Cloning and Characterization of the EAP30 Subunit of the ELL Complex That Confers Derepression of Transcription by RNA Polymerase II*

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The product of the human oncogene ELL encodes an RNA polymerase II transcription factor that undergoes frequent translocation in acute myeloid leukemia (AML). In addition to its elongation activity, ELL contains a novel type of RNA polymerase II interaction domain that is capable of repressing polymerase activity in promoter-specific transcription. Remarkably, the ELL translocation that is found in patients with AML results in the deletion of exactly this functional domain. Here we report that the EAP30 subunit of the ELL complex has sequence homology to the Saccharomyces cerevisiae SNF8, whose genetic analysis suggests its involvement in the derepression of gene expression. Remarkably, EAP30 can interact with ELL and derepress ELL’s inhibitory activity in vitro. This finding may reveal a key role for EAP30 in the pathogenesis of human leukemia.

The identification of genes at breakpoints of frequently occurring chromosomal translocations has been the basis for the discovery of novel cellular factors involved in oncogenesis. Many such proteins are transcription factors that regulate gene expression (1–3). The human ELL gene had been identified initially as a gene on chromosome 19p13.1 undergoing frequent translocations with the trithorax-like MLL gene on chromosome 11q23 in acute myeloid leukemia (AML) (4, 5). ELL is an 80-kDa RNA polymerase II (Pol II) transcription factor that can increase the catalytic rate of transcription elongation by Pol II from both promoter-dependent and promoter-independent templates (6–9). In addition to its elongation activity, ELL contains a novel type of Pol II interaction domain that can repress polymerase activity in promoter-specific transcription in vitro (6, 10). The addition of ELL to transcription reactions before the assembly of the preinitiation complex leads to a significant reduction in the yield of full-length run-off transcripts. This repression has shown to be due to the physical interaction of ELL with Pol II and the consequent disruption of preinitiation complex formation (10). Remarkably, the MLL-ELL translocation found in patients with AML results in the deletion of a portion of the functional domain required for inhibition of promoter-specific initiation by ELL (4, 5, 10). ELL mutants lacking the sequence deleted by the translocation are fully active in elongation; however, such mutants failed to inhibit initiation by Pol II (10).

The partner of ELL in the chimeric protein produced by the MLL-ELL translocation in patients with AML is the product of the MLL gene (1, 11–13). This gene encodes a large, multidomain 3,968-amino acid protein containing an N-terminal A-T hook DNA-binding domain, a methyltransferase-like domain, and a C-terminal trithorax-like region (11–13). The MLL gene is a recurring target for translocation in a variety of clinically distinct leukemias (12). The breakpoints of every MLL translocation create a putative oncogene that encodes nearly the entire translocation partner fused to the N terminus of the MLL protein. Although all these translocations occur within the same region of MLL, each translocation is associated with a clinically distinct form of leukemia, suggesting that MLL translocation partners, such as ELL, play a major role in determining the leukemic phenotype. In light of this data, recently it was demonstrated that the replacement of the normal MLL gene with an MLL-AF9 chimera led to the development of leukemia in mice, suggesting that translocation causes the development of AML (14).

Recently, we purified ELL in complex with three other cellular proteins from total rat liver extract (15). Biochemical characterization of this ELL-containing complex demonstrated that the ELL complex is capable of increasing that catalytic rate of transcription elongation; however, unlike ELL alone, the complex was unable to repress initiation of transcription by RNA polymerase II. This lead to a model that suggested that one or more of the ELL-associated proteins interact or at least renders nonfunctional the N-terminal domain of the ELL protein, which is shown to be important for ELL’s transcriptional inhibitory activity (10). In this view, the interaction of ELL with one of the EAPs normally regulates the transcriptional inhibitory activity of ELL, and deletion of this functional domain of ELL (i.e. MLL-ELL translocation) bypasses this regulation. Here, we report the cloning, expression, and biochemical characterization of the EAP30 subunit of the ELL complex, which has sequence homology to the S. cerevisiae SNF8, a protein whose genetic analysis suggests its involvement in the derepression of gene expression (18). Recombinant EAP30 can interact with ELL, and this interaction leads to the derepression of ELL’s transcriptional inhibitory activity in vitro.

EXPERIMENTAL PROCEDURES

Materials—Ultrapure ribonucleoside 5’-triphosphates were purchased from Amersham Pharmacia Biotec. [α-32P]CTP was obtained from ICN. Leupeptin, antipeptin, phenylmethylsulfonyl fluoride, and heparin were obtained from Sigma. Bovine serum albumin (Pentex fraction V) and Western development reagents were obtained from ICN.

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‡ The abbreviations used are: AML, acute myeloid leukemia; Pol II, polymerase II; EAP, ELL-associated protein; MALDI-MS, matrix-assisted laser desorption Ionization mass spectrometry; HPLC, high performance liquid chromatography; ORF, open reading frame.

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ImmunoBiologicals. Glycerol (spectro-analyzed grade), potassium chloride, Hepes, Tris, ammonium sulfate, and ultrapure sucrose were purchased from Fisher. The chromatographic columns Superose-12 HR and Superose-6 were purchased from Amersham Pharmacia Biotech.

**Purification of the ELL-Complex**—Rat liver extract was prepared by homogenization of 180 rat livers as described previously (15), with the exception that nuclear and cytosolic extracts were combined together after the removal of the lysosomal fraction. The purification of the ELL complex was performed at 4 °C, and the fractions were not frozen following the procedure as described previously (15).

**Cloning and Expression and Analysis of EAP30**—Total rat liver extract was prepared as described previously (15), and the ELL complex was purified following the previous procedure (15). Approximately 25 pmol of the purified EAP30 was reduced, S-carboxymethylated, digested with trypsin, and then either analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or fractionated with microbore HPLC. Peptides isolated by microbore HPLC were sequenced from the expressed sequence tag clone. The EAP30 ORF was sequenced from the expressed sequence tag clone. The EAP30 ORF was obtained by polymerase chain reaction amplification using a 5′ primer (5′-ggggtgcagacgtaacaggctgaggggagtgt-3′) and a 3′ antisense primer (5′-gggttggctctctcagggaggcagattcggcgt-3′) for subcloning into a M13 expression vector.

The construct for the expression of histidine-tagged EAP30 in bacteria was prepared as described below. First, the DNA fragment encoding the full-length EAP30 was obtained by polymerase chain reaction amplification and was introduced into the SalI and BamHI sites of M13mpET, which contains the complete pET T7 transcription-translation regions as well as the sequence encoding His tag (17). Then, the expression vector containing the entire EAP30 protein was expressed in Escherichia coli JM109 (DE3). About 500 ml of JM109 (DE3) culture was grown to an absorbance (at 600 nm) of 0.3 with gentle shaking in Luria broth medium containing 2.5 mM MgCl₂ and MgSO₄ at 37 °C. Cells were infected with M13mpET carrying the EAP30 ORF at a multiplicity of 100. After 4 h at 37 °C, infected cells were induced with 100 mM isopropyl-β-D-thiogalactopyranoside for another 4 h. Cells were harvested by centrifugation at 2,500 × g for 30 min at 4 °C. Inclusion bodies were solubilized by resuspension in 7 ml of ice-cold 6 M guanidine HCl with 50 mM Tris-HCl (pH 7.9) and recombinant EAP30 was purified by nickel chromatography on ProBond resin. The mutation of EAP30 to remove its His tag was performed by site-directed mutagenesis using the Bio-Rad Muta-gen kit. The EAP30 without His tag was expressed as above, and the interaction of EAP30 with His-ELL was carried out using nickel chromatography on ProBond resin. Polyclonal antibodies to either peptide sequence FAQDVSQDDLIR or the peptide (FAQDVSQDDLIR; left panel) obtained from the purified EAP30 or with silver staining (right panel). Co-chromatography of ELL with EAP30 using monoclonal antibodies raised against ELL (αELL) and polyclonal antibodies raised against EAP30 (αEAP30) is demonstrated above. Also, column fractions were tested for transcriptional elongation activity. Column fractions were added to transcription reactions containing RNA polymerase S. cerevisiae SNF8 protein.

**Acrylamide, 7 M urea.** The synthesis of the 135-nucleotide transcript is indicated by the arrow.

**Identification, cloning, and expression of human EAP30 protein, a component of the ELL complex.** A, sequence analysis of EAP30 from the ELL complex purification with Edman degradation and/or MALDI analysis. B, cloning and sequence comparison of EAP30 with S. cerevisiae SNF8 protein. C, Western and silver stain analysis of recombinant human EAP30. Human EAP30 protein was cloned and expressed as described under “Experimental Procedures” and analyzed either with polyclonal antibody raised against the peptide (FAQDVSQDDLIR) (left panel) or with silver stain (right panel). D, co-chromatography of human EAP30 with the purified ELL complex. Superose-6 PC fractions from the final purification step of the ELL complex purification (15) were analyzed for the presence of human EAP30 with polyclonal antibody raised against recombinant EAP30 in mouse (upper panel). Co-chromatography of ELL with EAP30 using monoclonal antibodies raised against ELL (αELL) and polyclonal antibodies raised against EAP30 (αEAP30) is demonstrated above. Also, column fractions were tested for transcriptional elongation activity. Column fractions were added to transcription reactions containing RNA polymerase II and oligo(dC)-tailed template pCpGR220 S/P/X. Transcription was initiated by the addition of three ribonucleoside triphosphates (50 μM ATP, 50 μM GTP and [α-32P]CTP) without UTP and incubated at 30 °C for 10 min. Transcripts were analyzed by electrophoresis through 7% (w/v) acrylamide, 7 M urea. The synthesis of the 135-nucleotide transcript is indicated by the arrow.
eluted as a 100-kDa protein in buffer A (40 mM Tris-HCl (pH 7.9), 10% glycerol, and 400 mM KCl) at 50 μl/min, and the EAP30 alone eluted very broad (ranging from 30 to 80 kDa) protein under the same conditions. These two polypeptides can be easily distinguished from each other on Superoxide-12. Renatured ELL alone, EAP30 alone, and ELL/EAP30 were applied to a Amersham Pharmacia Biotech Superoxide-12 PC column in buffer A at 50 μl/min, and fractions of 100 μl were collected. Each fraction was tested for the presence of EAP30 using polyclonal antibodies generated to recombinant EAP30.

The interaction of ELL and EAP30 was also tested by nickel trapping of His-ELL and EAP30 as described for His-ELL and RNA polymerase II (10). About 10 μg of recombinant His-ELL was renatured with 10 μg of recombinant EAP30 (in a 500-μl reaction mixture) in renaturation buffer (40 mM Hepes-NaOH (pH 7.9), 0.4 M KCl, 50 μM ZnSO4, 1 mM DTT and 10% (v/v) glycerol) for 3 h. Each reaction mixture was incubated with ProBond resin for 1 h. After an hour, ProBond resin was washed and eluted with 300 mM imidazole (pH 7.9) in renaturation buffer. Fractions were tested for the presence of EAP30 on SDS-PAGE and Western analysis using polyclonal antibodies raised against EAP30.

**RESULTS**

Cloning and Expression of EAP30—Although the ELL protein is the only MLL partner in leukemia whose biochemical function is known, its precise role in leukemia remains a mystery. Since the identification of ELL as a Pol II transcription factor (8, 9), several lines of evidence have suggested that ELL exists in a complex with other cellular factors. Recently, ELL was purified, together with three other proteins from total rat liver extract, in our laboratory (15). This complex can increase the catalytic rate of transcription elongation by Pol II; however, unlike the ELL polypeptide, the ELL complex is not capable of repressing polymerase activity in promoter-specific transcription (15). The identification of EAPs that derepress ELL's transcriptional inhibitory activity suggests that one or more of the EAPs interacts with, or at least renders nonfunctional, the N-terminal domain of the ELL protein, a domain proven to be necessary for ELL's transcriptional inhibitory activity (8–10). This hypothesis suggests a mechanism whereby the translocation could eliminate regulation of promoter-specific initiation of transcription, resulting in the loss of growth regulation. To investigate the role of the EAPs, we have purified the ELL complex in large quantities and have analyzed the N-terminal of several tryptic peptides of the EAPs using sequential Edman degradation and/or MALDI analysis (19). These analyses demonstrated that the EAP30 subunit of the ELL complex has sequence similarity to the *Saccharomyces cerevisiae* SNF8 protein (Fig. 1A).

The snf8 gene was identified initially by complementation from *S. cerevisiae* as a 27-dalton protein whose mutations impaire derepression of the *SUC2* gene (18). Genetic analysis of snf8 null mutations with *spf6/ssh20* and *ssh6* suppressor distinguished SNF8 from SNF1, SNF2, SNF4, SNF5, and SNF6 (18). Remarkably, snf8,ssh6 double mutants were extremely sick, and it has been postulated that SNF8 functions via regulation of gene expression. Genetic analysis has also indicated that snf8 in *S. cerevisiae* contributes to derepression of *SUC2* and may, along with other unknown proteins, provide a function that is essential for derepression (18). No information is available about the mammalian SNF8 protein.

To investigate the possibility that EAP30 can interact with ELL and thus regulate the transcriptional repressory activity of ELL, we cloned and expressed the full-length human EAP30 (Fig. 1B). Recombinant EAP30 protein was purified to homogeneity from guanidine solubilized inclusion bodies and was demonstrated to have a relative molecular mass of about 30 kDa on SDS-PAGE (Fig. 1C). This recombinant protein is recognized by polyclonal antibodies raised against one of the peptides obtained from sequential Edman degradation of EPA30 (Fig. 1C) (15). In addition, polyclonal antibodies raised against the recombinant EAP30 protein recognized purified EAP30 (Fig. 1D). Taken together, these data suggest that cloned recombinant EAP30 is the same as EAP30 purified from total rat liver extract.

**ELL Can Interact with ELL in Vitro**—The interaction of recombinant EAP30 with recombinant ELL was tested by two methods. First, we renatured recombinant ELL or recombinant EAP30 alone or with each other. To identify interaction between ELL and EAP30, we took advantage of size exclusion chromatography on an Amersham Pharmacia Biotech Superose-12 PC column. ELL and EAP30 alone can be easily distinguished from each other on Superoxide-12 PC size exclusion chromatography. If EAP30 interacts with ELL, it should elute as a larger protein when renatured with ELL. Upon renaturation, size exclusion chromatography, and Western analysis of ELL, EAP30, or ELL with EAP30 on Superose-12, it was observed that when EAP30 was renatured with ELL, it eluted as a larger protein with sharper elution profile than when renatured alone (Fig. 2A). We note that recombinant renatured EAP30 alone shows molecular mass ranging from 30 to about 100 kDa on size exclusion. We believe that recombinant EAP30 alone is not renatured properly, and therefore, it behaves with a broad elution profile from size exclusion column. However, when EAP30 is renatured with ELL, it behaves with a much sharper elution profile, due to its proper folding. We also noticed that the total yield of ELL recovered when ELL was renatured with EAP30 was much higher than when ELL was renatured alone (data not shown).

Our second strategy to investigate the possibility that EAP30 protein can interact with ELL took advantage of the ability of histidine-tagged ELL to retain untagged EAP30 on nickel agarose as described under “Experimental Procedures.” When EAP30 was renatured alone and then applied to nickel chromatography, virtually no EAP30 was detected in the nickel
bound fraction as tested with polyclonal antibodies raised against EAP30 (Fig. 2B, lane 5). However, when EAP30 was renatured with His-ELL, almost all EAP30s were found in the nickel-bound fraction (Fig. 2B, lane 6). These experiments indicated that EAP30 is capable of direct physical interaction with ELL in about a 1:1 ratio.

EAP30 Confers Derepression of ELL’s Transcriptional Inhibitory Activity—We next sought to determine whether the interaction of EAP30 with ELL can regulate ELL’s transcriptional inhibitory activity (15). As demonstrated previously, Pol II and the general initiation factors will synthesize the trinucleotide CpApC at the AdML promoter when provided with the dinucleotide primer CpA and [$\alpha$-32P]CTP. The nontemplate strand sequence corresponding to the CpApC product is underlined. B, either no ELL (lanes 1–4), ELL (lanes 5–8), or ELL-EAP30 complex (lanes 9–12) were added to transcription reactions before the formation of preinitiation complexes (PIC). Synthesis of CpApC in the presence of no ELL, ELL, and ELL-EAP30 complex were initiated by the addition of 500 $\mu$M CpA, 10 $\mu$Ci of [$\alpha$-32P]CTP, and 5 $\mu$M dATP and reactions incubated for indicated time postinitiation. Transcripts were analyzed by electrophoresis through 25% (w/v) acrylamide, 7 M urea gels. C, either 50 ng of ELL (lane 1), no ELL (lane 2), or 50 ng of ELL renature with increasing concentration of EAP30 (lanes 3–7) was added to transcription reactions before the formation of PICs. Synthesis of CpApC was measured as mentioned above.

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FIG. 3. Human EAP30 confers derepression of ELL’s transcriptional inhibitory activity. A, nontemplate strand sequences surrounding the transcription start site of the AdML promoter. RNA polymerase II synthesizes the trinucleotide CpApC at the AdML promoter when provided with the dinucleotide primer CpA and [$\alpha$-32P]CTP. The nontemplate strand sequence corresponding to the CpApC product is underlined. B, either no ELL (lanes 1–4), ELL (lanes 5–8), or ELL-EAP30 complex (lanes 9–12) were added to transcription reactions before the formation of preinitiation complexes (PIC). Synthesis of CpApC in the presence of no ELL, ELL, and ELL-EAP30 complex were initiated by the addition of 500 $\mu$M CpA, 10 $\mu$Ci of [$\alpha$-32P]CTP, and 5 $\mu$M dATP and reactions incubated for indicated time postinitiation. Transcripts were analyzed by electrophoresis through 25% (w/v) acrylamide, 7 M urea gels. C, either 50 ng of ELL (lane 1), no ELL (lane 2), or 50 ng of ELL renature with increasing concentration of EAP30 (lanes 3–7) was added to transcription reactions before the formation of PICs. Synthesis of CpApC was measured as mentioned above.

We also tested if such derepression of the inhibitory activity of ELL by EAP30 is dependent on the concentration of EAP30. ELL and ELL with increasing concentrations of EAP30 were added to transcription reactions before the formation of preinitiation complexes to test the derepression of the inhibitory
activity of ELL by EAP30. In this experiment, when 50 ng of ELL was added to transcription reaction before the formation of preinitiation complex, it dramatically decreased the yield of CpApC synthesis (Fig. 3, lane 1). However, when 10 ng of EAP30 was renatured with ELL, it partially derepressed ELL’s inhibitory activity (Fig. 3, lane 3). We observed a maximum derepression at about 50 ng of EAP30 with 50 ng of ELL. We noted that the derepression of ELL's inhibitory activity by EAP30 is not 100%. This may be due in part to the absence of EAP20 and EAP45, which could possibly be required to in
crease the stability of ELL-EAP30 complex. These findings indicated that the derepression of ELL's inhibitory activity by EAP30 was renatured with ELL, it partially derepressed ELL's transcriptional inhibitory activity in vivo.

DISCUSSION

Genetic and molecular analysis of a large number of chromosomal abnormalities in human cancer has revealed that the MLL gene is a recurring target for translocation in a variety of phenotypically distinct leukemias (13). To date, genes encoding eight MLL translocation partners have been isolated by genetic analysis. No information regarding the biochemical activities of any of the MLL partners (besides ELL) is available. We identified the product of the ELL gene, a partner of MLL in AML, as Pol II transcription factor. The ELL protein can regulate both the transcriptional initiation and elongation activities of Pol II. ELL contains a novel type of Pol II interaction domain that can repress polymerase activity in promoter-specific transcription in vitro (10). This repression of transcription by ELL has been demonstrated to be due to its physical interaction with Pol II and the disruption of the formation of preinitiation complex (10).

The ELL domain required for this transcriptional inhibitory activity is lost in the MLL-ELL translocation found in patients with AML. Since its identification as a Pol II transcription factor, ELL has been demonstrated to interact with three unknown cellular factors in total liver extract to form the ELL complex. Unlike ELL alone, the ELL complex is unable to repress initiation of transcription by Pol II (15). It has been proposed that one or more of the ELL-associated proteins can interact with ELL and render nonfunctional the N-terminal domain of the ELL protein required for ELL’s transcriptional inhibitory activity (15). This hypothesis raises several questions: (i) what are the components of the ELL complex, (ii) which EAPs can interact with ELL and regulate its transcriptional repressory activity, and (iii) do the EAPs that interact with ELL regulate transcription in vivo? Here, we have identified the human EAP30 gene product to be a component of the ELL complex. The EAP30 protein can interact with ELL and derepress ELL’s transcriptional inhibitory activity. This observation is consistent with the genetic analysis of the S. cerevisiae SNF8 where it was proposed to be involved in derepression of gene expression (18). In principle, EAP30 could regulate the expression of specific genes by controlling the rate of transcriptional initiation by Pol II. In conclusion, the identification of EAP30 as a component of the ELL complex and the analysis of its interaction with ELL in the regulation of ELL’s transcriptional inhibitory activity may provide a model in which the EAP30 protein may play a key role in the regulation of cell growth and the pathogenesis of leukemia.

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