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Ecm10p localizes in yeast mitochondrial nucleoids and its overexpression induces extensive mitochondrial DNA aggregations

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Abstract

Ecm10p was initially identified as a cell wall synthesis-related gene product [Genetics 147 (1997) 435] and also reported as a mitochondrial protein which was partially capable of compensating the phenotypic defect by SSC1 gene mutation [FEBS Lett. 487 (2000) 307]. Here we report that ecm10p is localized in mitochondrial nucleoids as its major component and the targeting signal resides between amino acid residues 161 and 240. Overexpression of ecm10p induces extensive mitochondrial DNA aggregations, which might be due to aberrant mitochondrial DNA cleavages through an altered endonuclease activity in mitochondrial nucleoids.

Keywords: Heat-shock protein; Mitochondrial DNA; Mitochondrial nucleoids; Yeast; *Saccharomyces cerevisiae*

HSP70 is a family of heat-shock proteins with a molecular weight of 70 kDa, which is conserved among various species from bacteria to mammals. These proteins reside in various subcellular fractions, where they work as molecular chaperones essential for protein modifications. As the whole genome sequence has already been determined in *Saccharomyces cerevisiae*, it was revealed that the yeast strain carries 14 members of HSP70, and their subcellular localizations were determined as follows: nine in cytosol, two in endoplasmic reticulum (ER), and three in mitochondria. Amino acid sequences of these HSP70 are well preserved with regard to each other, especially at the N-terminal 60-kDa region which consists of about 45-kDa ATPase domain and about 18 kDa peptide-binding region. In contrast, amino acid sequences at about 10 kDa C-terminal region are variable.

Two novel mitochondrial heat-shock proteins of HSP70 family other than the well-known Ssc1p have been identified in yeast. Ssc1p is indispensable for mitochondrial translocation of precursor proteins, which is synthesized in cytosol, delivered to mitochondrial matrix, where it interacts with a mitochondrial inner membrane protein, TIM44 or ATP/ADP exchanging factor (MGE1), for folding, oligomerization, and degradation of various precursor proteins [3–7]. Ssh1p/Ssq1p, the second identified HSP70 family member, is homologous to flataxin, which is thought to be involved in the development of a human neurodegenerative disorder, Friedreich's ataxia, and its impairment induces iron deposits in yeast cells as well as in human neurons [8]. Ecm10p was finally identified as a cell wall synthesis-related gene product [1], which was also reported as a mitochondrial protein partially capable of compensating the phenotypic defect by SSC1 gene mutation [2].

Here we report that ecm10p localizes in mitochondrial nucleoids as its major component with mitochondrial nucleoid-targeting signal between amino acid residues 161 and 240. Overexpression of intact ecm10p induces extensive mitochondrial DNA aggregations, suggesting that ecm10p might be involved in mitochondrial DNA

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cleavages through modulating the *Sce*I activity [9], a heterodimeric site-specific endonuclease in mitochondrial nucleoids.

Materials and methods

Yeast strains and antibodies. *Saccharomyces cerevisiae* ATCC24657 [MATα mal [rho + CAN^S]] for mitochondrial nucleoid purification and ATCC96099 [MATα leu2-3 leu2-112 ura3-52 his3Δ200 trp1-Δ901 lys2-801 suc2-Δ9] for ecm10p or β-galactosidase expression were purchased from American Type Culture Collection (ATCC). Ecm10p-specific rabbit polyclonal antibodies were raised using a synthetic peptide corresponding to the C-terminal 13 amino acid (SDNPETKN GRENK) of ecm10p coupled to BSA and purified by affinity chromatography using the synthetic peptide-conjugated folmyl-cellulofine (Seikagaku).

Yeast expression plasmid constructions. To express ecm10p in *S. cerevisiae*, ecm10p gene was amplified by PCR from yeast genomic DNA (ATCC96099 cells) using primers 5′-CCGGAT CCAACATGTTACCATCATGGAAAGCCT-3′ (ecm10F) and 5′-CCCTCGAGTTATTTATTTTCTCTCCCGTTCTTA-3′, digested with *Bam*HI and *Xho*I, and inserted into the *Bam*HI–*Xho*I site in a galactose-inducible centromeric vector p416GALS or in a galactose-inducible multicopy vector p426GALS (ATCC). To construct GFP-expression plasmid in yeast (p414GALS-GFP), the gene for GFP was amplified by PCR from a modified pGFPmut3.1 (Clontech), whose *Eco*RI site in the ORF was mutated by PCR-directed mutagenesis, using primers 5′-ACGCGTCGACCGGTGGAGGCTCTGGAGGC GGTTCTGGAGGCGGTTCTATGCGTAAAGGAGAAGAACTT TTCACTGGA-3′ and 5′-CCGCTCGAGCTAAAGGAACAGATGG TGGCGTCC-3′, digested with *Eco*RI and *Xho*I, and inserted into the *Eco*RI–*Xho*I site in a galactose-inducible centromeric vector p414GALS. The genes for deletion mutants (residues 1–23, 1–80, 1–160, 1–240, and 1–360) and full-length (residues 1–644) of ecm10p were amplified by PCR from the yeast genomic DNA using ecm10F and 5′-ACGCG TCGACTTGGCTGGCTCACCAACTAAACGTT- 3′; 5′-A CGCGTCGACACGCTCTTCGCTAAGTAAGCCTCCG-3′; 5′-AC GCGTCGACACGCTCTTCGCTAAGTAAGCCTCCG-3′; 5′-ACG CGTCGACGATTTAACCTCAAAGATACCGTTAT-3′; 5′-ACGCG TCGACTTATTTTCTCTCCCGTTCTTAGTTTC-3′, respectively, digested with *Eco*RI and *Sal*I, and then inserted into the *Eco*RI–*Sal*I site in p414GALS-GFP. To construct vector plasmids for promoter assay in yeast, the β-galactosidase gene was amplified by PCR from pβgal-Basic (Clontech) and inserted into the multi-cloning site of p416GALS and the resultant plasmid was designated p416GALS-βgal. The promoter regions of ECM10 (∼0.7 kb) and SSC1 (∼0.4 kb) were amplified by PCR from the yeast genome DNA using primers 5′-CCGGAGCTCGTGCAACAGGTCAAGATTGCCTCTA AATGA- 3′ and 5′-CGGGATCCGTTACTTGAATTTAAGTTCTT AATTATGACCAGTG-3′, 5′-CCGGAGCTCACCGCAACTAACA TCTGCTTATATTAAACT-3′ and 5′-CGGGATCCCTTGGTGCGT ATAATATGAGATTTGT GTAG-3′, respectively, digested with *Sac*I and *Bam*HI, and then replaced with the *Sac*I–*Bam*HI fragment containing GAL1 promoter in p416GALS-βgal.

Ecm10p expression in yeast cells. For limited expression of ecm10p, yeast cells (ATCC96099) harboring p416GALS-ecm10p, or p414GALS-ecm10p::GFP derivatives were grown to ∼0.2 OD_{600} at 30 °C in a synthetic medium SR [0.67% yeast nitrogen base (without amino acids), 0.5% casamino acids, and 2% raffinose] supplemented with 40 μg/ml L-tryptophan, or with 20 μg/ml uracil, respectively, and the expression of ecm10p was induced by incubation in a final concentration of 0.5% galactose at 30 °C for 2 h. To overexpress ecm10p, cells harboring p426GALS-ecm10p were grown at 30 °C for >8 h in SR containing 20 μg/ml uracil and 2% galactose.

Fluorescence microscopy. Cells were fixed with 4% formalin at 30 °C for 1 h, washed with PBS, and spheroplasted with 20 μg/ml Zymolyase 20T (Seikagaku) in 1 M sorbitol, 20 mM KPi, pH 7.0, and 30 mM β-mercaptoethanol at 30 °C for 15 min. Washed spheroplasts were transferred onto a glass-bottom dish coated with poly L-lysine and then permeabilized by incubation in methanol at −20 °C for 6 min and in acetone at −20 °C for 30 s. Cells were air-dried and blocked with 3% BSA containing phosphate-buffered saline. Ecm10p was detected with anti-ecm10p antibody as the first antibody and Alexa Fluor 588 goat anti-rabbit IgG antibody as the second antibody. Cells harboring plasmids containing full-length or C-terminally truncated ecm10p-GFP fusion proteins were grown in SR supplemented with 40 μg uracil. To stain the nuclear and mitochondrial DNA or mitochondria, cells were incubated with 1 μM of 4′,6-diaminidino-2-phenylindole (DAPI, Sigma) for 10 min or MitoTracker CM-H_2XROS (Molecular Probes) for 30 min. Microscopic observations were performed by Delta Vision Microscopy system (Applied Precision).

Promoter assay for ECM10 and SSC1. Cells harboring p416ECM10-βgal or p416SSC1-βgal were grown to OD_{600} = 0.4–5 in SD [0.67% yeast nitrogen base (without amino acids), 0.5% casamino acids, and 2% glucose] supplemented 40 μg/ml L-tryptophan, and these promotor activities were measured as previously described [10].

Isolation of mitochondrial nucleoids. Mitochondria were isolated from yeast ATCC24657 and purified by Nycodenz gradient centrifugation as previously described [11] except that the cells were grown to stationary phase. Mitochondrial nucleoids were purified according to Miyakawa et al. [12].

Results

Localization of ecm10p in mitochondrial nucleoids

Amino acid sequence of ecm10p has been reported [2] and it has 82% homology and 92% similarity with that of Ssc1p, which is most abundantly detected in yeast mitochondria. They are well conserved except for the mitochondrial targeting signal sequence of about 20 residues at the N-terminus and the variable region of about 15 residues at the C-terminus. Thus, anti-ecm10p specific polyclonal antibody was made against the C-terminal region, which exclusively recognizes ecm10p but does not cross-react with Ssc1p. Western blot analysis with the antibody revealed that ecm10p was exclusively detected in mitochondrial matrix fraction (Fig. 1) as previously reported [2].

Indirect immunofluorescent microscopy with anti-ecm10p antibody failed to detect any signals in yeast cells, because ecm10p is not an abundant protein as previous experiments suggested. Thus, about 700 bp promoter regions upstream of ORF of ecm10p as well as approximately 400 bp upstream of SSC1 were subcloned and promoter activities were compared. As a result, the promoter activity of ecm10p was about 1/300 in comparison with that of Ssc1p (data not shown).

To investigate its subcellular localization of ecm10p more in detail, we established yeast cell line stably expressing ecm10p at a low level. To suppress a possible cytotoxic effect or an aberrant subcellular localization, conventional GAL1 promoter was partly modified in the

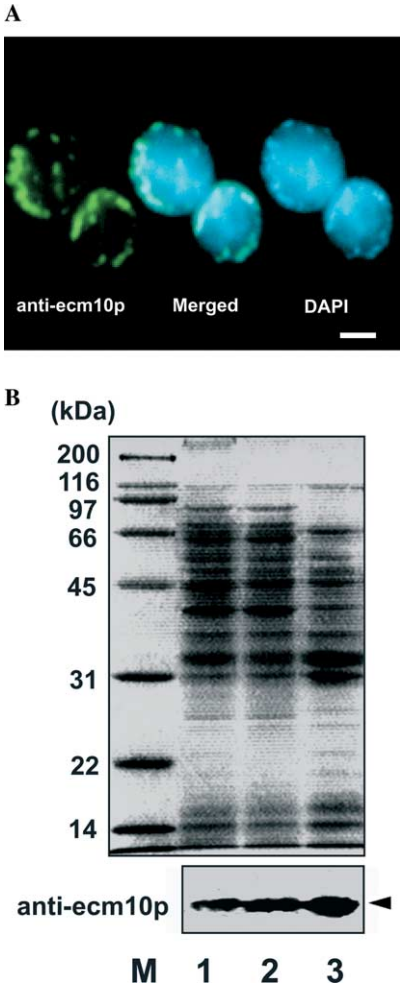

Fig. 1. Ecm10p colocalizes with mitochondrial nucleoids. (A) Ecm10p was expressed for 2 h under the control of the GAL1 promoter in yeast cells in a 0.5% galactose containing medium. Ecm10p was detected with anti-ecm10p antibodies as the first antibody and Alexa 488-conjugated anti-rabbit IgG as the secondary antibody (green). Nuclear and mitochondrial DNAs were stained with DAPI (blue). Left, anti-ecm10p; center, merged. Right, DAPI. Bar is 2 μm. (B) Mitoplasts were prepared by osmotic shock from highly purified mitochondria, solubilized in 2% Nonidet P40, and subjected to the stepwise sucrose density gradient ultracentrifugation. Nucleoids were collected from the interphase between 20% and 40% sucrose. Each fraction was separated by a 12.5% SDS–PAGE gel and visualized with CBB staining (upper panel) or anti-ecm10p antibody using ECL-plus (Amersham Bioscience) in Western blot (lower panel). Lane 1, mitochondria (20 μg); lane 2, mitoplasts (20 μg); and lane 3, mitochondrial nucleoids (5 μg). M shows molecular weight marker proteins.

expression plasmid and the expression was induced by 0.5% galactose. Yeast cells were fixed, spheroplasted, permeabilized with ice-cold methanol and acetone, and observed by an indirect immunofluorescent microscope. Ecm10p did not disperse over the string-shaped mitochondrial matrix but was focally distributed in a dotted pattern (Fig. 1A). Since such distribution pattern resembles that of mitochondrial DNA, DAPI staining was also performed. Subsequently, both staining patterns were completely merged, indicating that ecm10p localizes in the mitochondrial DNA complex designated nucleoids.

To confirm such subcellular localization of ecm10p biochemically, fraction of mitochondrial nucleoids was purified from yeast cells. Briefly, purified yeast mitochondria were solubilized in Nonidet P40 and its supernatant after high-speed centrifugation was further fractionated through a sucrose gradient centrifugation. Mitochondrial nucleoids recovered in the interface between 20% and 40% sucrose were diluted and further precipitated in pellets by another ultracentrifugation. SDS–PAGE and Coomassie brilliant blue (CBB) staining detected approximately 20 different polypeptides in the final fraction as previously reported [12,13] (Fig. 1). We estimated that mitochondrial nucleoids were highly purified in the final fraction, in which mitochondrial porin was not detected by Western blot analysis (data not shown). Finally, Western blot analysis with anti-ecm10p antibody exclusively detected ecm10p in the mitochondrial nucleoid fraction (Fig. 1) and confirmed that the band at 70 kDa was ecm10p in the CBB-stained gel.

Mitochondrial nucleoid-targeting signal in the ATPase domain

Such subcellular distribution of ecm10p immediately prompted us to identify a mitochondrial nucleoid-targeting signal, if any, of ecm10p. GFP-fused chimeric constructs with the full cDNA sequence or serially truncated ecm10p were expressed in yeast cells and their subcellular localizations were observed by a fluorescent microscopies. As the N-terminal region of ecm10p bears a mitochondrial targeting signal to be cleaved off in mature form, we are tempted to assume that the mitochondrial nucleoid targeting of ecm10p seems to depend on about 15 residues in the C-terminus which are highly variable among ecm10p and Ssc1p. This assumption is initially supported by the fact that 23 residues at the N-terminus of ecm10p targeted GFP-fusion proteins to mitochondrial matrix in a dispersed pattern. Inversely, the truncated ecm10p without the 23 residues at the N-terminus resided in cytosol, suggesting that this region works as a mitochondrial targeting signal but not a mitochondrial nucleoid-targeting signal. To further identify the responsible region for nucleoid localization, we constructed a series of deletion mutants (Fig. 2). To our surprise, all GFP-fused truncated ecm10p constructs with residues 1–644, 1–320, and 1–240 successfully localized in mitochondrial nucleoids, but those with residues 1–160 and 1–23 were targeted to mitochondrial matrix, indicating that the mitochondrial nucleoid-targeting signal resides not at the C-terminal variable region but at amino acid residues between 161 and 240 in the ATPase domain (Fig. 2).

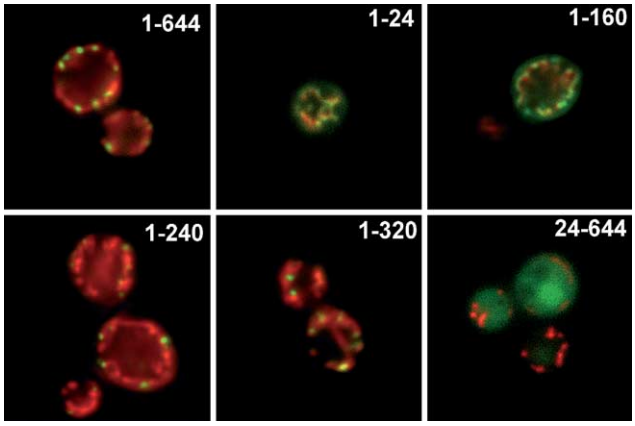

Fig. 2. Ecm10p bears a mitochondrial nucleoid-targeting signal. GFP-fused ecm10p constructs were limitedly expressed in yeast cells at 30 °C for 2 h under GAL1 promoter. Live cells were incubated with 1 μM MitoTracker Red CM-H_2XROS for 30 min and observed using Delta vision microscopy system (Applied Precision). Green: ecm10p-GFP derivatives, red: mitochondria. Scale bar is 2 μm.

Aggregation of mitochondrial nucleoids in ecm10p overexpressing yeast cells

Investigation of ECM10 gene-disrupted strain displayed no obvious alterations of mitochondrial nucleoids in vivo except that the growth rate was reduced by 85%. On the other hand, ECM10 gene-overexpressing strain formed extensive aggregations of mitochondrial nucleoids in the cells (Fig. 3). In such ecm10p overexpressing cells, only several mitochondrial meganucleoids were observed, whereas several dozens of mitochondrial nucleoids were usually observed in normal cells.

Discussion

Yeast mitochondria display a string-like profile at the proliferative phase, and 30–40 spheric mitochondrial nucleoids with about 5 μm in diameter were dispersed in each yeast cell. One yeast mitochondrial nucleoid is known to bear 4–5 copies of mitochondrial DNAs with about 20 kinds of yet poorly uncharacterized polypeptides [12,13]. Among them, a 20-kDa polypeptide was identified as ABF1, a basic DNA-binding protein involved in stabilizing mitochondrial DNA [14]. In addition, MIP1 (mitochondrial γ-DNA polymerase) [15], MSH1 (exonuclease) [16], PIF1 (mitochondrial DNA helicase) [17], MGT1 (mitochondrial DNA recombinase) [18], and MGM101 (mitochondrial DNA repair enzyme) [19] have also been identified.

We demonstrate for the first time that ecm10p localizes in mitochondrial nucleoids as its major component. Indirect immunofluorescent microscopy revealed that ecm10p distributed in a dotted pattern which merged with mitochondrial DNA, and GFP-fused ecm10p constructs also successfully localized in mitochondrial nucleoids. In addition, Western blot analysis with anti-ecm10p antibody further confirmed that ecm10p was concentrated in the highly purified mitochondrial nucleoid fraction.

We also located the mitochondrial nucleoid-targeting signal at amino acid residues 161–240 in its ATPase domain based on the experiments with a series of deletion mutants. Although another mitochondrial HSP70 family member, Ssc1p, has a highly homologous ATPase domain structure, only ecm10p is selectively targeted to mitochondrial nucleoids. Tim44 in mitochondrial inner

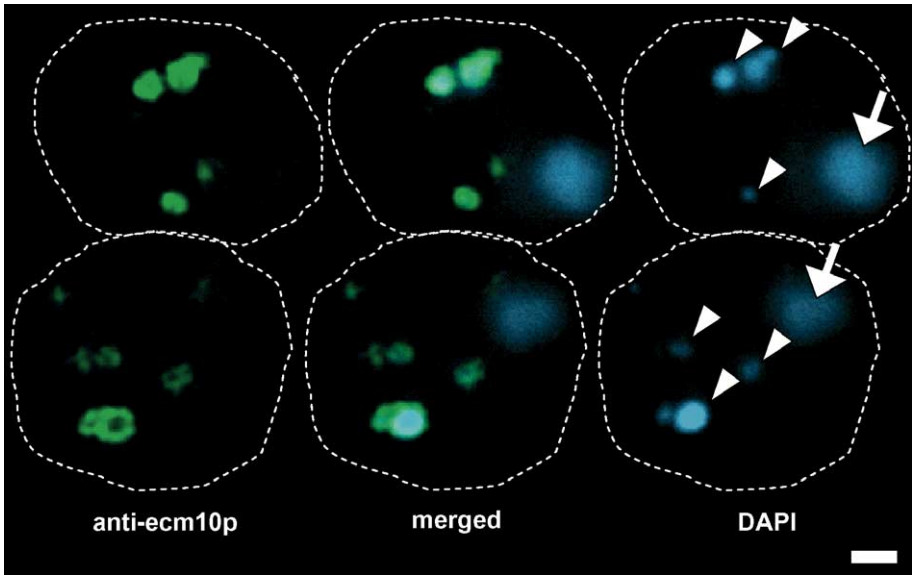

Fig. 3. Overexpression of ecm10p causes an aggregation of mitochondrial nucleoids. Ecm10p was overexpressed in yeast cells at 30 °C for >8 h under GAL1 promoter. Ecm10p and nucleoids were detected as shown in Fig. 1. Green: ecm10p, blue: nuclear DNA (arrow), and mitochondrial nucleoids (arrow head). Scale bar is 1 μm.

membrane is known to bind the ATPase domain of Ssc1p, whereas it cannot bind ecm10p since the peptide-binding domain of ecm10p as well as Ssq1p works against the interaction with Tim44 [20]. This might be relevant to such distribution of ecm10p in mitochondrial nucleoids. There are seven different residues between ecm10p and Ssc1p (A174S, I186V, D207E, E210N, P211S, I214V, and I235V) in the ATPase domain region. Hence, these residues especially at A174S and/or P211S, where most drastic substitutions occur, might provide the mitochondrial nucleoid-targeting signal.

When ecm10p was overexpressed in yeast cells, extensive aggregations of mitochondrial nucleoids were observed. This might be probably because overexpression of ecm10p induces aberrant mitochondrial DNA cleavages to cause and accelerate such aggregations. Morishima et al. [9] reported that *Sce*I of *S. cerevisiae* exhibits a heterodimeric site-specific endonuclease activity through its ATPase domain. Actually, the larger subunit of *Sce*I is identical to that of Ssc1p (mitochondrial matrix protein), which is also highly homologous to ecm10p (mitochondrial nucleoids protein) at the ATPase domain. Taking into account the fact that *Sce*I localizes in mitochondrial nucleoids, ecm10p rather than Ssc1p might be involved in modulating the activity of *Sce*I. However, it is difficult to confirm such assumption because Ssc1p is an extremely abundant protein, of which the deletion is lethal, whereas ecm10p is not. Finally, ecm10p might function as a molecular chaperone as it is a member of the HSP70 family, which is yet to be further investigated.

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