Phorbol Ester Treatment of K562 Cells Regulates the Transcriptional Activity of AML1c through Phosphorylation*

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We find that phorbol ester (PE) treatment of K562 cells greatly stimulates promoters (T cell receptor β, myeloperoxidase, macrophage colony-stimulating factor receptor, and granulocyte macrophage colony-stimulating factor receptor) containing AML1 transcription factor binding sites. This stimulation of AML1c transcriptional activity is mediated by direct phosphorylation of the AML1c molecule on multiple phosphorylation sites. Eleven AML1c (S/T)P sites in the transcriptional activating domain are phosphorylated at a basal level in untreated K562 cells; treatment of the K562 cells with PE results in increased phosphorylation at five of these sites (serines 276, 293, 303, 462, and threonine 300). Mutation of these five sites to alanine inhibits PE-induced transcriptional activity; mutation of the sites to an acidic amino acid, aspartic acid, stimulates constitutive activity. Single mutations in four amino acids or double mutations (serines 276 and 293 or threonine 300 and serine 303) have little effect on AML1c transcriptional activity. Inhibitor assays suggest that the ERK family of protein kinases is activated by PEs to phosphorylate the (S/T)P sites within the AML1c molecule and markedly enhance the transcriptional activity of AML1c.

Treatment of hematopoietic cells with phorbol esters, such as phorbol 12-myristate 13-acetate (PMA),1 causes them to undergo differentiation to more mature cell types. The U937, HL-60, NB-4, and PBL leukemic cell lines differentiate into monocyte-like cells, whereas the K562 cell line differentiates into megakaryocyte-like cells (reviewed in Ref. 1). The ability of phorbol esters to induce differentiation of leukemia cells has suggested that additional compounds that activate protein kinase C might be used as treatments for human leukemia (2). It is possible that PMA-induced differentiation occurs through the regulation of a limited number of key proteins, for example transcription factors, that are essential for hematopoiesis. The AML1 transcription factor is necessary for definitive hematopoiesis in embryonic stem cells (3) and for commitment to the hemangioblast stage of development (4). AML1 proteins, including AML1α, AML1β, and AML1c (AML1c is also known as AML1B), are generated from one gene by alternative splicing (5). The AML1c protein is composed of a DNA binding domain (also known as the runt domain) located in the amino terminus of the molecule (amino acids 85–204 in AML1c) and a transcriptional activating domain that occupies a significant portion of the carboxyl terminus. 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 (6). Mice that contain a homozygous knock-out for AML1 or CBFβ are embryonic lethal (7) with absent hematopoiesis and hemorrhage in the central nervous system. The importance of AML1 to normal hematopoietic development is further identified by the frequent disruption of AML1 secondary to translocations (AML/ETO, AML/EVI1, and TEL/AML), deletions, or point mutations (8) in more than 30% of human leukemias. Mutations that destroy one of the AML1 alleles are associated with familial platelet disorders (9), demonstrating that a specific level of AML1 expression is required for normal hematopoiesis. AML1 proteins regulate the transcription of a number of important hematopoietic genes including the T cell receptor α and β (TCRα,β), granulocyte macrophage colony-stimulating factor, myeloperoxidase and neutrophil elastase proteins, and colony-stimulating factor-1 receptor (10–14). Transfection of AML1 leukemic fusions into hematopoietic cells inhibits differentiation and enhances progenitor cell self-renewal (15). Thus, AML1 is a protein that plays a critical role in the control of hematopoietic differentiation.

Evidence suggests the possibility that PMA-induced differentiation of hematopoietic cells could in part be regulated by stimulation of the transcription factor AML1. First, this differentiation program is associated with increases in transcription of mRNAs, e.g. CSF-1 receptor, myeloperoxidase, and elastase that are similarly regulated by AML1 (16–18). Second, transfection of U937 cells with the dominant repressor protein AML1/ETO, a leukemic fusion, blocks the ability of PMA to induce differentiation of these cells (19), suggesting that AML1 activity is necessary for the differentiation process. Third, the knock down of AML/ETO in the human leukemic cell line Kasumi sensitizes these cells to PMA-induced differentiation (20). Thus, the control of AML1 activity by PMA could be essential to the induction of differentiation.

The activity of AML1 appears to be controlled by multiple transcriptional activators and repressors. This protein has been shown to bind to the histone acetylases, p300, MOZ, and MORF, and to the transcriptional activator HES-1 (13, 21–23). The YAP coactivator, which contains a WW protein interaction domain, interacts with a specific PY domain in the carboxyl-terminal half of the AML1 protein (24). The corepressor groucho/transducin-like enhancer of split (Gro/TELE) interacts with...
Phosphorylation of the Transcription Factor AML1c

RESULTS

Phorbol Esters Stimulate AML1 Activity during K562-induced Differentiation—To examine the ability of PMA to regulate the activity of AML1, we transfected K562 cells with both the T cell receptor-β (TCR-β) promoter and an artificial promoter containing four multimerized AML1-binding sites preceding a minimal portion of the thymidine kinase promoter (27). PMA treatment of the transfected K562 cells resulted in a 20-fold increase in the activity of the TCR-β promoter and an 18-fold increase in the activity of the 4XAML1 promoter (Fig. 1A). In both cases, deletion of the AML1-binding sites markedly reduced the PMA induction observed. This result suggests that PMA induction of the TCR-β and 4XAML1 promoters is mediated at least in part through the endogenous AML1 transcription factors expressed in K562 cells.

The precise identity of the endogenous AML1 proteins expressed in K562 cells has not been determined. AML1b is identical to AML1c (also called AML1B) except for 32 amino acids on the amino terminus of AML1c. Because AML1c was the largest protein containing potentially the greatest number of regulatory motifs, we chose to use AML1c in further studies.

To confirm that the biologic effects of PMA on AML1c regulation of the TCR-β and 4XAML1 promoters involve the transactivation and not the DNA binding domain of AML1, a GAL4-AML1c fusion protein containing AML1c amino acids 201–480 (encoding only the transactivation domain) was constructed and placed in the pMEP expression plasmid (Fig. 1B). The pMEP plasmid drives expression through the metallothionein promoter, which can be induced by treating cells with zinc or cadmium. Unlike the cytomegalovirus promoter, the metallothionein promoter is not stimulated by PMA treatment, so PMA treatment does not affect the level of protein expressed with the pMEP plasmid. To measure the activity of the GAL4-AML1c protein, the expression plasmid was cotransfected into K562 cells with a GAL4-luciferase reporter plasmid (containing promoter-binding sites for the GAL4 protein). Transfected cells were then divided into two samples, one treated with CdSO₄ alone to induce expression of the GAL4-AML1c protein and the other treated with CdSO₄ and PMA. As shown in Fig. 1B, the carboxyl terminus of AML1c contains a PMA-responsive activation domain.

PMA treatment of K562 cells activates the ERK kinase pathway, as well as other protein kinases (29). Because the ERK kinase has been shown to bind and phosphorylate AML1 (28), the carboxyl-terminal VWRPY amino acid sequence, and transcriptional repressors mSin3 and N-Cor interact with the AML1 and with histone deacetylases (12, 25). AML1 interacts with other DNA binding factors. It can bind to the ETS family of transcription factors in vitro, and it can synergize and interact with PU.1, another ETS family member in vitro to activate the macrophage colony-stimulating factor promoter (26). Interaction with this ETS factor along with ALY, ATF/CREB, and the macrophage colony-stimulating factor promoter (26). Interaction with PU.1, another P-40), 50 mM Tris, pH 7.5, 150 mM NaCl, 20 mM EDTA, 20 mM NaF, 1 mM vanadate, 10 mM benzamidine, 40 mM β-glycerol phosphate, and protease inhibitors for 30 min at 4 °C. Insoluble proteins, including the nuclear matrix, were pelleted by spinning for 10 min in a microcentrifuge at 4 °C, and the soluble protein supernatant was then prepared for Western blot analysis by the addition of an equal volume of 2× SDS buffer (20% glycerol, 4% SDS, 100 mM Tris-HCl, pH 6.8, and 200 mM dithiothreitol) and heating at 100 °C for 5 min. Western blots themselves were performed as described previously (36). Anti-AML1 antibodies were purchased from Active Motif and Oncogene Research Products.

Luciferase Assays—Luciferase assays were carried out as described previously (36). For luciferase assays using K562 lysate, a small amount of pCMV-Renilla-luciferase plasmid was added to the AML1c expression plasmid and the 4X-AML1 luciferase or other reporter plasmid. A separate small portion of lysate was then assayed for Renilla luciferase activity by using a kit from Promega to normalize the 4X-AML1 luciferase values.

Immunoprecipitation of ³²P-Labeled Proteins—K562 cells transfected with expression vectors for FLAG-tagged AML1c proteins were incubated 5 h in medium containing 200 μCi/mL ³²P orthophosphate. For immunoprecipitation, 10 μl of anti-FLAG-M2 agarose (Sigma) was added to each sample, followed by an overnight incubation on a rocker at 4 °C. Immunoprecipitates were then washed three times in lysis buffer, followed by heating at 100 °C for 5 min in SDS buffer for SDS-PAGE.
FIG. 1. Transcriptional activation by AML1c is stimulated by PMA and blocked by MEK inhibitors. A, plasmids containing either the wild type TCR-β promoter or a promoter with mutated AML1-binding sites was transfected into K562 cells. Transfected cells were divided into two samples, and one sample was treated with 200 nM PMA. After ~16 h, the samples were lysed and assayed for luciferase activity. Solid bars indicate activity in untreated samples; open bars indicate activity in PMA-treated samples. The same experiment was performed with either the 4XAML1 reporter plasmid or plasmid with the AML1-binding sites deleted. The plasmid used is indicated above the graphs, and both reporters are diagrammed at top. All experiments were repeated a minimum of five times, and the S.D. from the mean is shown.

B, diagrams of the AML1c and GAL4-AML1c proteins are shown at top. K562 cells were transfected with 10 μg of GAL4-luciferase reporter and 20 μg of either empty vector (pMEP) or pMEP-GAL4-AML1c expression vector as indicated below each set of bars. Approximately 6 h after transfection, cells were treated with 1 μM CdSO₄. After a further 2 h, cells were either treated with 200 nM PMA or left untreated (solid bars = CdSO₄ only, open bars = CdSO₄ + PMA). Sixteen to twenty four hours after transfection, cells were lysed for luciferase assays. All experiments were repeated a minimum of five times and the S.D. from the mean is shown.

C, K562 cells were transfected with the TCR-β-luciferase reporter plasmid or the 4XAML1 luciferase reporter plasmid with or without 4XAML1 reporter plasmid. After transfection, cells were treated with PMA and/or inhibitors as indicated. Luciferase activity was measured 16–24 h after transfection.
and PMA activates these kinases, we examined the role of inhibitors of MEK in regulating PMA-induced activation of promoters containing AML1-binding sites. K562 cells were transfected with the TCR-β-luciferase reporter plasmid, the 4XAML1 luciferase reporter plasmid, or with the pMEP/GAL4-AML1c expression plasmid and GAL4-luciferase reporter plasmid (Fig. 1C) and either pretreated with MEK inhibitors (PD98059 or U0126) or left untreated. One hour after treatment with inhibitors, samples were treated with PMA and incubated for 16 h (pMEP/GAL4-AML1c samples were treated with CdSO₄ just before PMA addition) before lysis for luciferase activity assays. As shown in Fig. 1C, the MEK inhibitors were then labeled by incubating the cells in media containing [³²P]orthophosphate. After 3–4 h labeling with [³²P]orthophosphate, some cell samples were pretreated with the MEK inhibitor PD98059 for 1 h and then treated an additional hour with PMA. Treatments are indicated above each lane. After treatment, the samples were lysed for immunoprecipitation. C, experiment was performed as in B, but without PMA treatment.

Fig. 2. AML1c is phosphorylated in untreated K562 cells; PMA treatment induces further phosphorylation. A, K562 cells were stably transfected with the pMEP vector expressing AML1c under the control of the metallothionein promoter. Cells were treated with CdSO₄ for 8 h to induce expression of AML1c and for various times with 200 nM PMA as indicated above the lanes. After treatment, cell samples were lysed for Western blotting with anti-AML1 antibodies. Lines to the right of A indicate the different forms of AML1c detected, a lower doublet (not well resolved) and an upper band enhanced by PMA treatment. B, K562/pMEP-AML1c cells were treated with CdSO₄ to induce expression of AML1c. The AML1c proteins were then labeled by incubating the cells in media containing [³²P]orthophosphate. After 3–4 h labeling with [³²P]orthophosphate, some cell samples were treated with the MEK inhibitor PD98059 for 1 h and then treated an additional hour with PMA. Treatments are indicated above each lane. After treatment, the samples were lysed for immunoprecipitation. C, experiment was performed as in B, but without PMA treatment.

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PMA Treatment of K562 Cells Stimulates the Phosphorylation of the AML1c Protein—The data shown in Fig. 1 suggest that PMA treatment of K562 cells may induce direct phosphorylation of the carboxyl terminus of AML1c. K562 cells clearly have sufficient endogenous AML1 protein to mediate PMA activation of promoters containing AML1-binding sites. However, the low level of endogenous AML1 makes interpretation of the Western blots somewhat difficult. To overcome these problems, K562 cells were stably transfected with the pMEP vector expressing AML1c under the control of the metallothionein promoter. As shown in Fig. 2A, treatment of this cell line with CdSO₄ induced high levels of AML1c expression that was expressed as a doublet band (not clearly separated) on SDS-polyacrylamide gels. After 15 min of treatment with PMA, a slower mobility form of AML1c appeared, creating a retarded band above the doublet, suggesting that PMA enhanced the phosphorylation of AML1c in K562 cells. This band was still visible 4 h after the start of PMA treatment, demonstrating this was a long-lived modification of this protein. Analysis of the lysates from these K562 cells with anti-phospho-ERK antibodies that identify activated ERK kinase revealed that some active ERK is present in untreated cells, but PMA treatment causes a substantial increase in ERK phosphorylation and activity. Consistent with this result, immunoprecipitation of AML1c from K562 cells labeled with [³²P]orthophosphate, but not treated with PMA, revealed that the AML1c in untreated cells is phosphorylated (Fig. 2B, lanes 1 and 2). PMA treatment appears to result in an increase in the level of AML1c phos-

plasmid, as indicated above the two left-hand graphs. The transfected K562 samples were either pretreated with MEK inhibitors (PD98059 or U0126) or left untreated, as indicated below the graphs. One hour after treatment with inhibitors, samples were treated with PMA as indicated below the graphs. Samples were then incubated for 16 h before lysis for luciferase activity assays. The right-hand graph shows the reduction of GAL4-AML1c activity by MEK inhibitors. K562 cells were transfected with pMEP-GAL4-AML1c wild type expression plasmid and GAL4-luciferase reporter plasmid. Six hours after transfection, all samples were treated with CdSO₄ to induce expression of GAL4-AML1c, and some samples were treated with MEK inhibitors, as indicated below the graph. One hour later, samples were treated with PMA or left untreated, as shown below the graph. After incubation for a further 16 h, samples were lysed for luciferase assays. All experiments were repeated a minimum of five times and the S.D. from the mean is shown.
phorylation beyond that observed in untreated cells. Pretreatment with the MEK inhibitor PD98059 reduced the level of AML1c phosphorylation in both untreated (Fig. 2C) and PMA-treated cells (Fig. 2B, compare lanes 3 and 4). Thus, base-line ERK activity in untreated K562 cells may be sufficient to partially phosphorylate AML1c, and PMA treatment enhances both ERK activity and AML1c phosphorylation.

Mapping of AML1c Phosphorylation Sites and Identification of Sites Subject to PMA-induced Phosphorylation—The data shown in Fig. 2 indicate that AML1c is subject to a complex pattern of phorbol ester-induced phosphorylation in K562 cells. There is some basal phosphorylation, and phosphorylation at some or all sites is enhanced by PMA treatment. The AML1c protein contains 14 serine or threonine residues followed by proline, the minimal consensus sequence for ERK phosphorylation. Three of these sites are located at the amino terminus of the protein (amino acids 1–230); the remaining 11 sites are distributed over the length of the carboxyl-terminal activation domain (see Fig. 3A).

To determine which Ser-Pro or Thr-Pro sites in the activation domain are phosphorylated in vivo, and which sites exhibit increased phosphorylation after PMA treatment, a series of proteins were expressed in K562 cells containing only the carboxyl terminus of the AML1c protein. The AML1c carboxyl-terminal sequences were placed in a plasmid where expression was under the control of the chicken β-actin promoter, which gives a very high level of expression in K562 cells. These constructs contained only one wild type Ser-Pro or Thr-Pro site and 10 mutated sites (Fig. 3A). Each construct was transfected into K562 cells, which were then divided and either treated with PMA or left untreated. As shown in Fig. 3B, all 11 Ser-Pro or Thr-Pro sites are phosphorylated at a low level in untreated K562 cells. However, only five sites (serines 276, 293, 303, 462, and threonine 300) show increased phosphorylation after PMA treatment. Because threonine 300 is at times the most difficult to see, this experiment was repeated in triplicate, and a representative example of phosphorylation of this amino acid is shown in Fig. 3C.

To determine whether the Ser-Pro/Thr-Pro sites account for all AML1c phosphorylation in K562 cells, constructs with all 11 sites mutated, or just the 5 PMA-induced sites mutated, were compared with a wild type construct (diagrammed in Fig. 4A). As shown in Fig. 4B, mutation of the 11 Ser-Pro/Thr-Pro sites eliminates almost all phosphorylation. Mutation of the 5 sites (serines 276, 293, 303, 462, and threonine 300) eliminates PMA-induced phosphorylation.

Mutation of Potential ERK Phosphorylation Sites Inhibits PMA-stimulated AML1c Transcription—The AML1c Ser-Pro or...
Thr-Pro sites that displayed enhanced phosphorylation after PMA treatment of K562 cells should play the greatest role in the PMA enhancement of AML1c transcriptional activity. To test this hypothesis, Ser-Pro/Thr-Pro site mutations were introduced into full-length AML1c (diagrammed in Fig. 5A). The serine or threonine residues were mutated to alanine at two Ser-Pro/Thr-Pro sites (276 and 293 or 300 and 303), at four sites (276, 293, 300, and 303), or at five sites (276, 293, 300, 303, and 462). In the 5-site mutant construct, all PMA-induced phosphorylation sites have been mutated. A final AML1c protein with all 11 Ser-Pro/Thr-Pro sites mutated was also generated. All constructs were then tested for activity in K562 cells.

By using the 4XAML1 luciferase plasmid to assay AML1c activity in K562 cells, it was observed that mutation of serines 276 and 293 in AML1c-2 M (276/293) had little effect on AML1c activity (Fig. 5B). The 4XAML1 luciferase reporter displays substantial activity in K562 cells without cotransfected AML1c expression vector. This is presumably because of endogenous AML1 proteins. Cotransfection of the reporter with wild type AML1c enhances activity in PMA-treated cells about 2-fold. Mutation of serines 300 and 303 in AML1c-2M (300/303) resulted in a slight inhibition of activity (Fig. 5B). Likewise, mutation of individual sites including 276, 293, 300, and 462 demonstrated no significant change from wild type AML1c, whereas 303 mutation alone showed similar results to the AML1c (300/303). However, mutation of all four sites (276, 293, 300, and 303) resulted in the loss of all transcriptional activation in K562 cells due to cotransfected AML1c (Fig. 5B). However, unlike the 11M (see below), this protein does not appear to function as a dominant-negative transcription factor.

It is possible to mimic the effect of phosphorylation at serine or threonine residues by mutating the serine or threonine to glutamic acid or aspartic acid (38). This places a negative charge at the site in the same manner as the presence of phosphoserine or phosphothreonine. In AML1c-4M (Ser → Asp), the four serines and threonines are mutated to aspartic acid. As shown in Fig. 5B when the cells are treated with PMA, this mutant AML1c protein has greater activity than wild type. This increase is clearly due to the placement of the charged amino acids at the four residues. We do not see a great deal of difference between the activity of AML1c-5M (Ser → Asp) and AML1c-4M (Ser → Asp) (Fig. 5B) treated with PMA. AML1c-5M (Ser → Asp) contains the 4M (Ser → Asp) mutations plus serine 462 mutated to aspartic acid.

We have observed that AML1c-11M has less transcriptional stimulatory activity on the 4X-AML1 promoter than AML1c-4M in K562 cells (Fig. 5B). This suggests the loss of basal phosphorylation at the remaining Ser-Pro or Thr-Pro sites has some effect on the function of this transcription factor.

As mentioned earlier, wild type or mutant AML1c expressed in K562 cells has to compete with endogenous AML1 proteins for the AML1-binding sites on the 4X-AML1 luciferase reporter, complicating the analysis of the mutant proteins (12). The endogenous protein induces background activity and ambiguity when full-length AML1c is assayed for activity in K562 cells. To avoid this problem, and to confirm results with full-length AML1c, we constructed the series of GAL4-AML1 fusion proteins with (S/T)P site mutations diagrammed in Fig. 5C. AML1c amino acids 201–480 (encoding the AML1c transcription domain) were fused to the GAL4 DNA binding domain (GAL4 amino acids 1–147). The AML1c amino acid sequence was either wild type or carried some of the mutations analyzed in the previous section. DNA binding by the GAL4 domain will not be affected by mutations in the AML1c protein sequence. Therefore, the possibility that AML1c mutations affect activity by interfering with DNA binding is eliminated.

To compare the activity of the wild type and mutant GAL4-AML1c proteins, the expression plasmids were cotransfected into K562 cells with a GAL4-luciferase reporter plasmid containing promoter-binding sites for the GAL4 protein. Transfected cells were then divided into two samples, one treated with CdSO4 alone to induce expression of the GAL4-AML1c protein, and the other treated with CdSO4 and PMA. As shown in Fig. 5D, PMA treatment of K562 cells stimulates the activity of wild type GAL4-AML1c by 5–6-fold. Introduction of the 4M mutations (serines 276, 293, and 303 and threonine 300 mutated to alanine, see Fig. 5C) abolished the PMA-induced activity of GAL4-AML1c but had little effect on basal activity (Fig. 5D). The introduction of further mutations in GAL4-AML1c-5M (the 4M mutations plus serine 462 mutated to alanine) and GAL4-AML1c-11M had little additional effect on activity.

In the GAL4-AML1c-5M (Ser → Asp) protein, the 4M residues and serine 462 were mutated to aspartic acid. Without any treatment, these mutations to aspartic acid alone cause a 3–4-fold increase in GAL4-AML1c activity in untreated K562
cells (Fig. 5D), demonstrating that acidic charges at these positions are sufficient to activate transcription. As demonstrated (Fig. 5B) for full-length AML1c, PMA treatment further enhanced the transcriptional activity of the 5M (Ser → Asp) protein. Overall, the effects of (S/T)P site mutations in the GAL4-AML1c protein confirm the results obtained by analysis...
of mutations in full-length AML1c. The serine and threonine residues mutated in AML1c-4M and GAL4-AML1c-4M drastically reduce activity when mutated to alanine and stimulate activity when mutated to aspartic acid. This clearly suggests that phosphorylation of these residues has a large role in the regulation of AML1c activity.

AML1c-4M and AML1c-11M Suppress the Activity of Several Different Promoters Containing AML1-binding Sites—The 4M (S/T)P site mutations clearly regulate the transcriptional activity of AML1c on the 4X-AML1 luciferase promoter and of GAL4-AML1c on a GAL4 luciferase promoter (Fig. 5). AML1-binding sites are found in the promoters of a number of genes involved in hematopoietic differentiation. We have selected four additional gene promoters for analysis with mutant AML1c proteins. Each gene promoter has been inserted upstream of luciferase coding sequences to create a reporter plasmid, and these reporter plasmids were cotransfected into K562 cells with AML1c expression vectors (Fig. 6). The reporter plasmids were cotransfected with empty vector or vectors expressing wild type AML1c, AML1c-4M, or AML1c-11M.

When K562 cells were cotransfected with empty expression vector and the various reporter plasmids, substantial stimulation of reporter activity by PMA treatment was still observed (Fig. 6, bars labeled empty vector). Stimulation of the empty vector by PMA could be secondary to effects on other basal transcription factors. The fold increase stimulated by PMA varied depending on the basal activity of the individual promoters. With the exception of the TCR-β promoter, the transcriptional activity of AML1c-4M, unlike expression of wild type AML1c, was not stimulated by PMA. This indicates that on most, but not all, promoters phosphorylation of the serines and threonine mutated in AML1c-4M is necessary for PMA-induced AML1c activity. In comparison, on the TCR-β promoter the 11 mutations were necessary to knock out PMA inducible activity.

The results shown in Fig. 6 suggest that specific phosphoryl-
Phosphorylation of the Transcription Factor AML1c

Phosphorylation sites on AML1c may be important for normal AML1c activity on some promoters but not on others. Because each promoter binds a unique combination of transcription factors, AML1c will be subject to a specific set of protein-protein interactions. Phosphorylation at a particular site on AML1c may promote some interactions, inhibit others, or have no effect at all.

**DISCUSSION**

The phosphorylation of AML1c is clearly a complex process. The protein is phosphorylated at multiple sites that display different responses to PMA stimulation. Phosphorylation of four closely spaced serine and threonine residues (serines 276, 293, 303, and threonine 300) plays a key role in the regulation of AML1c activity. Unlike most other Ser-Pro or Thr-Pro sites in the AML1c activation domain, these four sites all exhibit increased phosphorylation in response to PMA treatment. Mutation of these four sites to alanine reduces AML1c transcriptional activity; mutation of the four sites to aspartic acid enhances transcriptional activity.

It is not yet clear whether each of these sites has a specific function or whether the sites act together, with the function of individual sites being somewhat redundant. Our experiments show that single mutation of a phosphorylation site or a double mutation out of four sites (either serines 276 and 293 or threonine 300 and serine 303) has little or no effect on transcriptional activation by AML1c. This suggests that all four sites may regulate the same process and that retention of at least two sites leaves AML1c functional.

Mutation of the other seven Ser-Pro/Thr-Pro sites in the AML1c activation domain, individually or in pairs, appears to have little effect on AML1c transcriptional activation. However, mutation of all 11 AML1c Ser-Pro or Thr-Pro sites results in a protein with less transcriptional activity than the 4M protein, at least on some promoters. This suggests that the four PMA-induced Ser-Pro/Thr-Pro sites in the AML1c activation domain may be the strongest regulators of activity, but the remaining sites may have subtle effects on activity, perhaps through different mechanisms. We cannot rule out the possibility that the 11M mutant destroys the tertiary structure of the carboxyl terminus preventing any binding of transcriptional activators.

We find that mutation of five (S/T)P residues to aspartic acid converts AML1c to a constitutive activator of transcription. This finding again suggests the importance of these sites to the control of transcription by this protein. In addition, we find that PE further stimulates the activity of this protein even when the five sites are mutated. This result might be explained by our previous observation that PE treatment of leukemic cells can modulate other proteins involved in the transcription process, for example the activity of the TATA box-binding protein (39). Alternatively, it cannot be ruled out that PE treatment is also causing changes in the phosphorylation of other sites that enhance the effects of the major phosphorylation targets.

The phosphorylation pattern we have observed for AML1c appears to be different from those observed with other AML proteins. Previous studies on the AML1b protein (28) have identified only two phosphorylation sites, on AML1b serines 249 and 266. Phosphorylation of these sites was induced by overexpression of ERK and epidermal growth factor treatment of COS-7 cells or by interleukin-3 treatment of BAF3 cells. AML1b and AML1c are products of alternately spliced mRNAs from the AML1 gene and are identical except for 32 amino acids at the amino terminus. AML1b serines 249 and 266 are equivalent to AML1c serines 276 and 293, two of the residues we have identified as PMA-induced phosphorylation sites. It is unclear why AML1b and AML1c would have very different phosphorylation patterns; it is possible that the extent of phosphorylation at each site is cell type-specific or the extent of stimulation of the ERK pathway may regulate varying phosphorylation sites.

Phosphorylation sites have also been identified for the RUNX2/AML3 protein (40). RUNX2/AML3 shares extensive sequence homology with RUNX1/AML1. RUNX2/AML3 serines 14 and 451 were identified as major phosphorylation sites and serine 104 as a minor phosphorylation site when the protein was expressed in 293T, SAOS-2, or COS-7 cells. RUNX2/AML3 serine 451 corresponds to RUNX1/AML1c serine 424, which we identified as a phosphorylation site that does not respond to PMA in K562 cells. Mutation of RUNX2/AML3 serine 451 to alanine increased RUNX2/AML3 transcriptional activity, whereas mutation of this serine to glutamic acid had no effect (40). This suggested that phosphorylation of RUNX2/AML3 serine 451 suppressed activity. In contrast, mutation of RUNX1/AML1c serine 424 had little or no effect on transcriptional activity.2 This difference may be either protein-specific or cell type-specific.

The two phosphorylation sites in the RUNX2/AML3 amino terminus, serines 14 and 104, correspond to RUNX1/AML1c threonine 41 and serine 94. RUNX1/AML1c has an additional Ser-Pro site at serine 48; in RUNX2/AML3 this serine has been replaced by glutamine. Mutation of RUNX2/AML3 serine 104 to glycine or glutamic acid reduced RUNX2/AML3 activity and appeared to destabilize the protein. Mutation of serine 104 to glutamic acid also appeared to inhibit the interaction between RUNX2/AML3 and its heterodimerization partner, CBF-β, although mutation of serine 104 to glycine did not have this effect. It is therefore possible that phosphorylation of RUNX2/AML3 serine 104 inhibits the interaction between RUNX2/AML3 and CBF-β, but it is also possible that the effects of mutations at serine 104 are conformational. We find that mutation of RUNX1/AML1c serine 94, comparable with RUNX2/AML3 serine 104, has little or no effect on RUNX1/AML1c activity.2

Mutation of the other RUNX2/AML3 amino-terminal phosphorylation site, serine 14, had no detectable effect on RUNX2/AML3 activity (40). Likewise, we find that mutation of RUNX1/AML1c threonine 41 and serine 48 eliminates phosphorylation of the amino terminus but has little effect on RUNX1/AML1c activity.2 The functional significance of RUNX1/AML1c amino-terminal phosphorylation therefore remains unclear.

PE-induced differentiation of K562 and U937 cells has been a model for examining how small molecules might regulate protein kinases to inhibit the growth of leukemic cells. PE stimulates the mitogen-activated protein kinase family of protein-tyrosine kinases to inhibit the growth of leukemic cells. Phosphorylation at each site is cell type-specific or the extent of phosphorylation patterns; it is possible that the extent of phosphorylation at each site is cell type-specific. PE stimulates the mitogen-activated protein kinase family of enzymes and regulates the activity of a number of transcription factors, including c-JUN and ETS. Here we demonstrate that PE activation of the ERK pathway has profound effects on the activity of AML1. These findings shed light on how PE regulates genes that are essential for the control of hematopoietic differentiation.

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