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Direct interaction between EFL1 and SBDS is mediated by an intrinsically disordered insertion domain

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ABSTRACT

Removal of anti-association factor, Tif6 (eIF6), by elongation factor-like 1 (EFL1) and Shwachman–Bodian–Diamond syndrome (SBDS) protein is a critical step in the late stage of ribosome maturation. Although EFL1 is known to have GTPase activity that is stimulated by SBDS, how they cooperatively trigger dissociation of Tif6 from the ribosome remains to be elucidated. In the present study, the interaction between EFL1 and SBDS was analyzed by size exclusion chromatography, gel shift assay, and isothermal titration calorimetry (ITC). The results showed that EFL1 interacted directly with SBDS. ITC experiments using domain-truncated mutants showed that the interaction between EFL1 and SBDS is governed by the insertion domain of EFL1 and domains II–III of SBDS. Circular dichroism spectroscopy showed that the insertion domain of EFL1 has a random structure in the absence of SBDS, whereas the disadvantageous entropy change observed on ITC suggested a fixed conformation coupled with complex formation with SBDS. Based on these observations together with those reported previously, we propose roles of EFL1 and SBDS in ribosomal maturation.

1. Introduction

Shwachman-Diamond syndrome is an autosomal recessive disorder characterized by hematological dysfunction, pancreatic exocrine insufficiency, skeletal abnormalities, and short stature [1]. Approximately 90% of Shwachman–Diamond syndrome cases are caused by mutations in the Shwachman–Bodian–Diamond syndrome (*SBDS*) gene [2], which encodes a protein of approximately 250 amino acid residues. Orthologs of SBDS have been found in archaea, plants, and other eukaryotes. High-throughput affinity-capture mass spectrometry experiments identified potential interactions between SBDS and ribosome biogenesis factors [3], one of which was GTPase elongation factor-like 1 (EFL1). Genetic studies in *Saccharomyces cerevisiae* indicated that SBDS and EFL1 function cooperatively in a pathway to release the essential nucleolar factor, Tif6, from the late cytoplasmic pre-60S ribosomal subunit [4]. The removal of Tif6—the yeast homolog of mammalian eukaryotic translation initiation factor 6 (eIF6)—is critical for late cytoplasmic maturation of the 60S ribosomal subunit [5]. Tif6 acts as a ribosomal anti-association factor, which binds to the pre-60S subunit to inhibit subunit joining by steric hindrance [6–8]. Therefore, dissociation of Tif6 from pre-60S ribosome is essential for enabling assembly into the 80S subunit. Biochemical analysis showed that 60S-ribosome dependent GTP hydrolysis of EFL1 was stimulated by SBDS, and SBDS and EFL1 directly catalyzed Tif6 removal by a mechanism that required hydrolysis of GTP by EFL1 [9]. However, it remains to be elucidated how EFL1 and SBDS cooperatively trigger dissociation of Tif6 from the ribosome.

SBDS is composed of three domains (Supplementary Fig. S1), and missense mutations of SBDS associated with Shwachman-Diamond syndrome were identified in all three domains [8]. Nuclear magnetic resonance (NMR) spectroscopy identified domain I of SBDS as an RNA binding site [10]. Moreover, it was reported that two mutants in domain II (R126T and K151N in human) were defective in triggering Tif6 release [9]. However, the roles of domains II and III were unclear. On the other hand, EFL1 shares 26.8% sequence identity with translation elongation factor 2 (EF2), and these two proteins share a ribosome binding site [11]. EFL1 triggers release of Tif6 from the pre-60S ribosome, whereas EF2 assists in the translocation of tRNA and mRNA from the A-site to the P-site of the ribosome. There is a marked difference in domain composition between these proteins, i.e., insertion of an extra ~150-residue domain in EFL1. ELF1 and EF2 are commonly composed of domains G, G′, and II–V, but only EFL1 has the insertion region within domain II (Fig. S2) [12]. Although the insertion domain is expected to be an important determinant of the function, the details are still unclear.

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Here, we performed interaction analysis of EFL1 and SBDS. The results showed that EFL1 binds directly with SBDS, in which the insertion region of EFL1 and domain II–III of SBDS dominate the interaction. The results of spectroscopic analysis taken together with the thermodynamic properties suggested conformational changes in the insertion region of EFL1 coupled with the interaction. Based on these observations, we discuss the significance of the interaction between EFL1 and SBDS for Tif6 removal.

2. Materials and methods

2.1. Plasmid construction

The gene encoding EFL1 was amplified by PCR from *S. cerevisiae* genomic DNA using the primers EFL1-S-1 and EFL1-AS-1 (Supplementary Table S1), and inserted into the *Sbf*I–*Asc*I sites of a modified pET26b vector (pECO-H2). In the resultant plasmid, a His-tag was attached to the N-terminus of the EFL1 gene. The plasmid for EFL1-ΔIns (deletion mutant of the insertion region encoding residues 418–577) was constructed by the inverse PCR method using the EFL1 expression vector as the template and primers EFL1-S-2 and EFL1-AS-2. The DNA fragment for the insertion domain of EFL1 (EFL1-Ins; encoding residues 419–577) was amplified using the primers EFL1-S3 and EFL1-AS-3, followed by insertion into the *Sbf*I–*Asc*I sites of a modified pET26b vector (pECO-GH1). In the resultant plasmid, a GST-tag followed by a TEV protease site and a His-tag were fused at the N-terminus and C-terminus, respectively.

The gene encoding SBDS was amplified by PCR from *S. cerevisiae* genomic DNA using the primers SBDS-S-4 and SBDS-AS-4, and inserted into the *Nde*I–*Xho*I sites of a modified pET28b vector (pDBHT-2), in which a His-tag was fused at the N-terminus. The coding sequences of SBDS domain I (encoding residues 1–94), domain II (encoding residues 95–172), domain III (encoding residues 173–250), domain I–II (encoding residues 1–172), and domain II–III (encoding residues 95–250) were amplified separately by PCR with the expression vector of SBDS as the template and the primers shown in Supplementary Tables S1 and S2. The amplified DNA fragments encoding domains I, II, and I–II were inserted into the *Nde*I–*Xho*I sites of a modified pET28b vector (pET28M), in which His-tag was fused at the N-terminus. The DNA fragments encoding domain III and II–III were inserted into the *Nde*I–*Xho*I sites of the pET26b vector, in which His-tag was attached to the C-terminus.

2.2. Protein expression and purification

Escherichia coli strain B834 (DE3) harboring the expression vector and pRARE2 was grown at 37 °C in LB medium supplemented with 25 μg/mL kanamycin and 34 μg/mL chloramphenicol until the OD_{600} reached 0.6. To induce expression of the desired protein, IPTG was added at a final concentration of 0.25 mM. After incubation at 25 °C for a further 18 h (exceptionally, for the expression of EFL1, 15 °C for 24 h), cells were harvested by centrifugation at 4500×*g* for 10 min at 4 °C. Cells expressing EFL1 and the mutants were resuspended in 50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 1 mM $MgCl_2$, 10% (v/v) glycerol, 1 mg/mL lysozyme, and 0.1 mg/mL DNase I. Cells expressing SBDS and the mutants were resuspended in 50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 10% (v/v) glycerol, 1 mg/mL lysozyme, 0.1 mg/mL DNase I, and 0.1 mg/mL RNase A. Resuspended cells were disrupted by sonication, followed by centrifugation at 40,000×*g* for 1 h at 10 °C.

EFL1 and EFL1-ΔIns were purified on a HisTrap HP column (GE Healthcare) and HiLoad 16/60 Superdex 200-pg column (GE Healthcare). EFL1-Ins was purified on a HisTrap HP column (GE Healthcare), followed by removal of the GST-tag by digestion with TEV protease. EFL1-Ins without the GST-tag was further purified on a HisTrap HP column and HiLoad 16/60 Superdex 200-pg column.

SBDS and its truncated mutants were purified on a HisTrap HP column and HiLoad 26/60 Superdex 75-pg column. Exceptionally, SBDS-domain I was purified by three steps using a HisTrap HP column, HiTrap Heparin HP column, and HiLoad 26/60 Superdex 75-pg column.

2.3. Gel filtration analyses

Aliquots of 150 μL consisting of 2.5 nmol EFL1 and 8.5 nmol SBDS were loaded onto a HiLoad 10/300 Superdex 200-pg column (GE Healthcare) pre-equilibrated with 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 5% (v/v) glycerol, and 5 mM β-mercaptoethanol. Control experiments using each protein were also performed under the same conditions. Peak fractions were analyzed by SDS–PAGE, followed by staining with Coomassie brilliant blue R-250.

2.4. Gel shift assay

Gel shift assay was performed in 5-μL reaction mixtures containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, and the desired amounts of EFL1 and SBDS. Reaction mixtures were loaded onto a 3–10% native gradient polyacrylamide gel (PAGEL NPG-310L; ATTO). Electrophoresis conditions were as follows: temperature, 4 °C; power voltage, 100 V; and electrophoresis buffer, 50 mM Tris-MES (pH 8.0) and 10 mM Mg $(OAc)_2$. Proteins were visualized using SYPRO® Ruby Protein gel stain.

2.5. Isothermal titration calorimetry

All isothermal titration calorimetry (ITC) measurements were carried out with a VP-ITC System (MicroCal). Proteins were dialyzed against a buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 10% (v/v) glycerol, and 5 mM β-mercaptoethanol at 4 °C. All measurements were conducted at 30 °C and protein solutions were degassed under vacuum prior to use. The cell was filled with ~5 μM full-length EFL1, EFL1-ΔIns or ~2.5 μM EFL1-Ins, and a syringe was filled with ~50 μM full-length SBDS or each truncated SBDS. The solution of SBDS was injected 25 times in portions of 10 μL over 20 s. The data were analyzed using the program ORIGIN (MicroCal).

2.6. Circular dichroism measurements

Circular dichroism (CD) spectra were measured on a JASCO J-720 spectropolarimeter (JASCO) in a quartz cell with an optical path length of 2 mm. The CD spectra were obtained by taking the average of four scans made from 300 to 190 nm and normalized to molar ellipticities by protein concentrations.

2.7. Model building of EFL1-Tif6-bound ribosome

The binding position of Tif6 on EF2-60S complex was obtained by superposing 60S ribosome subunits of eIF6(Tif6)-60S (PDB code: 4A18 [13]) and EF2-60S complex structure (PDB code: 1S1H and 1S1I [14]) using the program PyMoL (The PyMOL Molecular Graphics System, Schrödinger, LLC, New York, NY). The final model of the EFL1-Tif6-bound 60S ribosome subunit was built by superposing the crystal structure of Tif6 (PDB code: 1G62 [6]) onto the Tif6-EF2-60S model [15].