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Functional characterization of a mammalian transcription factor, Elongin A

Takashi Yasukawa [a], Kazuto Sugimura [b], Mizue Fukuda [c], Katsuhisa Yamazaki [d], Shigetaka Kitajima [c], Katsuzumi Okumura [b], Teijiro Aso [a,*]

[a] Department of Functional Genomics, Kochi Medical School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan
[b] Laboratory of Molecular and Cellular Biology, Faculty of Bioresources, Mie University, 1515 Kamihama, Tsu, Mie 514-8507, Japan
[c] Department of Biochemical Genetics, Medical Research Institute, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan
[d] Microbial Chemistry Research Foundation, Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

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Abstract

Elongin A is the transcriptionally active subunit of the Elongin complex that strongly stimulates the rate of elongation by RNA polymerase II (pol II) by suppressing the transient pausing of the polymerase at many sites along the DNA template. We have recently shown that Elongin A-deficient mice are embryonic lethal, and mouse embryonic fibroblasts (MEFs) derived from Elongin $A^{-/-}$ embryos display not only increased apoptosis but also senescence-like phenotypes accompanied by the activation of p53. To further understand the function of Elongin A *in vivo*, we have carried out the structure–function analysis of Elongin A and identified sequences critical to its nuclear localization and direct interaction with pol II. Moreover, we have analyzed the replication fork movement in wild-type and Elongin $A^{-/-}$ MEFs, and shown the possibility that the genomic instability observed in Elongin $A^{-/-}$ MEFs might be caused by the replication fork collapse due to Elongin A deficiency.

Keywords: Elongin A; Transcription elongation; RNA polymerase II; Replication; Genomic instability

Eukaryotic messenger RNA synthesis by RNA polymerase II (pol II) is regulated by the concerted action of a set of general transcription factors that control the activity of pol II during the initiation and elongation stages of transcription. At least six general transcription initiation factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) have been identified in eukaryotic cells and found to promote the selective binding of pol II to promoters, and to support a basal level of transcription [1]. In addition, a diverse collection of elongation factors that promote the efficient elongation of transcripts by pol II on naked DNA templates *in vitro* has been identified biochemically [2–4]. These elongation factors fall into two broad functional classes based on their ability to either reactivate arrested pol II or suppress the transient pausing of pol II. The first class is composed of members of the SII family [2,5]. The second class is comprised of a collection of elongation factors, including TFIIF [6], Elongin [7,8], ELL [9], and Cockayne syndrome B (CSB) protein [10], which increase the overall rate of mRNA chain elongation by decreasing the frequency and/or duration of the transient pausing of pol II at sites along the DNA template.

Elongin was initially identified as a heterotrimeric protein composed of A, B, and C subunits of ~770, 118, and 112 amino acids, respectively [7,8,11,12]. Elongin A is the transcriptionally active subunit, whereas Elongins B and C are positive regulatory subunits that can form an isolable Elongin BC subcomplex [8,13,14]. Recently, we have generated Elongin A-deficient mice by gene targeting [15]. Elongin $A^{-/-}$ embryos exhibited severely retarded growth

* Corresponding author. Fax: +81 88 880 2281.
E-mail address: asot@med.kochi-u.ac.jp (T. Aso).

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and died at days between 10.5 and 12.5 of gestation, most likely due to excessive apoptosis. Moreover, mouse embryonic fibroblasts (MEFs) derived from Elongin $A^{-/-}$ embryos displayed not only increased apoptosis but also senescence-like growth defects accompanied by the activation of p53.

As part of our effort to understand the function and regulation of Elongin A *in vivo*, we have carried out the structure–function analysis of Elongin A and identified sequences critical for its nuclear localization and direct interaction with pol II. In addition, we have analyzed the rate of replication fork movement in wild-type and Elongin $A^{-/-}$ MEFs, and demonstrated the possibility that the genomic instability observed in Elongin $A^{-/-}$ MEFs might be caused by the replication fork collapse due to the lack of Elongin A.

Materials and methods

Plasmid construction. DNA constructs expressing Elongin A mutants were generated by oligonucleotide-directed mutagenesis of pcDNA3.1-FLAG-Elongin A [16] using the QuickChange site-directed mutagenesis kit (Stratagene). For the expression of glutathione *S*-transferase (GST)-Elongin A fusion protein, cDNA encoding the entire rat Elongin A was amplified by PCR from pSVL-Elongin A [13], and subcloned into the *Bam*HI and *Sal*I sites of pGEX-4T1 (Amersham). Subsequently, constructs expressing GST fused to various Elongin A mutants were generated by oligonucleotide-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene).

Expression of recombinant proteins in Escherichia coli and in vitro binding assay. GST and GST-Elongin A fusion proteins were expressed in *E. coli* DH5α and purified using glutathione–sepharose affinity chromatography (Amersham) as described [16] according to manufacturer's instructions. pol II was purified as described from rat liver nuclear extracts [17]. Immobilized GST fusion proteins (~15 μg) were mixed with purified pol II and incubated for 1 h at 4 °C. The beads were then washed five times with buffer containing 20 mM Hepes–NaOH (pH 7.9), 500 mM NaCl, 2.5 mM $MgCl_2$, 50 mM $ZnCl_2$, 0.1 mM EDTA, 0.05% Nonidet P-40, and 0.5 mM DTT. Aliquots of loaded and bound fractions were subjected to SDS–polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinylidene difluoride membrane (Millipore), and probed with anti-pol II (8WG16) antibody (Babco). Horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham) was used as a secondary antibody. Detection was performed using SuperSignal West Dura Extended Duration Substrate (Pierce).

Immunofluorescence staining. Immunostaining of cells was performed as previously described [16,18]. Briefly, COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and were transiently transfected with the pcDNA3.1/Hygro-FLAG-wild-type or mutant Elongin A using FuGENE 6 (Roche) according to manufacturer's protocol. The cells grown in a chamber slide were fixed by immersion in cold acetone/methanol (1:1) for 10 min, then rinsed with 70% ethanol, 50% ethanol and finally phosphate-buffered saline (PBS). After blocking in PBS containing 2% bovine serum albumin, 0.2% Tween 20, and 6.7% glycerol at 4 °C overnight, the cells were incubated for 1 h at 4 °C with rabbit polyclonal anti-FLAG antibody (1:1000) and mouse monoclonal anti-HA antibody. Reacting antibodies were stained with fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG_{2b} (Santa Cruz). Fluorescence microscopy of fixed cells was performed using an Olympus BX-50 confocal laser scanning microscope.

Generation of Elongin A-deficient mice. Elongin A-deficient mice were generated as previously described [15]. Briefly, Elongin A mutant ES cell clones were microinjected into blastocysts for the generation of chimeric mice. Chimeric males were crossed with C57BL/6 females, and agouti-colored offspring were analyzed for the germline transmission of the Elongin A mutation. Heterozygous animals were intercrossed to generate homozygous mutant animals.

PCR genotyping. Genomic DNA was isolated from embryonic yolk sacs, and genotyping was performed using PCR as described [15].

Preparation of MEFs. MEFs were prepared from E10.5 embryos as described [15]. Briefly, fetal tissue samples were rinsed in PBS and then were mechanically dissociated in DMEM with 10% FBS. The cells were then plated out on six-well plates. These cells were considered to be passage 1 MEFs.

Quantitative analysis of apoptosis in MEFs. For the quantitative analysis and to determine the cytoplasmic histone-associated DNA fragments, which are indicative of on-going apoptosis, the Cell Death Detection $ELISA^{PLUS}$ (Roche Applied Science) was used according to the manufacturer's instructions.

Replication labeling. Cells were labeled with biotin-16-dUTP and digoxigenin-11-dUTP (Roche Applied Science) at 20- or 30-min intervals using hypotonic shift procedure with slight modifications [19]. Cells grown on a 35 mm-diameter dish were washed with 1 ml KH buffer (30 mM KCl, 10 mM Hepes, pH 7.4) and then 10 μl KH buffer supplemented with 250 μM biotin-16-dUTP or digoxigenin-11-dUTP (Roche Applied Science) was added. After incubation at 37 °C with 5% CO_2 for 10 min, the samples were washed with serum-free medium and cultured in normal medium for 20 or 30 min to introduce modified nucleotides into nascent DNA. DNA fiber preparation was performed as described previously [20,21]. Replication-labeled cells and 10- to 20-fold unlabeled cells were mixed before sample preparation because DNA fibers were extended straight and each replication-labeled DNA fiber was well separated. Cells were fixed with methanol/acetic acid (3:1) solution, dropped onto a slideglass, and then dipped into lysis buffer (0.5% SDS, 200 mM Tris–HCl, pH 7.4, and 50 mM EDTA) for 10 min at 25 °C. DNA fibers were allowed to drop down from nuclei, dried and fixed with methanol/acetic acid (3:1) solution for 3 min at 25 °C. Biotin- and digoxigenin-labeled DNAs were detected using Alexa488-streptavidin (Invitrogen) and anti-digoxigenin conjugated with rhodamine (Roche Applied Science), respectively.

Microarray analysis. Total RNA was isolated from Elongin $A^{+/+}$ and Elongin $A^{-/-}$ MEFs according to the manufacturer's protocol using an RNeasy Mini kit (Qiagen). One-microgram aliquots of total RNA were labeled using the Agilent Linear Amplification/Labeling kit (Agilent Technologies) according to the manufacturer's instructions. After checking the labeling efficiency, 1-μg aliquots of Cy3-labeled Elongin $A^{+/+}$ cRNA and Cy5-labeled Elongin $A^{-/-}$ cRNA were mixed, then hybridized to an Agilent Whole Mouse Oligo Microarray using the manufacturer's hybridization protocol. After the washing step, the microarray slide was analyzed with an Agilent Microarray scanner and software (scanner model G2505B; software G2565BA). A data analysis was performed using the Agilent Feature Extraction software (Version A.6.1.1).

Results and discussion

Identification of the nuclear localization signals of Elongin A

We have previously shown that Elongin A and its related family proteins are predominantly localized in the cell nucleus [16,18]. In this study, we analyzed the sequences of Elongin A required for its nuclear localization using immunostaining assays. COS7 cells were transfected with constructs expressing wild-type or various deletion mutants of Elongin A with FLAG-tag at their NH_2-terminus, and stained with anti-FLAG antibody. As shown in Fig. 1, the Elongin A mutant composed of residues 400–589 was clearly localized in the nucleus.