Phosphorylation of AML1/RUNX1 Regulates Its Degradation and Nuclear Matrix Association

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Abstract
The acute myeloid leukemia 1 (AML1) transcription factors are key regulators of hematopoietic differentiation. Cellular AML1c protein is found in the nucleus and can be separated into two fractions, one soluble in buffers containing salt and nonionic detergent and the other insoluble and tightly bound to the nuclear matrix. We find that the AML1c protein is modified by both phosphorylation and ubiquitination. Our studies show that the majority of the ubiquitinated AML1c is associated with the insoluble nuclear matrix. Treatment of cells with the proteasome inhibitor PS341 (Velcade, Bortezomib) increases the levels of ubiquitinated AML1c. Mutation of the four phosphorylation sites necessary for transcriptional regulation (serine 276, serine 293, serine 303, and threonine 300) mimics the effects of the proteasome inhibitor, increasing the levels of ubiquitinated, matrix-bound AML1c. We find that the soluble and insoluble forms of AML1c are degraded at a similar rate. However, mutation of these four serine/threonine residues statistically increases the half-life of the matrix-associated AML1c. Thus, phosphorylation of AML1c on specific serine/threonine residues controls both transcriptional activity and rate of degradation.

(Mol Cancer Res 2005;3(7):391–401)

Introduction
The importance of acute myeloid leukemia 1 (AML1; RUNX1) to normal hematopoietic development is suggested by AML1 knockout mouse models and the frequent disruption of AML1 secondary to translocations (AML/ETO, AML/EVI1, and TEL/AML), deletions, or point mutations in ~30% of human myeloid leukemias and myelodysplastic syndrome patients (1-4). Mutations that destroy one of the AML1 alleles are associated with familial platelet disorders (5), which often lead to leukemia, demonstrating that a specific level of AML1 expression is required for normal hematopoiesis. Transfection of AML1 leukemic fusions into hematopoietic cells inhibits differentiation and enhances progenitor cell self-renewal (6). Thus, modulation of the AML1 protein plays a critical role in the development of leukemia.

The AML1 proteins, including AML1a, AML1b, and AML1c (AML1c is also known as AML1B), are generated from one gene by alternative splicing (7). This gene has been given the names RUNX1, AML1, CBFB, and PEBP2a/b1. The AML1c protein is composed of a DNA binding domain (or runt domain) located on the NH2 terminus of the molecule (amino acids 85-204 in AML1c) followed by a transcriptional regulatory domain. AML1c is slightly larger than AML1b (8), containing 32 different amino acids at its NH2 terminus. AML1a contains only the runt domain and has no known transcriptional regulatory domain. The runt domain of the AML1 proteins dimerizes with core binding factor β (CBFβ) and together they bind a specific DNA sequence and play a critical role in regulating definitive hematopoiesis (1, 9-14). Furthermore, it has been shown that AML1 is necessary for commitment to the hematangioblast stage of development (15, 16).

AML1 proteins regulate the transcription of a number of important hematopoietic genes including the T-cell receptors α and β (8), granulocyte macrophage colony stimulating factor (17), myeloperoxidase and neutrophil elastase proteins (18), and colony stimulating factor-1 receptor (19). AML1, by controlling the transcription of cyclin-dependent kinase 4, seems to regulate the time the cell spends in the G1 phase of the cell cycle (20). AML1 modulates transcription through interactions with multiple molecules including histone acetylases p300, monocytic leukemia zinc finger protein, and monocytic leukemia zinc finger protein–related factor (21, 22), transcriptional activators CAAT/enhancer binding protein α protein (19), HES-1 (23), and PU.1 (24), and corepressors groucho/transducin-like enhancer of split and sin3A (25, 26).

Previous work has suggested that AML1 activity may be regulated by both phosphorylation (27, 28) and ubiquitination (29). Phosphorylation at specific serine-proline or threonine-proline sites in AML1b and AML1c seems to be necessary for normal activity (28). Previously, studies on AML1b ubiquitination suggested that heterodimerization of AML1b with CBFB/β blocked AML1b ubiquitination and stabilized AML1b (29). Imai et al. (30) provided evidence that phosphorylation released AML1b from an association with the nuclear matrix mediated by sin3A, in turn leading to both increased activity and an increased rate of degradation.
We have recently reported that phosphorylation of AML1 via phorbol ester stimulation enhances AML1 transactivation (27). We have now observed that inhibition of proteasome activity leads to increased levels of ubiquitinated AML1c associated with the nuclear matrix. Similarly, mutation of specific AML1c phosphorylation sites from serine or threonine to alanine also causes increased association of ubiquitinated AML1c with the nuclear matrix, suggesting that phosphorylation regulates movement of AML1c along the proteolytic pathway. The fact that mutations of identical serine or threonine to alanine also greatly reduce AML1c transcriptional activation suggests that there is a link between AML1c transcriptional activity and its degradation.

Results

The Proteasome Inhibitor PS341 (Velcade, Bortezomib) Induces Accumulation of Ubiquitinated AML1c in the Nuclear Matrix

It has been previously reported that in hematopoietic cells and transfected fibroblasts, including Jurkat, ROS17/2.8, p19, and COS-7 cells, both AML1b and AML1c are nuclear proteins (31, 32). Furthermore, they are partially soluble and partially associated with the nuclear matrix in a manner that resists extraction with detergents or high salt. We obtained identical results when AML1c is expressed in 293T cells (data not shown). This suggests that transfection of this protein into 293T cells may be used as a model to study the location of this transcription factor. AML1c may be divided into soluble and insoluble (matrix-associated) fractions. When cells are fractionated by hypotonic lysis without detergent into cytosolic and nuclear fractions, almost all AML1c is found in the nuclear fraction. Similarly, stained and subsequently fixed cells transfected with AML1c also show that AML1c is almost exclusively nuclear. Thus, the soluble and insoluble fractions of AML1c represent AML1c loosely associated with the nucleus and AML1c tightly bound to the nuclear matrix, respectively. Although the AML1c protein is overexpressed in 293T cells, its distribution seems similar to that observed in K562 hematopoietic cells.

Treatment of transfected 293T cells with the proteasome inhibitor PS341 (33) causes an increase in the level of higher molecular weight forms of AML1c in the nuclear matrix (Fig. 1A). To obtain soluble and insoluble (matrix-associated) fractions of AML1c, 293T cells were transfected with AML1c expression vector, then divided into two samples. One sample was left untreated, the other treated with 1 μmol/L PS341. After 24 hours, the 293T cells were lysed in a buffer containing 0.5% IGEPAL-630CA (a detergent similar to Nonidet P-40) and 150 mmol/L NaCl. Centrifugation separated the AML1c into soluble and matrix-associated fractions (see Materials and Methods). As shown in Fig. 1A, in untreated 293T cells, a fraction of cellular AML1c is associated with the nuclear matrix. The AML1 associated with the nuclear matrix includes both lower molecular weight form, which may be degraded proteins, and higher molecular weight form, which could be ubiquitinated (Fig. 1A). In all experiments, the amount of AML1c protein found in the matrix was increased with PS341 treatment.

To show that the retarded AML1c bands seen after PS341 treatment were due to ubiquitination, and to confirm that the ubiquitinated AML1c is predominantly found in the nuclear matrix, 293T cells transfected with FLAG-AML1c and hemagglutinin-ubiquitin were divided into two aliquots, and one sample was treated with 1 μmol/L PS341 for 24 hours. Both samples were then separated into soluble and matrix-associated fractions. The FLAG-AML1c in each fraction was immunoprecipitated using anti-FLAG agarose, then analyzed for the presence of ubiquitination by Western blot with anti-hemagglutinin antibodies. As shown in Fig. 1B, most ubiquitinated AML1c is associated with the nuclear matrix. Some ubiquitinated AML1c is observed in the soluble fraction after PS341 treatment, which may represent leakage from the matrix.

All AML1b/AML1c lysines that might serve as ubiquitination targets are located in the NH2 terminus (Fig. 2A). To confirm that ubiquitination of AML1c occurs at the NH2 terminus, His6-AML1c (1-230) and His6-AML1c (231-480) were coexpressed with hemagglutinin-ubiquitin in 293T cells. The His6-AML1c proteins were precipitated with Ni-NTA agarose, then eluted from the agarose and examined for ubiquitination by Western blot with anti-hemagglutinin antibodies. As shown in Fig. 2B, His6-AML1c (1-230) is clearly ubiquitinated, but His6-AML1c (231-480) shows no signs of ubiquitination. The high molecular weight hemagglutinin-positive bands seen in both gels are nonspecific.

Mutations in AML1c Phosphorylation Sites (Serine 276, Serine 293, Serine 303, and Threonine 300) Alter AML1c Degradation

AML1c is a known phosphoprotein (27, 28), and phosphorylation may regulate both the activity and degradation of transcription factors (28, 34). We therefore decided to examine the role of specific phosphorylation sites on degradation. In the COOH-terminal activation domain, AML1c contains 11 serine/threonine residues followed by proline that could act as potential mitogen-activated protein kinase phosphorylation sites. Full-length AML1c contains 14 serine/proline or threonine-proline phosphorylation sites (diagramed in Fig. 3A). Threonine 41 and serine 48 in the AML1c NH2 terminus are phosphorylated in 293T cells, but mutation of these sites has no effect on AML1c transcriptional activity. In hematopoietic K562 cells treated with phorbol esters, we have (27) identified serine 276, serine 293, serine 303, serine 462, and threonine 300 (all of which are followed by prolines) as AML1c phosphorylation sites and potential targets of the mitogen-activated protein kinase family of enzymes. In the COOH terminus, serine 276 and serine 293 have been shown to be potential AML1 phosphorylation sites in fibroblasts (28) and targets for serine-proline/threonine-proline–directed protein deg
kinases (i.e., extracellular signal-regulated kinase). In contrast to these two sites, threonine 300 and serine 303 are surrounded by amino acids that make them perfect consensus sites for mitogen-activated protein kinase family phosphorylation (Pro-X-Ser/Thr-Pro; ref. 35). Thus, based on our published results in hematopoietic cells, serine 276, serine 293, serine 303, and threonine 300 were chosen for mutation. In addition, in a preliminary study of the effect of single amino acid mutations on phosphorylation of AML1c in 293T cells, mutation of serine 462 to alanine caused a loss of the AML1c doublet on Western blots, suggesting that this amino acid was phosphorylated. Other single amino acid mutations had no effect on mobility (data not shown).

To confirm that the pattern of AML1c phosphorylation is similar in 293T and K562 cells, the phosphorylation sites previously identified in K562 cells were mutated, and

FIGURE 1. The proteasome inhibitor PS341 induces accumulation of ubiquitinated AML1c on the nuclear matrix. A. The 293T cells were transfected with plasmid expressing AML1c. The transfected cells were divided into two samples, one of which was treated for 24 hours with 1 μmol/L PS341, as indicated above the lanes. Soluble (S) and matrix-associated (M) protein fractions were prepared from both the untreated and PS341-treated samples (see Materials and Methods), and the AML1 content in each fraction determined by Western blot with anti-AML1 antibodies. B. The 293T cells were cotransfected with plasmids expressing hemagglutinin-ubiquitin and FLAG-AML1c. The cells were divided into two samples and treated with PS341 as above. After preparation of soluble and matrix-associated proteins from each sample, the FLAG-AML1c in each fraction was immunoprecipitated with anti-FLAG agarose. The immunoprecipitated FLAG-AML1c was then examined for ubiquitination by Western blot with anti-hemagglutinin antibodies and for distribution by Western blot with anti-AML1 antibodies.

FIGURE 2. The AML1c NH₂ terminus is ubiquitinated. A. The locations of lysine residues in the NH₂ terminus of AML1c (targets for ubiquitination) are diagramed. B. The 293T cells were transfected with His₆-AML1c (1-230) alone, with His₆-AML1c (1-230) plus hemagglutinin-ubiquitin, or with hemagglutinin-ubiquitin alone as indicated. Total protein (soluble plus matrix-associated) was prepared from each sample, and His₆-AML1c (1-230) precipitated from each with Ni-NTA agarose. The amount of ubiquitinated His₆-AML1c (1-230) present was determined by Western blot with anti-hemagglutinin antibodies, and the amount of total AML1c present determined by Western blot with anti-AML1 antibodies. The 293T cells were transfected with His₆-AML1c (231-480) alone or with His₆-AML1c (231-480) plus hemagglutinin-ubiquitin. Samples were analyzed as in A, except the amount of total His₆-AML1c (231-480) present was determined by Western blot with anti-AML1 (epitope 249-271) antibodies.

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32P incorporation into wild-type and mutant AML1c compared with 293T cells. Figure 3 shows the phosphorylation pattern of AML1c when expressed in 293T cells. To avoid background from NH2-terminal sites, plasmids expressing AML1c amino acids 231 to 480 were used to analyze COOH-terminal phosphorylation sites. In this experiment, plasmids expressing wild-type FLAG-AML1c (231-480) and AML1c (231-480) containing mutated serine-proline/threonine-proline sites were transfected into 293T cells. The mutant AML1c (231-480) proteins are diagrammed in Fig. 3A. Serine 276, serine 293, serine 303, and threonine 300 were mutated to alanine in AML1c (231-480)-4M. AML1c (231-480)-5M carries the first four mutations and an additional mutation of serine 462 to alanine. Finally, all COOH-terminal serine-proline or threonine-proline sites are mutated in AML1c (231-480)-11M.

After transfection of FLAG-AML1c (231-480) expression plasmids into 293T cells, the cells were labeled with [32P]orthophosphate. FLAG-AML1c (231-480) proteins were then immunoprecipitated using anti-FLAG agarose and eluted for analysis of 32P incorporation on SDS gels. As shown in Fig. 3B, the 4M mutations (serine 276, serine 293, serine 303, and threonine 300) eliminate a substantial amount of phosphorylation. Mutation of serine 462 in AML1c (231-480)-5M eliminates a specific retarded-mobility band, and the 11M mutations eliminate most remaining phosphorylation. These results were identical to those reported by our group for hematopoietic cells (27), again confirming the validity of using 293T cells for this analysis. This result suggests that in 293T cells these four closely spaced amino acids are phosphorylated.

We next determined the role of the four phosphorylation sites in AML1c ubiquitination and accumulation in the nuclear matrix. In Fig. 4A, 293T cells were transfected with plasmids expressing full-length, wild-type AML1c, full-length AML1c with the 4M sites (serine 276, serine 293, serine 303, and threonine 300) mutated to alanine, or full-length AML1c with the 4M sites mutated to aspartic acid. Each batch of transfected 293T cells was divided in half, and one half was treated with 1 μmol/L PS341 for 24 hours. Soluble and matrix-associated protein was prepared from both untreated cells and cells treated with PS341. Figure 4A shows that mutation of the 4M sites to alanine without any additional treatment results in a large increase in the amount of AML1c associated with the nuclear matrix in untreated 293T cells. In contrast, mutation of these sites to aspartic acid had the opposite effect of blocking the ability of the proteasome inhibitor to stimulate increases in ubiquitinated AML1c.

The untreated 293T cells transfected with AML1c-4M (S>A) in Fig. 4A seemed to contain more ubiquitinated AML1c associated with the matrix than cells transfected with wild-type AML1c. To confirm this, 293T cells were transfected with plasmids expressing hemagglutinin-ubiquitin and either FLAG-tagged wild-type AML1c or FLAG-tagged AML1c-4M (S>A). The samples transfected with wild-type AML1c and AML1c-4M (S>A) were each divided in half, and one half was treated with PS341 for 24 hours. After treatment, all FLAG-AML1c in each sample (soluble and matrix) was immunoprecipitated with anti-FLAG agarose. Immunoprecipitated FLAG-AML1c (231-480) proteins were analyzed by Western blotting with anti-hemagglutinin antibodies to detect the presence of ubiquitinated AML1c. As shown in Fig. 4B, cells transfected with wild-type AML1c contain little ubiquitinated AML1c unless treated with PS341. In contrast, cells transfected with AML1c-4M (S>A) have a substantial amount of ubiquitinated AML1c-4M, even without PS341 treatment. PS341 treatment does little to increase the amount of ubiquitinated AML1c-4M. The ubiquitination results are consistent with the levels of AML1c found in these cells (Fig. 4B).

After it was determined that mutation of AML1c serine 276, serine 293, serine 303, and threonine 300 affected the process of degradation, the role of individual sites was examined in
more detail. AML1c with single mutations in either S276 or S300 did not show consistent association with the nuclear matrix (data not shown). Because of the behavior of singly mutated AML1c proteins, we doubly mutated serine 276 and serine 303 to alanine (AML1c-2M). This mutant gave similar results to that obtained with AML1c-4M. This experiment was carried out with PS341 and MG132, another proteasome inhibitor (Fig. 5). Mutation of these two serines led to the same accumulation of AML1c in the nuclear matrix (Fig. 5). Twenty-four hours after transfection, both samples were divided in half, and incubated for another 24 hours. One half of each sample was then treated for 8 hours with the proteasome inhibitor MG132. Soluble (S) and matrix-associated (M) proteins were then blotted with anti-AML1 antibodies. B. The experiment was done exactly as described in A, but cells were treated with 1 μmol/L PS341 for ~16 hours.

Both the 4M Mutations and Treatment with PS341 Affect the Distribution of AML1c in the Nucleus as Observed by Immunofluorescent Staining of Cells

The results shown in Fig. 5 suggest that introduction of the 4M mutations into AML1c or treatment with PS341 induces an accumulation of AML1c in the nuclear matrix. FIGURE 4. Mutation of AML1c serine-proline/threonine-proline sites affects ubiquitination and association with the nuclear matrix. A. The 293T cells were transfected with wild-type AML1c (full-length), AML1c-4M (S>A), with serine 276, serine 293, serine 303, and threonine 300 mutated to alanine, or AML1c-4M (S>D), with the same four residues mutated to aspartic acid. Each sample was divided in half, and one half was treated with 1 μmol/L PS341 as indicated. Soluble (S) and matrix-associated (M) proteins were blotted with anti-AML1 antibodies. B. The 293T cells were transfected with hemagglutinin-ubiquitin and either FLAG-AML1c wild-type or FLAG-AML1c-4M (S>A), as indicated. Total protein (soluble plus matrix-associated) was prepared from each sample, and the FLAG-AML1c in each immunoprecipitated. The amount of ubiquinated AML1c present was determined by Western blot with anti-hemagglutinin antibodies, and the amount of total AML1c present determined by Western blot with anti-AML1 antibodies.

FIGURE 5. Mutation of serine 276 and serine 303 to alanine is sufficient to promote accumulation of AML1c on the nuclear matrix. A. The 293T cells were transfected with wild-type AML1c or AML1c-2M (serine 276 and serine 303 mutated to alanine). Twenty-four hours after transfection, both samples were divided in half, and incubated for another 24 hours. One half of each sample was then treated for 8 hours with the proteasome inhibitor MG132. Soluble (S) and matrix-associated (M) proteins were then blotted with anti-AML1 antibodies. B. The experiment was done exactly as described in A, but cells were treated with 1 μmol/L PS341 for ~16 hours.
The 4M Mutations Increase the Half-life of AML1c in the Insoluble (Matrix) Fraction

To directly examine the effects of phosphorylation and subcellular location on AML1c stability, the relative half-lives of soluble and insoluble AML1c (wild-type and 4M) were compared. 293T cells were transfected with either wild-type FLAG-AML1c or FLAG-AML1c-4M (S>A). The transfected cells were either left untreated or treated for ~24 hours with 1 μmol/L PS341, as indicated on left. The transfected cells on coverslips were fixed in formalin and stained with anti-FLAG monoclonal antibodies followed by anti-mouse antibodies labeled with FITC (see Materials and Methods). The coverslips were then mounted on glass slides using media containing 4',6-diamidino-2-phenylindole for analysis by fluorescent microscopy. Images show FITC fluorescence only, 4',6-diamidino-2-phenylindole staining only, or both combined, as indicated above the lanes. B, FITC fluorescence at several different intensities is shown for wild-type AML1c and AML1c-4M to aid in comparison.
hours. Densitometry values (assigning the lowest density reading a value of 1) from the Western blot were used to normalize the values obtained for $[^{35}S]$methionine incorporation levels. A typical experiment is shown in Fig. 7A, and the

![Western blot image](image)

**Figure 7.** Comparison of the half-life of wild-type AML1c and AML1c-4M (S>A) in the soluble and insoluble fractions. A. A typical experiment is shown. The 293T cells were transfected with either wild-type FLAG-AML1c or FLAG-AML1c-4M (S>A). Approximately 24 hours after transfection, the cells were washed and transferred to methionine-free DMEM to which 200 μCi/mL $[^{35}S]$methionine was added. After 5 hours of labeling, one half of each sample was set aside for immediate lysis. The other half was transferred to fresh media without $[^{35}S]$methionine and incubated an additional 16 hours before lysis. All lysates were separated into soluble and insoluble (matrix-associated) fractions, and FLAG-AML1c precipitated from each fraction. Duplicate samples were run on SDS gel; one set of samples was dried up for autoradiography and the other set was transferred to a filter for Western blotting with anti-AML1 antibodies. B. After each experiment, the $[^{35}S]$ autoradiograph was used for densitometry scanning to determine the amount of $[^{35}S]$ incorporated into AML1c in each sample. The Western blot was used for densitometry to determine the total amount of AML1c in each sample, and this value was used to normalize the numbers for $[^{35}S]$ incorporation. The corrected values for $[^{35}S]$ incorporation from three independent experiments were averaged to obtain the values shown graphically. The graph shows the percentage of AML1c retaining $[^{35}S]$ label after the 16-hour chase with unlabeled media. Values are shown for wild-type AML1c and AML1c-4M (S<A); as indicated above, open columns represent the soluble fraction and closed columns the insoluble fraction.

The 4M Mutations Do Not Prevent AML1c Binding to DNA

To determine whether the 4M mutations affect AML1c binding to DNA, electrophoretic mobility shift assay was done.

For the electrophoretic mobility shift assay comparing the abilities of wild-type AML1c and AML1c-4M to bind to DNA, an oligonucleotide containing the AML1c binding site from the M-CSFR (c-fms) gene promoter was used (19). The $[^{32}P]$-labeled oligonucleotide was incubated with control lysate containing no AML1c, with lysate from cells overexpressing wild-type AML1c, or with lysate from cells overexpressing AML1c-4M. The AML1c expression plasmids were always cotransfected with plasmid expressing CBFβ. In the presence of CBFβ, the ability of AML1c-4M to bind DNA is clearly comparable to that of wild-type AML1c (Fig. 8). Anti-AML1 antibodies were able to supershift the complexes formed by both wild-type AML1c and AML1c-4M (Fig. 8).

Discussion

Consistent with previous studies, our data indicate that almost all AML1c expressed in 293T is located in the nucleus (29, 31, 37). These previous studies found most AML1b/AML1c to be associated with the nuclear matrix and resistant to solubilization by high-salt/detergent buffers. However, we have found that AML1c falls into two categories: protein, which can be solubilized with high-salt/detergent buffer, and AML1c, which is resistant to solubilization. Identical results were found for endogenous AML1 in K562 cells.

We have observed that the half-lives of soluble and insoluble AML1c are similar, but that most detectable ubiquitinated AML1c is found in the insoluble fraction, presumably associated with the nuclear matrix. One possible explanation for this observation is that the majority of the AML1c may be degraded by a mechanism that does not require ubiquitination. Studies on the subcellular location of proteasomes have identified proteasomes in both cytoplasm and nucleus (38, 39). Fractionation of nuclei has produced conflicting results. In one study, investigators found proteasomes tightly associated with the nuclear matrix (40); another laboratory found them

average values and SD obtained from three independent replicates of the experiment are shown in Fig. 7B. The data indicate that there is no significant difference in the half-life of soluble and insoluble forms of wild-type AML1c; both are degraded at similar rates. However, the four serine/threonine to alanine mutations increase the half-life of insoluble AML1c-4M, but have much less effect on soluble AML1c-4M (Fig. 7B). Data were analyzed first by ANOVA. Pairwise comparisons were then done with Bonferroni $t$ test (36). The difference in half-life between insoluble wild-type AML1c and AML1c-4M is statistically significant ($P = 0.003$), whereas the difference between the soluble fractions of these two proteins is not statistically different ($P = 0.2$). This result supports the idea that phosphorylation at serine 276, serine 293, serine 303, and threonine 300 is necessary to maintain the normal rate of matrix-associated AML1c degradation.
greater effect on degradation occurring in the matrix. It was therefore cause a buildup of ubiquitinated protein in the nuclear matrix, and increases the half-life of this insoluble AML1c. Mutation of the four sites to aspartic acid (which mimics phosphorylation) results in an increase in the amount of ubiquitinated AML1c in the matrix, and increases the half-life of this insoluble AML1c. Mutation of the four sites to aspartic acid has the opposite effect, decreasing the amount of ubiquitinated AML1c associated with the matrix. The mutations have little effect on the levels of soluble AML1c. The mutations do not prevent the binding of AML1c to DNA to approximately the same degree as wild-type AML1c. However, we have not ruled out the possibility that the mutations may have subtle effects on the ability of AML1c to interact with DNA.

One possible model to explain these observations is that phosphorylation might be necessary for the normal process of both proteasome degradation and transcriptional activation. Mutation of the phosphorylation sites to alanine would therefore cause a build up of ubiquitinated protein in the nuclear matrix (for the same reason, proteasome inhibitors cause a backup). It also seems that phosphorylation exerts a much greater effect on degradation occurring in the matrix. It was proposed that AML1c associated with the basal transcription complex at specific domains in the nuclear matrix. It is possible that these domains are the same sites visualized by immunofluorescent staining of AML1c accumulating in the nucleus after treatment with PS341 (Fig. 6).

There is growing evidence for the association between transcriptional activation and proteasomal degradation. In a recent study, Molinari et al. (42) noted a striking correlation between proteasome-mediated degradation of transcriptional activators and potency of the activation domain in vivo. This work showed that the strongest transcriptional activators were most rapidly degraded by the proteasome, and that mutations that abolished transcriptional activation also protected the protein from degradation. It was proposed that the proteasome recognizes and targets the complex of transcriptional activator and basal transcription factor (42). Since this initial study, evidence for the role of ubiquitination/proteosomal degradation events in gene expression has grown (reviewed in refs. 43, 44). Recent work on the transducin β-like 1/transducin β-like–related 1 proteins (45) suggests that these proteins recruit ubiquitin/19S proteasome complexes to transcription complexes, and that this recruitment is necessary for transcriptional activation. It was suggested that the proteasome complex activates transcription by mediating the degradation of corepressors such as nuclear receptor corepressor and silencing mediator of retinoid and thyroid hormone receptor (45). Others have proposed a model in which proteasomes must degrade activators to allow transcription elongation to occur, then the proteasome dissociates to allow another round of transcription initiation (44). Our results suggest that in the case of AML1c, this activity occurs in the nuclear matrix. Our phosphorylation site mutations seem to block degradation, but not ubiquitination, because there was ubiquitination of AML1c-4M, which accumulates in the matrix. This suggests that phosphorylation is necessary for the final degradation that occurs after ubiquitination. The mechanism of degradation may be similar to that of the c-Fos protein; previous studies have shown that c-Fos is degraded by the proteasome independently of ubiquitination, in a phosphorylation-dependent manner (34). It is not clear whether degradation of AML1c requires only phosphorylation, or both ubiquitination and phosphorylation.

A recent study of AML1b by Imai et al. (30) showed that mutation of serine 249 and serine 266 to alanine stabilized AML1b and reduced activity, whereas mutation of these two residues to glutamic acid destabilized the protein and enhanced activity. Experiments were also presented by this group to suggest that phosphorylation of these two serines inhibited binding to sin3A and association with the nuclear matrix. From these results, a model was suggested in which phosphorylation released AML1b from a sin3A-mediated association with the nuclear matrix, making the soluble AML1b available for both transcriptional activation and degradation. This model seems to be largely consistent with our observation that transcriptional activity is associated with degradation, although we might suggest that degradation occurs in the matrix.

The mechanism that regulates association of AML1c with the nuclear matrix is unclear. Previous studies of AML1
association with the matrix (31, 32) have identified what were termed nuclear matrix–targeting signals. These are amino acid sequences located in the COOH terminus of AML1c, which mediate association with the matrix. Zeng et al. (31) identified a nuclear matrix–targeting signal located between AML1c amino acids 351 and 381, whereas Kanno et al. (32) identified nuclear matrix–targeting signal sequences located between AML1c amino acids 318 to 358 and amino acids 399 to 480. Neither site matches the binding site of sin3A (AML1c amino acids 208-237). There could be at least three possible mechanisms for interaction between AML1 and the nuclear matrix: AML1 may associate with the nuclear matrix through the nuclear matrix–targeting signal sequences, possibly by protein-protein interaction; it may associate with sin3A (and the matrix) in a phosphorylation-dependent manner; and AML1 may associate with the matrix through a third mechanism dependent on ubiquitination and entry into the degradation pathway. All three mechanisms may be independently regulated and may interact to determine the precise amount of AML1c associated with the matrix.

Clearly, there is a complex interaction between AML1c phosphorylation, degradation, transcriptional activity, and association with the nuclear matrix. With the exception of TEL-AML1, leukemic translocations delete the COOH terminus of the AML1 protein, suggesting that for those proteins their half-life may be regulated by a different mechanism. Further study is needed to clarify the precise mechanisms of these interactions and their role in regulating transcription, differentiation, and transformation in hematopoietic cells.

**Materials and Methods**

**Cell Culture and Transfection**

293T cells were grown in DMEM medium supplemented with 10% fetal bovine serum at 37°C in 5% CO2. 293T cells were transfected using Effectene (Qiagen Valencia, CA).

**Plasmids**

The pCMV-AML1c wild-type expression plasmid was a gift from S.W. Hiebert (8). The 4XAML-1 luciferase reporter plasmids were a gift from A.D. Friedman (46). The hemagglutinin-ubiquitin plasmid was a gift from M. Treier. Plasmids expressing mutant AML1c were previously described (27). AML1c (1-230) and AML1c (231-480) were created by PCR using the appropriate primers and full-length AML1c plasmid as templates.

**Protein Fractions and Western Blots**

To examine total cell proteins, 293T cells were pelleted, washed in PBS, and boiled in 1× SDS sample buffer. For the purpose of separating soluble proteins from nuclear matrix–associated proteins, cells were lysed in 0.5% IGEPAL-CA630 (similar to Nonidet P-40), 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 20 mmol/L EDTA, 20 mmol/L NaF, 1 mmol/L sodium vanadate, 10 mmol/L benzamidine, 40 mmol/L β-glycerophosphate, and protease inhibitors (Sigma, St. Louis, MO) for 30 minutes at 4°C. Insoluble proteins, including the nuclear matrix, were pelleted by spinning for 10 minutes in a microcentrifuge at 4°C, and the soluble protein supernatant was drawn off. The insoluble pellet was washed once with PBS, then both soluble and insoluble fractions were then prepared for Western blot analysis by the addition of an equal volume of 2× SDS buffer (20% glycerol, 4% SDS, 100 mmol/L Tris-HCl pH 6.8, and 200 mmol/L DTT) and heating at 96°C for 5 minutes. Western blots were done as previously described (47). Anti-AML1 antibodies were purchased from Active Motif (Carlsbad, CA) and Oncogene Research Products (now part of Calbiochem, La Jolla, CA). For treatment with PS341 (Velcade, Bortezomib), cells were transfected with the appropriate expression vectors and incubated for 24 hours at 37°C. Samples were then divided in half and incubated for 12 to 24 hours before beginning the treatment. PS341 was added to a final concentration of 1 μmol/L followed by incubation for 24 hours. Treatment with MG132 (Sigma) was done in the same way, except that the cells were treated for 8 hours at an MG132 concentration of 4 μmol/L.

**Electrophoretic Mobility Shift Assay**

For electrophoretic mobility shift assays, an oligonucleotide containing the AML1c binding site from the M-CSFR (c-fms) promoter was used (19). The assays were done as previously described (24).

**Immunoprecipitation of AML1c**

For immunoprecipitation of FLAG-tagged proteins from 293T cell protein fractions, agarose linked to M2 anti-FLAG antibodies was purchased from Sigma. Cell protein fractions or total protein was incubated with the anti-FLAG agarose overnight at 4°C. Immunoprecipitates were washed thrice in lysis buffer, then at 96°C for 5 minutes in SDS buffer for SDS-PAGE. For immunoprecipitation of matrix-associated AML1c, the matrix fraction was boiled for 10 minutes in 10% glycerol, 2% SDS, and 50 mmol/L Tris-HCl (pH 7.5). This lysate was then diluted 1:10 with 0.5% IGEPA buffer described above for immunoprecipitation. For immunoprecipitation from total cell protein, cell pellets were boiled in glycerol/SDS, then the total cell lysate diluted with IGEPA buffer. For immunoprecipitation of 32P-labeled AML1c proteins, 293T cells transfected with expression vectors were incubated for 5 hours in phosphate-free DMEM medium to which 200 μCi/ml [32P]orthophosphate was added. Immunoprecipitation was then done as previously described. For precipitation of His6-AML1c, cells were lysed in glycerol/SDS as above, then the lysate diluted with 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% IGEPA for incubation with Ni-NTA agarose. For immunoprecipitation of 35S-labeled proteins, 293T cells transfected with expression vectors were incubated for 5 hours in DMEM medium without methionine to which 200 μCi of [35S]methionine were added. For half-life experiments, half of each 35S-labeled sample was transferred to fresh media (DMEM + 10% fetal bovine serum with no cold methionine) and incubated an additional 16 hours.

**Immunofluorescent Staining**

293T cells were seeded onto glass coverslips. After ~24 hours, the cells were transfected with plasmid expressing AML1c. If desired, treatment with PS341 was started 6 to 16
hours after transfection. Twenty-four to 48 hours after transfection, cells on coverslips were fixed for 15 minutes at room temperature in formalin solution, 10% neutral buffered (Sigma). The coverslips were washed thrice with PBS, then blocked overnight in PBS plus bovine serum albumin and 0.5% Tween 20 at 4°C. Coverslips were incubated for 2 hours with anti-FLAG monoclonal antibodies in PBS plus bovine serum albumin and Tween 20, washed thrice with the same solution, then incubated for 1 hour with anti-mouse-FITC antibody. After three final washes in PBS with bovine serum albumin and Tween 20, the coverslips were mounted on glass slides with 4',6-diamidino-2-phenylindole—containing mounting medium (Vectashield from Vector Laboratories, Burlington, CA).

Acknowledgments
We thank Scott W. Hiëbert (Vanderbilt, University, Nashville, TN) for the wild-type AML1c expression plasmid, Mathias Treier (European Molecular Biology Laboratory, Heidelberg, Germany) for the hema/latin-ubiquitin plasmid, and Alan D. Friedman (Johns Hopkins University, Baltimore, MD) for the 4XAML-luciferase reporter plasmid. PS341 (Velaide, Bortezomib) was a gift from Millennium Inc. (Cambridge, MA).

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