A Conserved Cysteine Residue in the runt Homology Domain of AML1 Is Required for the DNA Binding Ability and the Transforming Activity on Fibroblasts*

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The AML1 gene encodes DNA-binding proteins that contain the runt homology domain and is found at the breakpoints of t(8;21), t(3;21), and t(12;21) translocations associated with myelogenous leukemias. AML1 heterodimerizes with PEBP2α/β/CBFβ, resulting in the enhanced affinity with DNA. The runt homology domain is responsible for binding with DNA and heterodimerizing with PEBP2α/β/ CBFβ. AML1 is suggested to perform a pivotal role in myeloid cell differentiation, whereas it can cause neoplastic transformation when overexpressed in fibroblasts. In this study, we demonstrated that the reducing reagent, dithiothreitol (DTT), markedly enhances the DNA binding of AML1 expressed in COS7 cells. Oxidation by diamide or modification by N-ethylmaleimide of the free sulfhydryl residues inhibited the interaction of AML1 with DNA. The diamide effect was irreversible with excess of DTT, whereas DTT could not restore the DNA binding of AML1 treated with N-ethylmaleimide. Site-directed mutagenesis of the amino acid residue 72, a highly conserved cysteine in the runt homology domain of AML1, to serine almost completely abolished DNA binding without altering the interaction with PEBP2α/β/ CBFβ. This substitution also impaired transactivation through the consensus DNA sequence and transformation of fibroblasts induced by AML1b. These data indicate an essential role of the conserved cysteine residue in DNA binding of AML1, and it is possible that the redox state of AML1 could contribute to the regulation of its function.

AML1 was originally identified as the gene on chromosome 21 which is disrupted in the t(8;21)(q22;q22) translocation associated with human acute myelogenous leukemia (1). It is also involved in human leukemias carrying t(3;21) or t(12;21) translocation (2–6), suggesting it plays an important role in leukemogenesis. AML1 encodes site-specific DNA-binding proteins that share a high homology within the 128-amino acid region and the murine transcription factor, PEBP2α (8–10). This region is evolutionally conserved and termed the runt homology domain. PEBP2, also called a core binding factor (CBF) (11), is composed of two structurally independent subunits, α and β (10–12). PEBP2α/β/CBFα, which encodes the α subunit of PEBP2, has two related genes, PEBP2αA and PEBP2αB/ CBFα (10, 13), with the latter being a murine counterpart of AML1. AML1 and PEBP2α/CBFα bind to DNA in a site-specific manner (11, 13) and recognize the consensus sequence, (R/T)ACCCRA (10, 14, 15), designated the PEBP2 site, which is found in the enhancer regions of T-cell receptor genes and the myeloperoxidase gene (16, 17). On the other hand, the β subunit (PEBP2β/CFBβ) does not bind to DNA but associates with the α subunit, enhancing the affinity of the α subunit to DNA. The human PEBP2β/CFBβ gene has been reported to be disrupted and fused to a smooth muscle myosin gene in inv(16)(p13q22), which is associated with acute myelogenous leukemia (18).

Three isoforms of AML1 proteins, AML1α, AML1β, and AML1c, are produced by alternative splicings from the AML1 gene (19). AML1α is a 250-amino acid protein that possesses the runt homology domain (1). AML1β is a longer 453-amino acid protein which, downstream of the runt homology domain, contains a proline-, serine-, and threonine-rich region that is absent in AML1α (20). This region is called the PST region. AML1c differs only 32 amino acids in the N-terminal from AML1b (1) and is presumed to have a similar function as AML1b. The runt homology domain is known to be essential for DNA binding properties of the proteins (15, 21), whereas the PST region is thought to contain a transcriptional activation domain (20, 22). Previously, we showed that AML1α and AML1b act antagonistically for transactivation and myeloid cell differentiation (22), suggesting that transcriptionally active AML1 is essential for myeloid cell differentiation.

Reduction-oxidation (redox) is one of the mechanisms that post-translationally modulate the activities of transcription factors, reduction enhances binding of a subset of transcription factors to DNA. These facts suggest that cysteine residues could play an important role in the DNA binding process. To examine the regulatory mechanisms for the DNA binding of AML1, we have investigated the effect of the redox state of AML1 on DNA binding. These studies were prompted by the recognition that DNA binding of AML1 was enhanced by the presence of dithiothreitol (DTT). All of the known runt protein family members contain conserved cysteine residues in the DNA-binding domain. To define the involvement of specific cysteines in AML1 in DNA binding, we have examined the effect of chemical modification and site-specific mutagenesis of electrophoretic mobility shift assay; NEM, N-ethylmaleimide; WT, wild type.
cysteine residues in AML1b on its DNA binding activity. We have found that the most well-conserved cysteine in the runt homology domain is required for the optimal DNA binding and transactivation through the PEBP2 site induced by AML1. This cysteine did not affect the association between the AML1 protein and its heterodimeric partner, PEBP2β/ICBFβ. We have also shown that this cysteine residue is necessary for fibroblast transformation exerted by AML1, suggesting a pivotal role of the cysteine in the in vivo regulation of the AML1 function.

EXPERIMENTAL PROCEDURES

Cell Culture—COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). NIH3T3 cells were maintained in DMEM with 5% FCS. P19 cells were cultured on gelatin-coated dishes in DMEM (high glucose formulation) with 10% FCS (23).

Plasmid Construction—The human AML1b cDNA was inserted into the EcoRI site of pME18S, an Srα promoter driving expression plasmid (24), as described elsewhere (22). A retrovirus construct that expresses AML1b was established using as a retroviral vector pSRαMSVtkneo which expresses the bacterial neomycin resistance gene, neo, driven from an internal herpesvirus thymidine kinase gene promoter (25), as described previously (26). To generate the AML1b site-directed mutagenesis (27) was carried out. Each of the three cysteine residues located in AML1b was individually changed to a serine residue. The substitutions of serine for cysteine were confirmed by sequencing. Each mutant was cloned into pME18S and pSRαMSVtkneo. For construction of pG8Tβ-b(60–189)WT, -b(60–189)C72S, and -b(60–189)C81S, internal Smal DNA fragments (248–638) (numbers in parentheses indicate nucleotide numbers from the start site of translation to the cutting site of the enzyme) of the wild-type and the two cysteine mutants of AML1b, which encode the regions between amino acids 60 and 189, were cloned into the Smal site of the yeast GAL4 DNA-binding domain vector pG8Tβ (Clontech). pGAD424-β was produced by inserting the murine PEBP2β/ CBFβ probe containing the GAL4 DNA-binding domain, pGAD424 (Clontech). For construction of pME-b(1–203)WT, -b(1–203)C72S, and -b(1–203)C81S, fragments from the PvuI site (680) within the AML1b cDNA to the SpeI site located downstream of the EcoRI site of pME18S were deleted from the wild-type and the two cysteine mutants of AML1b placed in pME18S, and the resultant fragments were religated. To generate pME-b(60–189)WT, -b(60–189)C81S, pME-b(60–189)C72S, and -b(60–189)C81S, internal EcoRI DNA fragments (248–638) (numbers in parentheses indicate nucleotide numbers from the start site of translation to the cutting site of the enzyme) of the wild-type and the two cysteine mutants of AML1b, which encode the regions between amino acids 60 and 189, were cloned into the SmaI site of the yeast GAL4 DNA-binding domain vector pG8Tβ (Clontech).

RESULTS

Reduction Activates DNA Binding of the AML1 Protein Produced by COS7 Cells—To study the DNA binding of AML1, we used nuclear extracts of COS7 cells transfected with the AML1-expressing vector. A full-length AML1b cDNA cloned into pME18S was transfected into COS7 cells, and nuclear extracts were prepared as described previously (26). We performed EMSA using a double-stranded oligonucleotide containing the PEBP2β site as a probe (M4 probe). When the probe was incubated with nuclear extracts of COS7 cells expressing AML1b, a distinct band was observed which was not recognized in the lane loaded with the mock transfectant (Fig. 1A, lanes 1 and 2), in agreement with the previous data (22). This band became undetectable when a cold probe (containing the same sequence as a labeled probe) was incubated with a labeled probe (Fig. 1A, lane 3). On the other hand, it was not affected when incubated with an unlabeled mutated probe in which the PEBP2β site was replaced by a sequence different from the PEBP2β site consensus (Fig. 1A, lane 4). These findings indicate that the band contains the AML1b protein and that the binding of AML1b to the probe specifically depends on the nucleotide sequence of the PEBP2β site.

To investigate whether the modification of free sulfhydryl groups alters the interaction of the AML1 protein with its DNA-binding site, nuclear extracts were treated with DTT. Nuclear extracts of AML1b-expressing COS7 cells were incubated with or without DTT in the binding buffer for 15 min at room temperature, and then the radiolabeled M4 probe was added and incubation continued for 30 min at room temperature. Samples were subsequently analyzed using EMSA. As shown in Fig. 1B, the DNA binding activity of AML1b was

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nuclear extracts were treated with 3 mM NEM, the DNA pudding NEM, which alkylates free sulfhydryl groups. When hydryl groups in DNA binding, we took advantage of the com-
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treated with reagents that oxidize free sulfhydryl groups. Diamide (NEM) severely reduced the DNA binding function of the AML1 protein produced in COS7 cells.

The AML1b protein contains three cysteine residues (Cys-72, Cys-81, and Cys-405), two of which (Cys-72 and Cys-405) are located in the runt homology domain. The two cysteines in the runt homology domain are highly conserved among the human AML1 proteins. To determine whether these three residues are responsible for redox modulation in the DNA binding property of AML1, the cysteine residues were individually replaced with a serine residue (Fig. 2A), since serine, which is structurally similar to cysteine, possesses a hydroxyl group instead of a sulfhydryl group. We prepared nuclear extracts from COS7 cells transfected with each mutant and compared the DNA binding abilities of the wild-type and the mutant AML1 proteins. The nuclear extracts of the wild-type and the mutant AML1 proteins prepared from COS7 cells were incubated with the M4 probe. The bc72S mutant showed decreased DNA binding as compared with the wild-type, the bc81S, or the bc405S mutant. C, expression of the AML1 mutants in COS7 cells. The nuclear extracts prepared from COS7 cells used in B were analyzed by the immunoblotting with the anti-AML1 antibody. The far left lane indicates the COS7 cells introduced with the empty pME18S vector. The equivalent amounts of each product are observed. The migrations of the proteins are marked by the arrows.
Cys-72 is important for the DNA binding activity of the AML1 proteins.

Cys-72 in AML1b Is Required for Both Transactivation through the PEBP2 Site and Fibroblast Transformation—To examine the effect of the cysteine to serine substitution on the binding of AML1 to the target DNA in vivo, we analyzed the cysteine mutant AML1b for transcriptional activation through nucleotide sequences containing the PEBP2 sites by employing the luciferase assay system. The P19 murine embryonal carcinoma cell line, whose endogenous PEBP2/CFB activity is very low when maintained in an undifferentiated state, was cotransfected with the PEBP2 site-containing reporter plasmid Tww-tk-Luc and a vector expressing the wild-type or the mutant AML1b. As shown in Fig. 4, we observed a 17-fold or more transactivation when wild-type AML1b was expressed. This transactivation depends specifically upon the PEBP2 sites because fold induction was reduced to less than 30% when we used Tmm-tk-Luc in place of Tww-tk-Luc, in which two PEBP2 sites were altered to different sequences from those of the PEBP2 consensus. Those cysteine-mutants of AML1b which retained DNA binding (bC81S, bC405S) transactivated the reporter gene as efficiently as did the wild-type of AML1b. In contrast, bC72S, whose DNA binding ability was markedly abrogated, showed the significantly reduced level of transactivation.

Recently, we have reported that overexpression of AML1b induces neoplastic conversion of NIH3T3 cells depending on the DNA binding activity (26). To study the role of Cys-72 and the other cysteine residues in the in vivo function of AML1, we compared transactivation activities of the wild-type and the cysteine mutants of AML1b. The cDNA coding each mutant was inserted into the retroviral pSR vector, and replication-deficient retroviruses were generated by hyperexpression of the corresponding plasmid in COS7 cells. NIH3T3 cells were infected with retroviruses containing each expression plasmid, and soft agar assays were performed on G418-resistant populations. Soft agar colonies resulting from infection of NIH3T3 cells with each of the cysteine mutants of AML1b are shown in Figs. 5 and 6. The two cysteine mutant proteins, bC82S and bC405S, produced macroscopic colonies similar in number to the wild type of AML1b, indicating mutation of Cys-82 or Cys-405 did not affect fibroblast transformation defined by anchorage-independent growth of the cells in soft agar. However, replacement of Cys-72 to serine in bC72S remarkably impaired the transactivating activity of AML1b, presumably because of the inability to bind DNA efficiently. These results suggest that Cys-72 which was necessary for the efficient DNA binding is also important for the biological activity of AML1 in vivo.

Substitution of the Cysteine Residues to Serine Does Not Alter the Interaction of AML1 with the β Protein—The β subunit is a heterodimeric partner of the AML1 protein. AML1 binds to DNA only weakly, whereas the β subunit increases the DNA binding affinity of AML1 without interacting with DNA by itself (10, 12). The AML1 protein expressed in COS7 cells would act as the AML1/PEBP2/β/CFB complex by heterodimerizing with the β protein which exists endogenously in COS7 cells. Therefore, it is plausible that the interaction of the β subunit with AML1 could determine the DNA binding property of the AML1 protein in nuclear extracts of COS7 cells. AML1 is known to heterodimerize with the β subunit through the runt homology domain (12). To examine whether cysteine residues included in AML1 might affect the heterodimerizing property of the β subunit with AML1, we analyzed the effects of the substitution of the two cysteines within the runt homology domain upon interaction with the β protein, using the yeast two-hybrid system. To clarify the specific interaction of the runt homology domain with the β protein and to eliminate nonspecific transactivation induced by AML1b in yeast, we employed the regions between amino acids 60 and 189 of AML1b variants (b(60–189)) which include the entire of the runt homology domain. Each fragment encoding these regions
was introduced into the yeast vector (pGBT9-b(60–189)) and expressed as GAL4 DNA-binding domain fusions (GAL4bd-b(60–189)) (Fig. 7). A protein consisting of the GAL4 activation domain fused to full-length PEBP2b/CBFb (GAL4ad-b) was coexpressed with GAL4bd-b(60–189) in the yeast strain SFY526. As shown in Fig. 8, the chimeras containing each cysteine mutant of the runt homology domain showed no significant differences in levels of β-galactosidase activity as compared with the wild-type runt homology domain. These interactions depend upon the specific interaction of the runt homology domain and the β subunit, because the three GAL4bd-b(60–189) fusion proteins did not interact with GAL4 activation domain alone at all.

To analyze further the interaction between the AML1 and the β proteins, we examined subcellular localization of these proteins employing immunofluorescence labeling of COS7 cells. To evaluate unequivocally the interaction of the proteins in cells, DNA fragments encoding the region between amino acids 1 and 203 (b(1–203)) derived from the wild-type and the cysteine mutants of AML1b were inserted individually into pME18S. As presented schematically in Fig. 7, these regions contain the entire runt homology domain and could interact with the β protein effectively. The resultant plasmids, pME-b(1–203)WT, -b(1–203)C72S, and -b(1–203)C81S, and the control vector were cotransfected with full-length PEBP2β/CBFβ in pME18S (pME-β) into COS7 cells. The protein products were detected by labeling with fluorescein-conjugated antibodies. Labeling with the anti-AML1 antibody shows the nuclear localization of b(1–203)WT expressed in COS7 cells (Fig. 9b). Substitutions of the cysteines in the runt homology domain to serine did not alter the nuclear localization of the proteins (Figs. 9, c and d). The β protein, which is known to localize in the cytoplasm when expressed in NIH3T3 cells (34), has been revealed to be mainly a cytoplasmic protein also in COS7 cells by labeling with the anti-β antibody (Fig. 9e). Furthermore, the β protein was translocated into the nucleus when b(1–203)WT was coexpressed (Fig. 9f). Similar colocalizations were observed when pME-b(1–203)C72S or pME-b(1–203)C81S was cotransfected (Fig. 9, g and h). Therefore, the cysteine residues in the runt homology domain do not affect the translocation of the β protein into the nucleus exerted by the runt homology domain of AML1.

**DISCUSSION**

Our data indicate that the DNA binding property of AML1 is sensitive to reduction and oxidation. Redox modulation is one of the post-translational control mechanisms for various factors. Growing numbers of transcription factors have proven to be subject to redox modulation. It has been shown that Fos/Jun binding to DNA is enhanced by treatment with DTT or the reductase Ref-1 (35, 36). The activity of NFκB is affected by thiol-oxidizing or -reducing agents (37, 38). The affinity of the upstream stimulating factor for DNA is decreased through formation of intermolecular and intramolecular disulfide bond (39). Furthermore, E2A helix-loop-helix proteins form homodimers by intermolecular disulfide bond resulting in increased DNA binding (40). By different mechanisms, redox manipulation may also affect the activities of other transcription factors, such as Myb and Ets (41–43).

We have demonstrated that modification of a free sulfhydryl can impair the DNA binding process of AML1 in vitro. Specifically, we have shown the ability of both alkylating (NEM) and oxidizing (diamide) agents to eliminate the binding. From the observation that addition of DTT to nuclear extracts could remarkably enhance the binding of AML1 to the PEBP2 site, it is suggested that a considerable part of AML1 expressed in COS7 cells is present in an inactive form that could be converted to an active form by DTT. There are several observations consistent with the hypothesis that redox modulation may be used as a means of cellular signaling or control of cellular metabolism. For example, reducing agents such as 2-mercaptoethanol support the growth of lymphoid cells (44), whereas depletion of glutathione from T-cells inhibits antigen-driven T-cell activation and proliferation (45). If reducing conditions are related to hemopoietic cell differentiation, AML1 might be stimulated to bind the regulatory DNA sequence and induce expression of target genes at an appropriate stage of differentiation. It is of interest to determine whether the regul-
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alter the heterodimerizing property of AML1 with the β protein nor subcellular localization of the β protein determined by AML1. Therefore, the reduced DNA binding of bC72S would reflect the altered interaction of AML1 itself with DNA rather than the lack of enhancing effect of the β protein. It has been suggested that the DNA-binding domain of Myb undergoes a conformational change upon binding DNA (50). The crystal structure of the papillomavirus E2 protein in complex with DNA demonstrated that a cysteine residue possesses a recognition function in sequence-specific DNA binding of proteins (51). A similar mechanism might be active in the case of AML1.

Further investigation is needed to determine the precise role of the cysteine residue in the DNA binding activity.

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Fig. 9. Immunofluorescence labeling of the AML1 mutants and the PEBP2β/CBF b protein expressed in COS7 cells. Transfected plasmids are as follows: a and e, pME and pME-β; b and f, pME-(1-203)WT and pME-β; c and g, pME-(1-203)C72S and pME-β; d and h, pME-(1-203)CBF b and pME-β. Immunofluorescence labeling with the anti-AML1 antibody (a–d) and with the anti-β antibody (e–h) is shown.

ulation of the reducing activity exists along with differentiation of hemopoietic cells and whether it can be used as a means for cellular signaling in the case of AML1.

We identified the critical cysteine residue (Cys-72) in the runt homology domain for the DNA binding property of AML1b. This cysteine was found to be important for DNA binding since its mutation to serine almost completely abolished this property. Substitution of Cys-72 to serine resulted in the decreased transactivational potency and the impaired transforming activity of AML1b, indicating a critical role for this cysteine in the integrated functions of the AML1 proteins. Prior studies have indicated that many transcription factors such as Fos, Jun, Rel, Ets, and NF-κB contain essential cysteine residues, modification of which affects the DNA binding affinities (35, 46–49). DNA binding of the Fos/Jun heterodimer was modulated by a single conserved cysteine residue in the DNA-binding domains of the two proteins (35). Replacement of Cys-154 in Fos and Cys-272 in Jun with a serine residue resulted in increased DNA binding activity (35). In contrast, replacement of Cys-130 in Myb with a serine residue resulted in loss of DNA binding and transactivation in vivo (41, 42). For v-Rel protein, the conserved cysteine in the RXXRXXXC motif is critical for the regulation of DNA binding (49). The molecular mechanism by which Cys-72 in AML1 contributes to site-specific DNA binding is not known. Our results suggest that Cys-72 does not
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