 and pcDNA6-HA-AML1, with the addition of 0.5 μ g or 2.5 μ g of p3XFlag-CBF β (left panels) or cotransfected with 1 μ g of p3XFlag-CBF β and 5 μ g pcDNA6-HA-AML1 with the addition of 5 μ g or 10 μ g of pRSV-cyclin D3 (right panels) and equilibrated to 25 μ g with the empty vector. HA-tagged AML1 protein was immunoprecipitated (IP)

FIG. 10. Models of the effect of cyclin D on the regulation of AML1 function. A. Cyclin D is able to compete with CBF β for AML1 binding. The loss of association with CBF β reduces AML1 transactivation activity through the lowering of its ability to bind DNA that could be due to conformational changes in the Runt domain. Alternatively, conformational changes in AML1 could also lead to variations in cofactors associated with carboxy-terminal domains of AML1. C/EBP α can partially rescue the negative effect of cyclin D. B. AML1 is known to promote cell cycle progression and differentiation in various hematopoietic cells. The positive regulation of cyclin D proteins could thereby regulate its expression directly by its association with AML1 during proliferative signals to prevent overproduction of cyclin D proteins. In addition, the proposed differentiation activity of AML1 could be counteracted by the displacement of CBF β or, alternatively, the coactivator p300 by cyclin D proteins, known to cooperate with AML1 during differentiation.

with anti-HA (α HA) antibody, and Western blotting (WB) was performed with the indicated antibodies (top panels). A straight Western blot of 10 μ g of each sample is shown (bottom panels). B. Cyclin D3 disrupts Runt and Runt-CBF β DNA binding. GST-Runt and GST-CBF β proteins were isolated from *E. coli*, and cyclin D3 was in vitro translated. Sixty ng of GST-Runt was mixed with 40 ng of GST-CBF β . Reticulocyte lysates of cyclin D3-HA and/or control pRc-CMV was used to a maximum of 5 μ l in the titrations, and the binding was performed on ice in the presence of the c-fms promoter AML1 binding site probe. The protein-DNA complexes were resolved on a 5% (29:1) 0.5 $\times$ Tris-borate-EDTA gel for 1 h at 15 mA at 4°C and dried for autoradiography. Arrows indicate the shifted complex of GST-Runt and the GST-CBF β /GST-Runt heterodimer. C. Dose-dependent competition between cyclin D3 and CBF β for association with AML1. 293T cells were transfected with 10 μ g of pcDNA6-HA-AML1 or 20 μ g of pRSV-cyclin D3. A pull-down assay with GST-CBF β was performed with HA-AML1 and increasing concentrations of cyclin D3 lysate. Western blot analysis was performed with the indicated antibodies (left panels). Straight Western blot with 15 μ g of each sample before the addition of GST-CBF β (right panels). D. Chromosome immunoprecipitation of AML1 and cyclin D3 on the MIP1 α promoter. K562 and Jurkat cells were subjected to CHIP assays with α -AML1 or α -cyclin D3 or rabbit IgG and beads alone as controls. CHIP was done on 2×10^6 cells with each antibody. PCR was performed with the primers described in Materials and Methods for the MIP1 α promoter region containing an AML1 binding site. The input represents DNA isolated from 2×10^5 cells.

scription factor and did not require TSA-sensitive HDAC activity, as was suggested for cyclin D1 regulation of the thyroid hormone receptor promoter (27). In addition, we show that cyclin D3 is associated with chromatin-containing AML1 sites in myeloid and lymphoid cell lines. A competitive mechanism for the negative regulation of AML1-CBF β association by cyclin D3 was observed. However, increased CBF β expression was unable to reverse cyclin D3 inhibition of AML1 in reporter assays (data not shown). This is due to the predominant cytoplasmic localization of CBF β , which requires AML1 for its nuclear translocation (47). Nevertheless our observations suggest a novel mechanism of AML1 regulation whereby cyclin D displaces CBF β from AML1, abrogating AML1's DNA binding potential to a target promoter(s), which is partly counteracted by C/EBP α (Fig. 10A). Alternatively, due to the multiple factors that can associate with (26) or modify (8, 55) AML1, it is still possible that the association between AML1 and cyclin D leads to conformational changes of AML1 which disrupt or enhance the interaction of AML1 with identified or unidentified coactivators and corepressors, such as p300/CBP or sin3a, respectively.

Finally, various reports implicate AML1 in the regulation of the G₁ phase of the cell cycle. Our observations of an association between AML1 and cyclin D leading to the inhibition of AML1 function in transactivation suggest a putative negative-feedback mechanism that promotes the shutdown of AML1 activity as the cells accumulate cyclin D proteins during the G₁/S phase (Fig. 10B). In addition, it is known that members of the cyclin D family block the granulocytic differentiation of a hematopoietic cell line (20), which requires AML1 during G-CSF-induced differentiation (48). Thus, cyclin D association with AML1 to down regulate the expression of critical genes, such as that for PU.1 (Fig. 6), for myeloid cell differentiation may be one of the mechanisms for the differentiation block observed by cyclin D in hematopoietic cells (Fig. 10B). Recent observations of AML1 involvement in megakaryocyte development (10, 16) in patients with the familial platelet disorder (43) and the ability of cyclin D3 not only to be upregulated in megakaryocytes (52) but also to promote polyploidy (58) in these cells suggest that they may regulate one another in the fine-tuning of megakaryocyte development. Our observation of associated AML1 and cyclin D3 protein in PMA-stimulated K562 cells, which promotes polyploidy in these cells (37), suggests the possibility of an as-yet-unidentified function of AML1-cyclin D3 association in megakaryocytes. In addition, during T-lymphocyte development, both AML1 (23, 29, 39) and cyclin D3 (42) are required. Thus, our observed association of AML1 and cyclin D3 in a T-lymphocytic cell line suggests similar mechanisms of mutual regulation. The general observation of this report and the known functions of AML1 and cyclin D suggest that the interaction between AML1 and cyclin D proteins during hematopoiesis regulate complex "timely balanced" events during the cell cycle and differentiation.

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