Down Regulation of DHFR Promoter Activity by PML Transcription Repressor

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Abstract

The PML gene encodes a nuclear protein with transcriptional regulatory activity. It directly interacts with the Sp1 transcription factor and abrogates its activation of the epidermal growth factor receptor (EGFR) gene promoter. Here, the effects of PML on the activity of dihydrofolate reductase (DHFR) promoter, mainly regulated by Sp1, were investigated. On functional analysis, transient transfection of PML into mammalian cells, U2Os and HeLa, resulted in a significant repression of the DHFR promoter. The DHFR promoter also contains binding sites for the E2F transcription factor. When the Sp1 DNA-binding site, but not the E2F-binding site of the promoter, was replaced with an unrelated binding site (GAL4), the repressive effects of PML were lost. Moreover, electrophoretic mobility shift assay (EMSA) using Sp1-containing oligonucleotide probes showed significant reduction in Sp1 binding in the presence of PML, confirming the necessity of the Sp1 DNA-binding sites for PML’s repressive effects. Analysis of DNA synthesis using [3H]thymidine incorporation assay showed a significant reduction in DNA synthesis in HeLa cells overexpressing PML. Together, the data demonstrated that PML could function as a negative regulator of the DHFR promoter, which may represent a novel mechanism for the known repressive effects of PML on cellular growth.

Keywords: Promyelocytic leukemia; Transcription repression; Dihydrofolate reductase; Cell cycle

Introduction

The PML gene was originally identified by cloning the translocation breakpoint of t(15q;17q), a consistent feature of acute promyelocytic leukemia (APL) [1-2]. PML encodes a nuclear protein with several domains resembling transcription factors. These domains include a RING-finger motif and two other cys/his domains designated the B boxes [3]. PML also contains an α-helical domain at its C-terminus, which is involved in PML/PML and PML/PMLRARα dimerization [3-4].

The PML protein is localized in the nucleus in form of nuclear bodies designated PML oncogenic domains (PODs) or PML nuclear bodies (PML NBs)[5]. Many

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proteins with transcription regulatory function have been identified to localize in the PODs. These include SP100, the ubiquitin-like protein modifier SUMU-1/PIC-1/sentrin, the interferon induced protein ISG20, immediate early viral proteins IE1 and IE4 and the transcription co-regulator CBP (CREB binding protein) [5-9,25]. Moreover, direct interaction of PML with several transcription factors including Sp1, pRB, PLZF and p53 have been reported [12,29,22,34,35]. These findings supported the notion that PML might be involved in transcription regulation.

In fact, direct role of PML in transcription regulation both as an inhibitor or activator, depending on the promoter tested, has been reported. It has been shown that PML could function as an activator of transcription of steroid hormone receptors and transcription mediated by Fos/AP-1 [9,10,13]. When fused to the GAL4 DNA-binding domain, PML acted as an inhibitor of transcription from the GAL4-responsive promoter [10]. Specific domains of the PML protein were involved in the transcription repressing events [10-12]. PML suppresses the promoter of EGFR by inhibition of EGFR’s Sp1-dependent activity [12].

PML also functions as a growth and transformation suppressor [14-16]. The PML gene knock-out study reported by Wang et al. [26] strongly supported a crucial role for PML in the control of cell growth. Recent studies demonstrated that PML is essential for multiple pathways of programmed cell death by using PML-/- mice and in the cells overexpressing PML [27].

In this study the effects of PML on dihydrofolate reductase (DHFR) promoter was investigated. The results showed that the promoter activity is significantly repressed in the presence of the PML protein through the Sp1 site, but not E2F site presented in the promoter region. Since DHFR promoter functions as a key regulator in DNA synthesis and cellular growth, its repression by PML represents a novel mechanism for the well documented growth inhibitory effects of PML [14-17]. Also, these data provide a link between growth suppression and transcription regulatory function of the PML protein.

Materials and Methods

Plasmids and Cell Lines

The pSG5/PML plasmid was constructed by subcloning of cDNA coding for the long isoform of the PML protein in the EcoRI site of the pSG5 vector [11]. The DHFR-Leu contained the DHFR promoter linked to the firefly Luciferase gene [12]. The plasmids GAL4-Sp1-Leu and E2F-GAL4-Luc contained the GAL-4-binding sites in place of the E2F- and Sp1-binding sites of the DHFR, respectively [12].

HeLa and U2OS cells were maintained in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin (GIBCO/BRL, Gaithersburg, MD) in 5% CO2 at 37°C in a humidified incubator.

Transfection and Luciferase Assays

Cells were cultured to semi-confluence and were transfected with the plasmids using the Superfect reagent (Qiagen, Germany). For a 5-cm tissue culture dish, 5 µg of total DNA was used. This contained 0.5 µg of the reporter and 1-3 µg of the expression plasmids (see the legends to the figures), and 0.5 µg of pCMV-β-GAL plasmid as an internal control. The latter expresses the bacterial β-galactosidase gene constitutively. The luciferase assay was performed with the Promega (Madison, Wis.) luciferase assay system according to the supplier’s instructions. The luciferase activity was measured with a Luminometer (Turner Design, Sunnyvale, Calif.) and normalized against β-galactosidase activity. The activity of each sample was measured and calculated relative to the activity exhibited by cells transfected with empty expression vector (pSG5), and is shown as the fold increase. The β-galactosidase activity was measured as described before [11].

Electrophoretic Mobility Shift Assay (EMSA)

DNA-protein interactions were analyzed using EMSA essentially as described previously [12]. Briefly, 5 µg nuclear proteins from HeLa cells first preincubated in a binding buffer (4% Ficoll 400, 20 mM HEPES [pH 7.9], 2 mM MgCl2, 1 µg of salmon sperm DNA with final concentration of KCl in the reaction mixture adjusted to 100 mM) for 10 min at room temperature in a total volume of 19 µl. In each reaction mixture, 10 fmol (1 µl) of the 32P-labeled (Amersham Bioscience, USA) probe was then added, and the reaction mixture was incubated at room temperature for 30 min. Double stranded Sp1 or E2F binding sites oligonucleotide probes were made by annealing the complementary strands of the following oligonucleotides:

Sp1: 5’-CATTGCATCGGGGGGCAGAG-3’;
E2F: 5’-TCCGTAATTTTCGCCTTAAATTTGAGAAGAGGCGGAAAATCTGGTCC-3’;

In competition assays, 100-fold molar excess of Sp1...
or E2F double stranded oligonucleotides were preincubated with the extracts for 10 min before addition of the labeled probes. Similarly in supershift assays, 1 µl monoclonal anti Sp1 antibody (Santa Cruz Biotechnology, Inc., USA) was pre-incubated with the extracts. The PML protein was a gift from Dr. ZM Mu (MD Anderson Cancer Center, Texas). The protein-DNA complexes were resolved on a 4% native polyacrylamide gel in 0.25 TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA), and visualized by autoradiography.

\[^{3}H\]thymidine Incorporation Assay

The DNA synthesis rate of HeLa cells which reflects cell proliferation and growth was determined using the \[^{3}H\]thymidine incorporation assay. Cells were plated at 1 × 10^4 cells/plate in 24-well plates and cultured in complete medium. Cells were then transfected with 5 µg of pSG5/PML vector. \[^{3}H\]thymidine (2 mCi) (Amersham Bioscience, USA) was added to each well at 24, 48, and 72 h intervals, and the cells were incubated for additional 2 h at 37°C. Cells were then washed in PBS and harvested as described previously [16]. Radioactivity of the incorporated \[^{3}H\]thymidine was determined in a scintillation counter.

Results

DHFR Promoter Activity Is Inhibited by PML

The ability of PML as a growth suppressor has been documented in several studies in vivo and in vitro [14-17]. However, the mechanism by which PML exerts its growth suppressive effects is not understood very well. In this study, the effects of PML on dihydrofolate reductase (DHFR) promoter were investigated. The cDNA encoding the PML protein in pSG5 vector (pSG5/PML) was cotransfected with a reporter promoter containing the DHFR promoter in front of the firefly Luciferase (the reporter) gene, into mammalian HeLa and U2OS cells. Analysis of the effects of PML on DHFR promoter activity in HeLa cells showed a sharp repression of the promoter activity in the presence of PML (Fig. 1A). As shown in Figure 1B, similar results were found in U2OS cells. However, the background level of the promoter activity in U2OS cells was lower compared to the activity in HeLa cells.

Sp1 DNA Binding Is Disrupted by PML

Given the inhibitory effects of the PML protein on the DHFR promoter through the Sp1 site of the promoter, we were prompted to analyze these effects by electrophoretic mobility shift assay (EMSA) using Sp1 and E2F oligonucleotide probes corresponding to the related region of the DHFR promoter. Nuclear extracts from HeLa cells were prepared and mixed with labeled Sp1 and E2F oligonucleotides. Analysis of the DNA-protein interaction through EMSA showed a clear change in the mobility pattern in the assays containing the Sp1 probe in the presence of PML (Fig. 1A). As shown in Figure 1B, similar results were found in U2OS cells. However, the background level of the promoter activity in U2OS cells was lower compared to the activity in HeLa cells.
Figure 1. Inhibition of DHFR promoter activity by PML. A) HeLa cells; B) U2OS cells. Cells were cultured and transfected with control plasmid (pSG5) and plasmid containing the PML gene (pSG5/PML) together with the Luciferase reporter plasmid (DHFR-Leu). After transfection the Luciferase activity was measured on the total cell extract. The activity of each sample was measured and calculated relative to the activity exhibited by cells transfected with empty expression vector (pSG5), and is shown as the fold increase.

Figure 2. Abrogation of Sp1 mediated activity of DHFR promoter by PML. HeLa cells were transfected with the DHFR reporter plasmid containing the Sp1 site (Sp1-GAL4-Leuc) or the reporter plasmid containing only the E2F site (GAL4-E2F-Leuc) together with the pSG5 and pSG5/PML plasmids. After transfection the Luciferase activity was measured on the total cell extract (see the legend to Fig. 1).
binding. In parallel assays, addition of the PML protein showed no effects on the mobility pattern of the E2F binding (Fig. 3, right panel, lanes 3-5). These data clearly showed that the inhibitory effects of PML on the DHFR promoter were exerted through the Sp1 site of the promoter. Since the Sp1 site functions as a major regulator of the DHFR promoter, its inhibition by PML suggests a significant role for the PML protein in regulation of the promoter. Moreover, given the key role of the DHFR promoter in DNA synthesis and cellular growth, its inhibition by PML protein could provide a mechanism for inhibitory effects of PML on the cellular growth.

**DNA Synthesis Is Significantly Reduced by PML**

The DHFR promoter functions as a key regulator in DNA synthesis [30,32,33]. Given the significant repression of the DHFR promoter activity by the PML protein, and the fact that PML functions as a negative regulator of the cell growth, it was interesting to investigate the effects of PML on the rate of the DNA synthesis. Therefore, the rate of DNA synthesis in HeLa cells was examined in the presence of PML. Cells were transfected with pSG5/PML or pSG5 plasmids, incubated with [3H]thymidine and analyzed at 24 h intervals. The incorporation of [3H] thymidine into DNA was measured as an index for the rate of the DNA synthesis. As shown in Figure 4A, the cells expressing PML showed much lower incorporation of [3H] thymidine, compared to that of the control cells (pSG5 transfected), suggesting a reduced DNA synthesis rate in the presence of PML.

**Discussion**

The promoter of the dihydrofolate reductase (DHFR) gene, contains Sp1 and E2F response elements in the vicinity of its transcription initiation region [31,33], and both of these elements have been shown to be involved in the promoter activity. Our previous studies showed that PML physically and functionally interacted with Sp1, disrupting the binding of the Sp1 protein to the promoter [12]. *In vivo* and *in vitro* experiments demonstrated that the association of the Sp1 and PML exerts a significant effect on the EGFR promoter activity, which is mainly regulated by Sp1 [12,18,19,23,24]. The data demonstrated that PML could function as a negative regulator of Sp1 transcriptional activity. In the present study, the association of PML and Sp1 was further investigated using the DHFR promoter. The data showed that PML could significantly affect the promoter activity of the DHFR promoter. Interestingly,
Sp1 binding to the promoter. The fact that PML inhibited the DHFR promoter by disrupting the
in regulating cell cycle progression [12,14,33,39].

E2F and synergistically activated transcription of G1/S
DHFR together with the reports that Sp1 interacts with
and inhibited its binding to the target promoters such as
progression. The finding that PML interacted with Sp1
normally plays a role in regulating cell cycle
overexpression of PML in HeLa cell led to the alteration
of DHFR promoter by PML might also involve HDAC.
In fact, the Sp1 mediated transcriptional activation of
DHFR promoter was found to be efficiently repressed
by HDAC1 [32,33].

One of the most characterized functions of PML is its
inhibitory role in cellular growth [14-17,20,21]. However, the mechanisms underlying this function are
poorly understood. The finding that PML is associated
with several transcription factors involved in cellular
growth including the retinoblastoma protein (pRb) and
p53 [22,25,29,34,35], as well as negative and positive
effects of PML on transcription regulation depending on
the promoter tested, clearly suggested that the growth
inhibitory and transcriptional regulatory activity of PML
might be related to each other [9,19,12,17,36,37]. The
findings from this study on the inhibition of DHFR
promoter activity by PML and its significant role in
suppression of DNA synthesis, provides a strong
support for this hypothesis.

Furthermore, it has been previously reported that
PML could functionally and physically interact with all
three isoforms of histone deacetylase enzymes (HDAC)
through specific domains [11,38]. Both the C-terminal
and the N-terminal of the PML protein were found to be
necessary for its efficient binding to HDAC. These data
demonstrated that PML transcriptional inhibitory
activity was associated with histone deacetylases that
were recruited to the promoter. Therefore, the inhibition
of DHFR promoter by PML might also involve HDAC.
In fact, the Sp1 mediated transcriptional activation of
DHFR promoter was found to be efficiently repressed
by HDAC1 [32,33].

Previous studies showed that overexpression of PML
induced a G1 cell cycle arrest both in cancer cell line
and in normal human lung fibroblasts and stable
overexpression of PML in HeLa cell led to the alteration
of cell cycle progression by lengthening G1 [14-17,28].
Based on these findings it was hypothesized that PML
normally plays a role in regulating cell cycle
progression. The finding that PML interacted with Sp1
and inhibited its binding to the target promoters such as
DHFR together with the reports that Sp1 interacts with
E2F and synergistically activated transcription of G1/S
checkpoint genes, suggested that PML could play a role
in regulating cell cycle progression [12,14,33,39].

Our study presented in this report clearly showed that
PML inhibited the DHFR promoter by disrupting the
Sp1 binding to the promoter. The fact that PML
interacted with HDACs to repress transcription strongly
support a significant role for PML in cell cycle
regulation. Therefore, it could be speculated that PML
normally regulates cell cycle progression by modulating
the functional activity of the Sp1/HDAC complex. In
this content, possibly disruption of PML function by
the t(15;17) in APL may contribute to the development of
acute leukemia.

Acknowledgements
We are grateful to Dr ZM Mu for his kind gift of the
PML protein. This work was part of a collaborative
study between the University of Isfahan and the
University of Texas MD Anderson Cancer Center.

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