Expression and Role of the ErbB3-Binding Protein 1 in Acute Myelogenous Leukemic Cells

Le Xuan Truong Nguyen, Li Zhu, Yunqin Lee, Lynn Ta, and Beverly S. Mitchell

Abstract

Purpose: The ErbB3-binding protein 1 (Ebp1) has been implicated in diverse cancers as having either oncogenic or tumor suppressor activities. The present study was undertaken to determine the effects of Ebp1 expression in AML cells and to determine the mechanisms by which Ebp1 promotes cell proliferation in these cells.

Experimental Design: The expression of Ebp1 was studied in mononuclear cells obtained from the peripheral blood of 54 patients with AML by Western blot analysis. The effects of Ebp1 expression on proliferating cell nuclear antigen (PCNA) expression and cell proliferation was measured using Western blot analysis, immunoprecipitation, in vitro ubiquitination, and colony-forming assays. The role of Ebp1 in promoting rRNA synthesis and cell proliferation was evaluated by measuring the level of pre-rRNA and the recruitment of Pol I to rDNA.

Results: Ebp1 is highly expressed in acute myelogenous leukemia (AML) cells and regulates the level of ribosomal RNA (rRNA) synthesis by binding to RNA Polymerase I (Pol I) and enhancing the formation of the Pol I initiation complex. Ebp1 also increases the stability of PCNA protein by preventing its interaction with Mdm2, for which it is a substrate.

Conclusions: These results demonstrate an important role of Ebp1 in promoting cell proliferation in AML cells through the regulation of both rRNA synthesis and PCNA expression. Clin Cancer Res; 22(13); 3320–7. ©2016 AACR.

Introduction

ErbB3-binding protein 1 (Ebp1), the human homologue of the mouse protein p38-2G4, is ubiquitously expressed in a variety of normal and malignant human tissues and cell lines (1,2) and has been shown to play important roles in the regulation of cell proliferation and differentiation (2,3). Ebp1 encodes two alternatively spliced isoforms, p48 and p42, that have different functions (4). The p48 isoform is located predominantly in the nucleolus and cytoplasm and promotes cell proliferation and cell survival (4–6), whereas the p42 isoform is predominantly cytoplasmic and inhibits cell growth (4,7). Ebp1 also interacts with a variety of other important regulatory proteins that include Akt and Nucleophosmin 1 (NPM1; 8,9).

Ribosomal RNA synthesis is a prerequisite for cellular proliferation and is tightly regulated in response to diverse growth and inhibitory stimuli (reviewed in 10, 11). It requires the recruitment of RNA polymerase I (Pol I) and several transcription initiation factors including Transcription Initiation Factor I (TIF-IA, upstream binding factor (UBF), and the TATA-binding protein (TBP)-containing factor TIF-IB/SL1 to the ribosomal DNA (rDNA) promoter to generate a productive transcription initiation complex. Although the p48 isoform of Ebp1 has been localized to the nucleolus and shown to bind to RNA, it had not previously been known to participate in the increasingly complex process of regulating rRNA synthesis. We have recently shown that Ebp1 p48 binds to TIF-IA to regulate its intracellular localization and activity (12). Our present studies demonstrate that Ebp1 is also involved in the formation of the rRNA transcription initiation complex, contributing to its ability to regulate rRNA synthesis.

Proliferating cell nuclear antigen (PCNA) is a ring-shaped heterotrimeric clamp that provides a platform for a variety of proteins that are involved in DNA replication and repair and is frequently increased in expression in malignant and cycling cells. PCNA binds to the E3 ligase HDM2 that regulates its turnover during the normal cell cycle (13, 14). Higher levels of PCNA expression have been found to correlate with more proliferative disease (15) and with a poorer response to treatment in acute myeloid leukemias (16) and in myelodysplastic syndromes (17).

In investigating the role of Ebp1 in acute myelogenous leukemia (AML), we found that it is expressed at high levels in the great majority of patient samples and that its expression correlates with and is directly responsible for increased expression of PCNA. In addition, Ebp1 expression is an important determinant of rRNA synthesis in these cells, demonstrating that it contributes to cell proliferation by two independent, but potentially complementary pathways.

Materials and Methods

Human patient samples

Mononuclear cells were isolated from the bone marrow or the peripheral blood of patients obtained under IRB-approved protocols using density-gradient centrifugation with Ficoll–Paque Plus (GE Healthcare). Cells at the interface were removed and washed with PBS. Cell pellets were viably frozen.
at −80 °C until use. All cultures were carried out in defined medium as previously referenced (18). The patient characteristics of 13 samples (SU37 to SU49) are shown on Supplementary Table S3.

Translational Relevance

Previous studies have indicated that Ebp1 plays a variety of roles in human cancer cells that are dependent in part on the type of cell in which it is expressed. Its role in acute leukemia has not been investigated. The results of the present study confirm that Ebp1 is expressed at high levels in the majority of AML clinical samples and further demonstrate that p48 Ebp1 regulates two parameters pivotal to cell proliferation, rRNA synthesis through direct interaction with the Pol I transcription initiation complex and PCNA expression through enhancing protein stability. These previously unrecognized effects of Ebp1 raise the possibility that it could constitute a new therapeutic target in the treatment of a subset of AML.

Figure 1.

Relative expression of Ebp1 and PCNA in primary AML cells as compared with normal marrow mononuclear cells. Western blot analyses of cell lysate (30 μg) from 12 normal bone marrows and 54 AML samples were probed with anti-Ebp1, anti-PCNA, and anti-tubulin antibodies as indicated by the arrowhead. The bottom panels were performed with same loading of cell lysate (30 μg) on different blots.

MLL-AF9 and MLL-AF10 mouse leukemia cells

C57BL/6 mice were obtained from Jackson Laboratory. All studies were performed in accordance with protocols approved by the Stanford University (Stanford, CA) Institutional Animal Care and Use Committee. Mouse bone marrow cells were harvested from leg bones and c-kit+ cells were purified by autoMACS using anti-CD117 microbeads (Miltenyi Biotec). Purified cells were transduced with recombinant retroviruses made from pMSCV-neo constructs encoding MLL-AF9 and MLL-AF10, and plated in methylcellulose media (M3231, Stemcell Technologies) containing SCF, IL3, IL6, and GM-CSF. Transformed cells (1 × 10⁶) from methylcellulose cultures were transplanted intravenously into lethally irradiated C57BL/6 mice (900 rads) with 2 × 10⁵ syngeneic bone marrow cells (19). Mice were subsequently sacrificed and bone marrow cells from control animals and from MLL-AF9 and MLL-AF10 mice were collected for analysis by Western blot analysis.

Synthetic siRNA oligonucleotides

The siGENOME SMARTpool for si-RNA was purchased from Thermo Scientific. Scrambled control RNA (SCR) was...
used as a control. The target sequences for Ebp1 p48 siRNA are shown in the Supplementary Table S1.

**ChIP assay**

Chromatin immunoprecipitation (ChIP) was performed as described by the manufacturer (Pierce). Precleared chromatin was incubated overnight by rotation with 4 μg of Pol I antibody or IgG antibody as a negative control. Immunoprecipitates were resuspended in 50 μL TE buffer. Inputs and immunoprecipitated DNA samples were quantified by qPCR on a 7900T Fast real-time PCR system (Applied Biosystems). Primers are listed in Supplementary Table S2.

**RNA labeling and analysis**

The cells were washed and incubated in phosphate-free DMEM (Gibco) supplemented with 10% FBS for 2 hours followed by one hour label with 0.5 mCi [32P] orthophosphate (PerkinElmer). Total RNA was extracted with TRIzol (Life technology) following the manufacturer’s protocol. Equal amounts of RNA (10 μg) were separated on a 1.2% MOPS formaldehyde gel. The gel was dried and visualized by autoradiography.

**Figure 2.**

Regulation of Mdm2-induced PCNA ubiquitination and degradation by Ebp1. A, effect of Ebp1 depletion on ubiquitination and expression of PCNA protein in K562 leukemic cells. Cells were cotransfected with HA-Ub and SCR or siEbp1 (20 nmol/L) for 36 hours. Top, after treatment with MG132 (10 μmol/L) for 8 hours, cell lysate was immunoprecipitated with anti-PCNA and immunoblotted with anti-HA antibodies; (bottom) cell lysate was immunoblotted with the antibodies shown. B, effects of Ebp1 overexpression on PCNA ubiquitination and protein expression in MEF cells (Mdm2+/+; p53−/−, HA Ub) and (Mdm2−/−, p53−/−, and Mdm2 C464, p53−/−). Cells were transfected with vector control or GFP-Ebp1 for 24 hours. The cells were treated continuously with MG132 for 8 hours (top) or untreated (bottom) and the cell lysate was immunoprecipitated and immunoblotted as shown. C and D, effect of the level of Ebp1 expression on the interaction of PCNA with Mdm2 in leukemic cells. C, K562 cells were transfected with SCR or siEbp1 (20 nmol/L) for 36 hours and the lysate was immunoprecipitated and immunoblotted as indicated. D, ten AML patient samples were divided into two groups with low (n = 5) and high (n = 5) expression of Ebp1 based on the immunoblot analysis shown in Fig. 1. Left, the combined cell lysates were immunoprecipitated and immunoblotted as indicated; (right) the cells were treated with MG132 (10 μmol/L) for 8 hours and the combined lysates were immunoprecipitated with anti-PCNA antibody. E, effect of Ebp1 overexpression on the interaction of Mdm2 with PCNA and on PCNA ubiquitination in MEF Ebp1−/− cells. The MEF Ebp1−/− cells were transfected with vector control or GFP-Ebp1 (left) or cotransfected with HA-Ub and vector control or GFP-Ebp1 (right) for 24 hours. The lysate was immunoprecipitated and immunoblotted with the antibodies shown.
Statistical analysis
Where indicated, results were compared using the unpaired Student t test with values obtained from at least three independent experiments. P < 0.05 was considered significant.

Results
Ebp1 expression correlates with the PCNA expression in AML cells
We compared the level of expression of Ebp1 protein in frozen normal mononuclear cells obtained from normal bone marrow by Ficoll separation with that in mononuclear cells obtained from the bone marrow or peripheral blood of 54 patients with AML. The expression of Ebp1 protein was significantly higher in the majority of AML samples (Fig. 1, second lanes; Supplementary Fig. S1A) and was associated with an increase in the expression of PCNA (Fig. 1, second and third lanes). Of note, however, a few SU samples expressed very little Ebp1 for unknown reasons and these samples had a correspondingly low level of PCNA expression (Fig. 1, second lanes; Supplementary Fig. S1A). There was also a relative increase in Ebp1 and PCNA expression in leukemic cells obtained from two murine AML models in which either of two MLL fusion proteins (MLL-AF9 or MLL-AF10) is expressed (Supplementary Fig. S1B; ref. 26). These results further suggest a relationship between the expression of these two proteins.

Regulation of PCNA ubiquitination and degradation by Ebp1
Previous studies have shown that p48 Ebp1 binds to and stabilizes the HDM2 E3 ligase through Akt signaling (20). PCNA is known to be regulated by HDM2 and previous studies have indicated that the interaction of a signaling protein, ERK8, with HDM2 prevents PCNA ubiquitination and degradation, thereby stabilizing PCNA levels (14). We therefore asked whether the interaction of p48 with HDM2 provided an explanation for the association of Ebp1 expression with that of PCNA. Depletion of Ebp1 in K562 leukemia cells using siRNA increased the ubiquitination of PCNA and decreased PCNA expression (Fig. 2A), whereas overexpression of Ebp1 in murine embryonic fibroblasts deficient in p53 (Mdm2+/−, p53−/−) inhibited PCNA ubiquitination, as well as its interaction with Mdm2 (Fig. 2B, left). In contrast, there was no change in the ubiquitination or expression of PCNA in the presence or absence of exogenously expressed Ebp1 in MEFs that were deficient in Mdm2 (Mdm2−/−, p53−/−) or expressed a catalytically inactive form of Mdm2 (Mdm2 C464, p53−/−; Fig. 2B, middle and right). K562 leukemic cells that were

Figure 3.
Regulation of rRNA synthesis and cell proliferation by Ebp1. A, quantitative expression of (1) Ebp1 mRNA, (2) pre-rRNA synthesis, (3) Pol I occupancy of the rDNA promoter, (4) OD absorbance by MTS assay, and (5) immunoblot analysis of Ebp1 and PCNA protein in bone marrow mononuclear cells from normal individuals and from patients with AML. The bar indicates the average value and the ends of the whiskers represent minimum and maximum values. Significance was determined using the Student t test. B, effects of Ebp1 depletion on rRNA synthesis and cell proliferation in K562 leukemic cells. Panel 1, cells were transfected with SCR or siEbp1 (20 nmol/L) for 36 hours. RNA was extracted for measurement of 5ETS pre-rRNA synthesis and RNA labeling with [32P]. Panel 2, recruitment of Pol I to the rDNA promoter; Panel 3, MTS and colony forming assays; Panel 4, Western blot analysis of K562 cells depleted of Ebp1.
Regulation of rRNA transcription and cell proliferation by Ebp1 in AML cells

The high level of Ebp1 expression in primary AML cells was reflected in highly significant increases in the expression of Ebp1 mRNA, as well as in increased levels of rRNA synthesis and cell proliferation as determined by MTS assays (Fig. 3A, 1-2, 4; Supplementary Fig. S2A). Depletion of endogenous Ebp1 expression in K562 leukemic cells strongly inhibited both PCNA expression and cell proliferation (Fig. 3B, 3 and 4; Supplementary Fig. S2B), suggesting that Ebp1 expression is integral to both these processes. In addition, those leukemic cells that expressed higher levels of Ebp1 demonstrated significantly higher levels of rRNA synthesis as determined by the level of 5'ETS pre-rRNA and Pol I recruitment to rDNA when compared with normal mononuclear cells (Fig. 3A, 1-3), whereas reducing Ebp1 expression in K562 leukemic cells significantly reduced rRNA synthesis (Fig. 3B, left) and cell proliferation, as determined by colony-forming assays (Fig. 3B, third). Similar results demonstrating increased levels of Ebp1 mRNA correlating with increased rRNA synthesis were obtained from murine MLL-AF9 and MLL-AF10 leukemic cells (Supplementary Fig. S3). Depletion of Ebp1 in K562 leukemic cells also slightly increased cell death, as measured by Annexin V staining (Supplementary Fig. S2C). Further analysis of the cell-cycle response to Ebp1 depletion revealed a decrease in the number of cells entering S phase and a corresponding increase in cells in G1 (Supplementary Fig. S2D).

To obtain additional data in primary AML cells, 24 AML samples were divided into low (n = 8) and high Ebp1 (n = 16) expression groups based on the Western blot analyses shown in Fig. 1. Those samples with higher expression of Ebp1 also displayed higher levels of rRNA synthesis, PCNA expression, and cell proliferation (Fig. 4). Similarly, MEF Ebp1+/+ cells had higher levels of rRNA synthesis and higher rates of proliferation than did MEF Ebp1−/− cells, whereas overexpression of Ebp1 in MEF Ebp1−/− cells significantly enhanced both rRNA synthesis and cell proliferation (Supplementary Fig. S4). Taken together, these data strongly suggest that the expression of Ebp1 plays an important role in regulating both rRNA synthesis and cell proliferation in AML cells.

### Interaction of Ebp1 with the Pol I complex and enhancement of the formation of the Pol I transcription initiation complex

Previous studies have indicated that the p48 but not the p42 isoform of Ebp1 can be found in the nucleolus as well as in the cytoplasm (4). Immunofluorescent and FISH staining of 293T cells demonstrated colocalization of p48 Ebp1 with Pol I, UBF, and rDNA, as shown in densitometry scans (Fig. 5A and Supplementary Fig. S5). To determine more specifically whether Ebp1 associates with the Pol I transcription initiation complex, Ebp1 was immunoprecipitated from AML cell lysate and the samples probed for Pol I, UBF, and PAF53. Each of these proteins coprecipitated with Ebp1 (Fig. 5B), strongly suggesting that Ebp1 is involved in the regulation of rRNA transcription. Decreasing the expression of Ebp1 in K562 leukemic cells (Fig. 5C, left) or in the primary AML cells with lesser expression of Ebp1 (Fig. 5C, right) decreased the binding of both UBF and TIF-IA to Pol I. MEF Ebp1−/− cells also demonstrated less binding of TIF-IA and UBF to Pol I than did wild-type MEF cells, while reexpression of Ebp1 increased the interaction of both proteins with Pol I (Fig. 5D). These results are consistent with the extent both of Pol I recruitment to rDNA and of rRNA synthesis in these cells (Supplementary Fig. S4). These data demonstrate that p48 Ebp1 is a component of the Pol I initiation complex and suggest that Ebp1 plays an integral and direct role in the regulation of rRNA synthesis. A schema that demonstrates the effects of Ebp1 on both PCNA stability and rRNA synthesis is shown in Fig. 6.

### Discussion

Our data show that Ebp1 is expressed at higher levels in AML cells than in normal bone marrow mononuclear cells and that
Figure 5.
Interaction of Ebp1 with the Pol I transcription initiation complex. A, colocalization of p48 Ebp1 with Pol I, UBF, and rDNA. 293T cells were transfected with GFP-p48 Ebp1 for 24 hours. Left, cells were immunostained with anti-Pol I (top) and anti-UBF (middle) antibodies. rDNA was labeled with an rDNA probe (bottom). Fluorescence intensity was measured along the line shown. Scale bar, 5 μm. B, interaction of Ebp1 with Pol I, UBF, and PAF53 in AML cells. Lysate from combined AML cells (n = 10) was immunoprecipitated with anti-IgG or anti-Ebp1 antibody and immunoblotted with anti-Pol I (top), anti-UBF (middle), or anti-PAF53 (bottom) antibodies, as shown. C, effects of Ebp1 expression on UBF and TIF-IA binding to Pol I in AML cells. Left, K562 cells were transfected with SCR or siEbp1 (20 nmol/L) for 36 hours; Right, 24 AML patient samples were divided into groups with low (n = 8) and high (n = 16) expression of Ebp1. Combined cell lysate was immunoprecipitated with anti-UBF (first lanes) or anti-TIF-IA (second lanes) antibody and immunoblotted with anti-Pol I antibody. D, effects of Ebp1 overexpression on UBF and TIF-IA binding with Pol I in MEF Ebp1+/− cells. Lysates from MEF Ebp1+/+, GFP-control and GFP-Ebp1-transfected MEF Ebp1−/− cells were immunoprecipitated with anti-UBF (left) or anti-TIF-IA (right) antibody and immunoblotted with anti-Pol I antibody.
cell proliferation depends on Ebp1 expression. In addition, and not previously recognized, Ebp1 appears to be a component of the Pol I transcription initiation complex and to facilitate rRNA synthesis.

The regulation of rRNA gene transcription is central to ribosome production and therefore to cell growth and proliferation and the growth-dependent regulation of rRNA synthesis is evolutionarily conserved (11). The synthesis of pre-ribosomal RNA that reflects the overall level of rRNA transcription is increased in various human cancers and indeed has been considered as one of the hallmarks of cancer (reviewed in ref. 21). The transcription of pre-ribosomal rRNA by Pol I requires the formation of a preinitiation complex containing the promoter selectivity factor (SL1) complex, UBF, and TIF-IA. There are many oncogenic events including protein overexpression, mutations, and translocation caused by the alterations of cellular signaling pathways such as PI3K/Akt/mTOR, Myc, and Rb that directly act on the proteins essential for rRNA transcription. For example, activated Akt enhances rRNA synthesis and consequently promotes cell proliferation in AML cells through the stabilization and activation of TIF-IA (22–25).

p48 Ebp1 is the predominant form of Ebp1 expressed in many cell types (4–6,8). Western blot analyses of AML cells using an antibody that also detects the p42 isoform show only a single band of 48 kDa. The use of an Ebp1 siRNA construct that targets the N-terminal sequence that is specific for the p48 isoform largely depletes Ebp1 protein, further suggesting that p48 accounts for the great majority of Ebp1 expression in AML cells. A number of studies have indicated that p48 may play an important role in oncogenesis. For example, p48 Ebp1 interacts with nuclear PKB/Akt to prevent apoptotic cell death in neuronal cells (8). High expression of Ebp1 in breast cancers predicts a poor clinical outcome, whereas tamoxifen treatment of MCF-7 breast cancer cells markedly decreases both the transcription and protein expression of p48 Ebp1 (26). It has also been shown recently that the phosphorylation of p48 Ebp1 by CDK2 is required for its tumorigenic function (6). Although further studies are needed to prove that p48 Ebp1 is an oncogenic protein, the findings to date strongly suggest it has tumor-promoting activities. The correlation of Ebp1 with PCNA expression and proliferation support a potential role for Ebp1 in the progression of AML. However, we have not found an association between the level of Ebp1 gene expression and overall survival in published databases from AML patients.

Previous studies have shown that p48 Ebp1 binds to and stabilizes Hdm2 (20) and enhances Hdm2-induced p53 degradation (5). In contrast, our results indicate that Ebp1 expression reduces the ubiquitination and degradation of PCNA by Mdm2 (Fig. 2). This study also suggests that the increase in PCNA protein induced by Ebp1 is independent of the mechanism by which Ebp1 downregulates p53 (5). By using MEF cells deficient in p53 or in both Mdm2 and p53, we have demonstrated that Ebp1 enhances the interaction of Mdm2 with PCNA in the absence of p53, indicating that p53 is not relevant to this interaction. Previous studies have similarly demonstrated that the chromatin-bound kinase ERK8 acts independently from p53 in preventing Hdm2-induced PCNA degradation (14). Although it remains possible that additional factors are involved in these interactions, the data obtained to date indicate that Ebp1 differentially regulates the expression of...
p53 and PCNA through Mdm2. Although technically difficult, the direct relationship between Ebp1 expression, PCNA expression, and cell proliferation could be further confirmed by depleting Ebp1 in cells overexpressing PCNA. Nonetheless, the present data strongly support a role of p48 Ebp1 as a promoter of cell proliferation and, potentially, as an enhancer of susceptibility to cellular transformation.

In summary, Ebp1 plays an important and previously unrecognized role in regulating rRNA synthesis and cell proliferation in AML cells. These results provide preliminary evidence that Ebp1 might constitute an additional therapeutic target for the treatment of AML.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: L.X. Truong Nguyen, B.S. Mitchell

Development of methodology: L.X. Truong Nguyen, L. Ta

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Zhu, Y. Lee

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.X. Truong Nguyen.

Writing, review, and/or revision of the manuscript: L.X. Truong Nguyen, L. Zhu, B.S. Mitchell

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Zhu

Study supervision: B.S. Mitchell

Grant Support

This work was supported by a translational research grant and by a SCOR award from the Leukemia and Lymphoma Society.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 18, 2015; revised December 7, 2015; accepted December 21, 2015; published OnlineFirst January 26, 2016.

References


Clinical Cancer Research

Expression and Role of the ErbB3-Binding Protein 1 in Acute Myelogenous Leukemic Cells

Le Xuan Truong Nguyen, Li Zhu, Yunqin Lee, et al.


Updated version
Access the most recent version of this article at:

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2016/01/26/1078-0432.CCR-15-2282.DC1

Cited articles
This article cites 26 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/22/13/3320.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.