Overexpression of the MEN/ELL Protein, an RNA Polymerase II Elongation Factor, Results in Transformation of Rat1 Cells with Dependence on the Lysine-rich Region*

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The MEN gene (also called ELL) encodes an RNA polymerase II elongation factor that has been implicated in t(11;19)(q23;p13.1) translocation in myeloid leukemias. The function of another elongation factor, elongin, is known to be inhibited by VHL tumor suppressor protein in vitro, suggesting the possible relationship of aberrant transcriptional elongation to oncogenesis. We overexpressed the MEN protein in Rat1 fibroblasts to evaluate its transforming activity. MEN-overexpressing cells acquired the capacity for anchorage-independent growth. In addition, the growth factor requirement was decreased in these cells. However, cells expressing a deletion mutant of MEN lacking the lysine-rich region did not exhibit such biological abilities. c-Fos protein expression and AP-1 activity were elevated in the MEN-expressing cells, which might be part of the mechanism responsible for the transformation. The c-fos mRNA, the expression of which is known to be regulated partly at the stage of transcriptional elongation, appeared earlier in the MEN-expressing cells than in cells transfected with an empty vector or the deletion mutant lacking the lysine-rich region after stimulation with epidermal growth factor. The RNA polymerase II elongation factor MEN may play an important role in the regulation of cell proliferation.

The MEN gene (also called ELL) was cloned from myeloid leukemia cells carrying the t(11;19)(q23;p13.1) chromosomal translocation (1, 2). Recently, Shilatifard et al. (3) have reported that MEN is an RNA polymerase II elongation factor, which agrees with the fact that MEN localizes in the nucleus (4, 5). RNA polymerase II elongation factors act on transcriptional elongation by the conversion of termination-prone transcription complexes into productive elongation complexes, the prevention of premature termination, or the suppression of transient pausing (6–8). To date, P-TEFb, SII, transcription factor IIF, elongin, and MEN are known to be included in the family (3, 6–9). The last three proteins exert their elongation activities by the third mechanism. Actually, MEN shares homology with rat elongin in a 16-amino acid domain in the lysine-rich region (4). However, the role of the lysine-rich region is still not clear.

The implication of an elongation factor in human diseases was first suggested in the von Hippel-Lindau disease, which predisposes individuals to a variety of cancers (10, 11). The product of the normal VHL tumor suppressor gene binds to B and C subunits of the elongin complex and inhibits the transcriptional elongation activity of the factor in vitro (10, 11). In contrast, the product of the mutated VHL gene does not bind to elongin (10, 11). These facts suggest the possible correlation between the activation of transcriptional elongation and oncogenesis. The MEN protein is the second elongation factor that is considered to take part in human diseases. The t(11;19)(q23; p13.1) translocation found in myeloid leukemia results in the formation of the MLL/MEN fusion protein (1, 2, 5). In the fusion protein, almost the entire MEN protein fuses to the N-terminal part of the MLL protein, which is disrupted between AT hook motifs and two zinc finger domains (1, 2). The MLL protein is a transcription factor involved in a variety of 11q23 chromosomal translocations. Some of the partner genes, which are fused to the MLL gene by the translocations, have been identified (12–14). MEN is the only partner with a known biochemical function. However, the elongation activity of the MLL/MEN protein is not yet known, and the mechanism of leukemogenesis by the fusion protein has not been discovered.

In this study, we overexpressed the MEN protein in Rat1 fibroblasts and analyzed its transforming activity to clarify the relationship between the transcription elongation factor and cell proliferation. We also overexpressed a deletion mutant of MEN lacking the lysine-rich region to evaluate the functional responsibility of the domain. Moreover, to clarify the molecular mechanism of the transformation by the MEN protein, we evaluated the expression of c-Fos protein and AP-1 activity.

MATERIALS AND METHODS

Plasmid Construction—Previously, we constructed the pME18S-MEN plasmid, which contains the entire coding region of MEN cDNA followed by the sequence coding for nine amino acids of the influenza hemagglutinin (HA) epitope (TACCCATACGACGTCCCAGACTACGCT) in the EcoRI site (5). The pME18S-MEN-ΔLR plasmid, which is a deletion mutant lacking the lysine-rich region, was constructed by digesting the pME18S-MEN plasmid with Apal, blunting with T4 DNA polymerase, and religating. The EcoRI fragments from the two constructs were cloned in the sense orientation into the EcoRI site downstream of the 5′-long terminal repeat of the retroviral vector pSRaMSVtkneo (15) and were named pSRaMSVtkneo-MEN and pSRaMSVtkneo-MEN-ΔLR. The empty pSRaMSVtkneo vector (pSRaMSVtkneo-Mock) was used as a negative control.

The p(9/wild TRE)x3-ΔLuc reporter plasmid, which contains three tandemly repeated 12-O-tetradecanoylphorbol-13-acetate-responsive elements (TREs) immediately upstream of the herpes simplex virus thymidine kinase promoter followed by firefly luciferase cDNA (16), was

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1 The abbreviations used are: HA, hemagglutinin; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; EGF, epidermal growth factor.
used to estimate AP-1 (Fos/Jun heterodimer and Jun/Jun homodimer) activity. The p.mut. TRE/3×3-tk-Luc reporter plasmid contains a two-nucleotide mutation in each TRE (16).

*Cell Culture*—COS-7 cells, NIH3T3 cells, and Rat1 cells including their transfected derivatives were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and the indicated concentration of fetal calf serum (FCS).

**Viral Infection**—To prepare the retrovirus stocks, 10 μg of pSRαMSVtkneo-Mock, pSRαMSVtkneo-MEN, and pSRαMSVtkneo-MEN-ΔLR constructs were transfected with 40 μg of pV packaging plasmid into 1 × 10⁸ COS-7 cells by the DEAE-dextran method (17). The culture medium containing viruses was harvested 96 h after transfection. Viral titers were determined and normalized. Viral infections were performed by exposing 5 × 10⁵ Rat1 cells to 1 ml of virus stocks for 8 h. G418-resistant populations were selected in medium containing 800 μg/ml G418 after an additional incubation for 48 h in medium without G418. The following experiments were performed with uncloned cell populations.

**Protein Analysis**—Cells for analysis were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% SDS, 1% Nonidet P-40, and 1 mM phenylmethylsulfonil fluoride), and protein concentrations were determined using protein assay dye (Bio-Rad). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Corp.). The membranes were blocked with 10% skim milk and treated with anti-HA-11 antibody (BAbCO) and anti-c-Fos antibody (Ab-2, Oncogene Research Products), which are rabbit polyclonal antibodies against the HA epitope (YPYDVPDYA) and the human c-Fos epitope (SGFNADYEASSRC), respectively. Thereafter, they were washed and reacted with goat anti-IgG antibody coupled to alkaline phosphatase (Promega). The blots were visualized by incubation with nitro blue tetrazolium and bromochloroindolyl phosphate (Promega).

**Subcellular Fractionation**—Nuclear and cytoplasmic proteins were separated by the method described previously (5). Anti-Rb antibody (Pharmingen) and anti-actin antibody (Boehringer Mannheim) were used to detect the controls of the nuclear and cytoplasmic fractions, respectively.

**Assays for Transformation**—For the soft agar assay, cells of each transfected derivative were trypsinized, suspended in DMEM containing 0.3% agar and 20% FCS, and plated on to a bottom layer containing 0.6% agar. Cells were plated at a density of 2 × 10⁵ cells/3.5-cm dish, and colonies >0.5 mm in diameter were enumerated after 14 days. All procedures were performed in three independent experiments, and similar results were obtained.

**Growth** was assessed by plating 1 × 10⁵ cells/well in six-well tissue culture dishes in triplicate and incubating in DMEM containing 5% or 0.1% FCS. Cells were harvested at 24-h intervals by trypsinization and counted using a hemocytometer.

**Luciferase Assay**—Cells for analysis were plated at a density of 1.4 × 10⁵ cells/6-cm dish in DMEM containing 5% FCS. Five μg of pSRαMSVtkneo-Mock, pSRαMSVtkneo-MEN, and pSRαMSVtkneo-MEN-ΔLR were used to transfect the cells. The probes for the measurement of AP-1 activity were constructed from the nuclear factor (NF)-κB consensus sequence (18). The luciferase assay was performed at 42 °C in hybridization solution (1

![Fig. 1](http://www.jbc.org/)  
**A**, schematic representation of the MEN protein. MEN has a lysine-rich region in the C-terminal portion. MEN-ΔLR is a deletion mutant of MEN lacking the lysine-rich region. These two proteins were tagged with the HA epitope. **B**, expression of the MEN and MEN-ΔLR proteins in Rat1 cells. Total cell lysates were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-HA-11 antibody. The arrowheads indicate the migration positions of the 80-kDa MEN protein and the 57-kDa MEN-ΔLR protein. C, subcellular localization of the MEN and MEN-ΔLR proteins. Both proteins localized in the nucleus. Anti-Rb and anti-actin antibodies were used to detect the controls of the nuclear (Nuc.) and cytoplasmic (Cyt.) fractions, respectively.

**Nuclear Run-on Assay**—Cells (1 × 10⁶ cells/transfectant) were stimulated for 30 min with EGF as described above. Harvested cells were washed three times with ice-cold phosphate-buffered saline, resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40), incubated on ice for 5 min, and centrifuged. The pelleted nuclei were washed twice with lysis buffer, resuspended in suspension buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA), and frozen in liquid nitrogen. The nuclei were mixed with reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 300 mM KCl, 0.5 mM ATP, 0.5 mM GTP, and 150 μCi of [α-32P]UTP (800 Ci/mmol; Amersham Corp.)) and incubated for 30 min at 30 °C. DNase I was added to a final concentration of 100 units/ml and incubated for 10 min at 30 °C. The samples were mixed with stop buffer (200 mM Tris-HCl, pH 7.4, 2% SDS, 10 mM EDTA, and 200 μg/ml proteinase K) and incubated for 30 min at 42 °C. After treatment with phenol/chloroform and chloroform, nuclear transcripts were separated from unincorporated nucleotides on a Sephadex G-50 column.

The probes used to detect the 5′- and 3′-end transcripts were the 322-base pair EcoRI-HindIII fragment and the 286-base pair StuI-PstI fragment from human c-fos cDNA, respectively. An oligonucleotide (TGATGGTACA TGACAAGGTG CGGCTCCCTA GGCCCCTCCC CTCTAA) complementary to the glyceraldehyde-3-phosphate dehydrogenase cDNA was synthesized to detect the glyceraldehyde-3-phosphate dehydrogenase transcript. The pUC vector was used as negative control. The probes were denatured in 0.2× NaOH, and 500 ng of each probe was loaded onto a nitrocellulose membrane (Hybond-C pure, Amersham Corp.) using a slot-blot machine and preincubated in hybridization solution.

The labeled RNAs were heat-denatured and hybridized to DNA probes for 48 h at a density of 1 × 10⁶ cpm/ml in hybridization solution. Filters were washed and exposed to autoradiography film as described above.

**RESULTS**

**Viral Infection**—We used the retroviral gene transfer method as a means of introducing MEN or MEN-ΔLR, a deletion mutant lacking the lysine-rich region (Fig. 1A), into Rat1 cells. Replication-deficient retroviruses were generated by transfecting the retroviral vector pSRαMSVtkneo containing the corre-
sponding cDNAs with Ψ⁻ helper plasmid in COS-7 cells. Subsequently, Rat1 cells were infected with the retroviruses, and infected cells were selected by G418. Expression of the 80-kDa MEN protein and the 57-kDa MEN-ΔLR protein was confirmed by Western analysis using anti-HA.11 antibody (Fig. 1B). The following experiments were performed with these uncloned cell populations. To examine the localization of the MEN mutant, nuclear and cytoplasmic proteins were separated and immuno-blotted with anti-HA.11 antibody. The nuclear localization of the MEN and MEN-ΔLR proteins was demonstrated (Fig. 1C).

MEN Transforms Rat1 Cells Depending on the Lysine-rich Region—To analyze the transforming ability of MEN and MEN-ΔLR, we evaluated the capacity for anchorage-independent growth and the growth factor requirement. Rat1 derivatives were seeded in DMEM containing 0.3% agar and 20% FCS, and colony formation was estimated as an anchorage-independent growth ability. Mock and MEN-ΔLR transfectants barely made colonies. In contrast, MEN-expressing cells formed many macroscopic colonies within 14 days (Figs. 2 and 3A).

To evaluate the serum requirement, we determined the growth curves for these transfectants. There was observed no difference in the growth rate when cultured in 5% FCS. However, as shown in Fig. 3B, cells expressing the MEN protein could steadily grow in 0.1% FCS, whereas the other transfectants slowly declined. Although morphological change and focus formation of MEN-expressing Rat1 cells were not observed (data not shown), some of the transformed phenotypes, such as the acquirement of the capacity for anchorage-independent growth and the decrease in growth factor requirement, were detected for these cells. The lysine-rich region seemed essential for the transformation because cells expressing MEN without the region did not exhibit any of the transformed phenotypes.

c-Fos Protein Expression and AP-1 Activity Are Increased in MEN-expressing Cells—To clarify the mechanism of transformation, we analyzed the product of the c-fos proto-oncogene because overexpression of c-Fos protein results in transformation of Rat1 cells (21), and the expression of c-fos mRNA is known to be regulated partly at the elongation stage (22, 23). Rat1 derivatives were cultured in DMEM containing 0.1% FCS because proliferation of MEN-expressing cells was prominent at this serum concentration. NIH3T3 cells were used as a positive control for the detection of c-Fos protein. Cell lysates
were subjected to SDS-polyacrylamide gel electrophoresis. Western analysis with anti-c-Fos antibody revealed higher expression of 62-kDa c-Fos protein in MEN-expressing cells than in other transfectants (Fig. 4A).

We then performed the luciferase assay to evaluate AP-1 activity using the p(wild TRE)x3-tk-Luc and p(mut. TRE)x3-tk-Luc reporters. Cells were transfected with the reporter plasmid and harvested after incubation in DMEM containing 0.1% FCS for 24 h. Luciferase activity with the wild-type reporter was significantly increased in cells expressing the MEN protein (Fig. 4B). The increase was canceled in cells transfected with the mutant reporter plasmid, indicating that the increase in luciferase activity depended on the sequence of TRE. Cells expressing MEN-ΔLR showed a slight elevation of AP-1 activity. These findings suggest that elevated c-Fos protein expression and AP-1 activity might be part of the mechanism responsible for cell transformation by the MEN protein.

Time Course of c-fos mRNA Expression after Stimulation with EGF—The time course of c-fos mRNA expression after stimulation with EGF was analyzed to examine whether the MEN protein alters the expression of c-fos mRNA. Cells were stimulated in DMEM containing 10 nM EGF after starvation in serum-free medium for 24 h. Cells were harvested just before and at 10-min intervals from 10 to 30 min after stimulation with EGF. Total RNAs were extracted and subjected to Northern hybridization with the c-fos probe. In mock and MEN-ΔLR transfectants, the 2.4-kilobase c-fos transcript was detected at 30 min after stimulation (Fig. 5A). On the other hand, the c-fos transcript was visible at 20 min in MEN-expressing cells. In addition, the c-fos transcript at 30 min after stimulation was detected with more intensity in MEN transfectants than in other transfectants. The c-myc transcript was also examined on the same filter, but there was no difference in the expression of c-myc mRNA among the three transfectants (data not shown). These findings suggest that the overexpressed MEN protein accelerated the generation of c-fos mRNA depending on the lysine-rich region.

Nuclear Run-on Assay of c-fos mRNA—To examine whether the change in the generation of c-fos mRNA results from altered elongation, we performed the nuclear run-on assay. The nuclei of Rat1 transfectants were harvested after stimulation with EGF. Nuclear RNAs extracted following in vitro transcription with [α-32P]UTP were hybridized to 3'- and 5'-probes of c-fos cDNA. The rate of elongation was assayed by the ratio of 3'- and 5'-signals. As shown in Fig. 5B, the elongation of c-fos mRNA was activated in MEN transfectants, but not in MEN-ΔLR transfectants. The increase in c-fos mRNA was therefore considered to result from accelerated elongation of the transcripts.
DISCUSSION

We have shown in this study that overexpression of the MEN protein transforms Rat1 cells depending on the lysine-rich region. MEN-expressing Rat1 cells formed colonies in soft agar and grew in low serum medium. This is the first report describing the direct association between the transcription elongation factor and cell proliferation, although the relationship between them has been suggested in the mechanism of von Hippel-Lindau disease (10, 11). Moreover, MEN-expressing cells disclosed higher expression of c-Fos protein and an increase in AP-1 activity, which could lead to transformation of Rat1 cells. The expression of c-fos mRNA appeared earlier in MEN transfectants than in other transfectants after stimulation with EGF. Results of the nuclear run-on assay suggested that the elongation of c-fos mRNA was accelerated by the overexpressed MEN protein.

We demonstrated the transforming activity of the overexpressed MEN protein by the soft agar assay and serum requirement analysis. However, neither morphological change nor focus-forming ability was observed. Therefore, the MEN protein is considered to be important for the regulation of cell proliferation, but is not directly associated with neoplastic transformation because MEN-expressing cells did not show the phenotype for full transformation.

The expression of c-Fos protein was increased in MEN-expressing cells, and it might be one of the mechanisms responsible for the transformation by the MEN protein. However, it has been reported that constitutive overexpression of c-Fos protein itself in Rat1 cells results in morphological transformation, but decreases the growth rate (21). Thus, there were phenotypic differences between Rat1 cells overexpressing MEN and c-Fos proteins, which might result from the fact that the expression of c-Fos protein in MEN-overexpressing Rat1 cells was not constitutive and/or that MEN could affect the expression of other genes that might be responsible for the differences. AP-1 activity was also elevated in MEN-overexpressing Rat1 cells. It has been demonstrated in P19 embryonal carcinoma cells that c-Fos expression is a crucial step for AP-1 activity (16). The higher expression of c-Fos protein might be associated with the increase in AP-1 activity in Rat1 cells, as is the case with P19 cells.

The c-fos mRNA, the expression of which is regulated partly at the elongation stage, was detected earlier in MEN-expressing cells than in other transfectants after stimulation with EGF. The nuclear run-on assay demonstrated that the elongation of the c-fos mRNA is accelerated by the MEN protein. In contrast, the time course of c-myc mRNA expression, which is also known to be regulated at the elongation stage (24), was not altered by MEN. Therefore, we speculated that the RNA polymerase II elongation factor MEN has specific functional targets.

We have also shown that the lysine-rich region is essential for cell transformation, elevated expression of c-Fos protein, and increase in AP-1 activity by the MEN protein. The deletion of the region resulted in the almost complete loss of these abilities, although the deletion mutant localized still in the nucleus. The 16-amino acid domain from positions 450 to 465 of MEN, which is included in the lysine-rich region, shares homology with a region of rat elongin (3). Taken together, the lysine-rich region might play an important role in transcriptional elongation. However, a slight elevation of AP-1 activity was observed in MEN-ΔLR-expressing cells, suggesting the existence of another important domain outside the lysine-rich region, which is not enough for cell transformation. In a recent report, Shilatifard et al. (25) demonstrated by in vitro analyses that the lysine-rich region is not essential for elongation activation (25). The discrepancy between their in vitro data and our in vivo findings raised the possibility that another molecule in Rat1 cells binds to the lysine-rich region and activates the function of the MEN protein. It is natural to consider that loss of the lysine-rich region does not affect the in vitro elongation analyses when there are excessive amounts of the factor in the analysis.

Our study highlighted the relationship between the control of cell proliferation and the transcription elongation factor. Functional targets of the MEN protein should be identified to reveal the molecular mechanism of the regulation of cell proliferation by the MEN protein. Identification of the proteins that interact with and regulate the function of the MEN protein is also required to clarify the whole mechanism of transcription regulation.

Myeloid leukemia carrying the t(11;19)(q23;p13.1) translocation is so far only a human disease involving the MEN elongation factor. The mechanism by which 11q23 abnormalities cause leukemias has not been determined. The first possible mechanism is that MLL loses its zinc finger domains, which might bind to specific DNA sequences or proteins. A second possible mechanism is that the function of the partner protein is modified by acquiring the N-terminal region of the MLL protein. The MLL/MEN fusion protein consists of the N-terminal part of MLL and almost the entire MEN protein. Thus, the fusion protein may have altered biological activity of transcription elongation. In addition, the in vivo stability of the MEN protein may be modified in the fusion protein. We are currently trying to analyze the elongation activity of the MLL/MEN fusion protein by in vitro analysis and to examine the in vivo function of the fusion protein in transgenic mice.

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