Defective Guanine Nucleotide Exchange in the Elongation Factor-like 1 (EFL1) GTPase by Mutations in the Shwachman-Diamond Syndrome Protein*

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**Background:** EFL1 and the protein mutated in the Shwachman-Diamond syndrome (SBDS) participate in the maturation of the 60S ribosomal subunit.

**Results:** SBDS increases the dissociation constant of EFL1 for GDP 60-fold. SBDS S143L disease mutation disrupts the interaction with EFL1.

**Conclusion:** SBDS favors GDP dissociation from EFL1 and disease mutations abolish this function.

**Significance:** SBDS disease mutations prevent the nucleotide exchange regulation on EFL1.

Ribosome biogenesis is orchestrated by the action of several accessory factors that provide time and directionality to the process. One such accessory factor is the GTPase EFL1 involved in the cytoplasmatic maturation of the ribosomal 60S subunit. EFL1 and SBDS, the protein mutated in the Shwachman-Diamond syndrome (SBDS), release the anti-association factor eIF6 from the surface of the ribosomal subunit 60S. Here we report a kinetic analysis of fluorescent guanine nucleotides binding to EFL1 alone and in the presence of SBDS using fluorescence stopped-flow spectroscopy. Binding kinetics of EFL1 to both GDP and GTP suggests a two-step mechanism with an initial binding event followed by a conformational change of the complex. Furthermore, the same behavior was observed in the presence of the SBDS protein irrespective of the guanine nucleotide evaluated. The affinity of EFL1 for GTP is 10-fold lower than that calculated for GDP. Association of EFL1 to SBDS did not modify the affinity for GTP but dramatically decreased that for GDP by increasing the dissociation rate of the nucleotide. Thus, SBDS acts as a guanine nucleotide exchange factor (GEF) for EFL1 promoting its activation by the release of GDP. Finally, fluorescence anisotropy measurements showed that the S143L mutation present in the Shwachman-Diamond syndrome altered a surface epitope for EFL1 and largely decreased the affinity for it. These results suggest that loss of interaction between these proteins due to mutations in the disease consequently prevents the nucleotide exchange regulation the SBDS exerts on EFL1.

Ribosomes are the molecular ribonucleoprotein machines responsible for protein synthesis. The eukaryotic ribosome is composed of two subunits, the large 60S subunit and the small 40S subunit. The yeast large ribosomal subunit consists of 46 ribosomal proteins and three rRNA (5.8S, 5S, and 25S), whereas the small subunit contains only one rRNA (18S) and 33 ribosomal proteins. Cells commit a large amount of resources to assemble ribosomes, as not only the four rRNAs and the 79 proteins that conform the mature ribosome are necessary. In yeast, an additional 200 accessory proteins and 75 small ribonucleoproteins have been shown to participate in the process (1–3). Ribosome biogenesis is closely linked to growth and cell proliferation and dysregulation of the process causes several diseases collectively known as ribosomopathies such as the Shwachman-Diamond syndrome (SBDS)3 (4, 5). The SBDS protein mutated in this disease participates with EFL1 in the cytoplasmatic maturation of the 60S subunit. SBDS couples the energy liberated from the hydrolysis of GTP by EFL1 to release eIF6 from the surface of the 60S ribosomal subunit (6, 7). The eIF6 protein prevents the premature association of the ribosomal subunits by binding to the B6 intersubunit bridge and additionally contacting the sArc-rinic loop, Rpl23 and Rpl24 (8, 9). EFL1 belongs to the P-loop family of GTPases and is homologous to the elongation factor EF-G/EF-2 (10). EFL1 probably removes eIF6 from the 60S surface through a conformational change similar to that triggered by EF-2 during elongation in the protein synthesis. Additionally, EFL1 also tests the functionality of the newly synthesized 60S subunits before they enter the pool of translating ribosomes by means of a translational-like activity (11). As a GTPase, EFL1 has a conserved G-domain in domain 1 composed of the five structural motifs (G1–G5) responsible for the binding of guanine nucleotides. The G1 motif binds strongly to the α- and β-nucleotide phosphate, whereas the G4 and G5 regions contact the guanine moiety and the ribose ring. The G2 (switch 1) and G3 (switch 2) motifs allow the GTPase to discriminate between GTP and GDP. In GTPases, these regions undergo large conformational

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3 The abbreviations used are: SBDS, Shwachman-Diamond syndrome; Gpp(NH)p, guanosine 5′-O-(β,γ-imido)triphosphate; mant, 2′,3′-O-(N-methyl-antraniloyl); GEF, guanine nucleotide exchange factor.
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changes during the functional cycle of the enzyme that alternates from an inactive state bound to GDP to an active state in complex with GTP (12, 13). In addition, EFL1 has four other domains expected to be very similar to those described for EF-2 except for an unstructured insertion of 160 residues within domain 2 that participates in the interaction with SBDS (14). The function of these domains are unknown but they might undergo large conformational changes similar to those observed for EF-2 during translocation (11). The activity of the GTPases can be modulated by effector biomolecules such as those that accelerate the hydrolysis rate of GTP know as guanine nucleotide activating proteins or by guanine nucleotide exchange factors (GEFs). The latter can be further subdivided in 1) factors that favor the release of bound GDP and 2) factors that stabilize the interaction with GTP (15). Recently, we have shown that the interaction of EFL1 with SBDS resulted in a decrease of the Michaelis-Menten (\(K_m\)) constant for GTP and thus SBDS acts as a GEF for EFL1 (16). However, that work did not address which binding constant the SBDS protein modifies. Besides, the \(K_m\) is a constant composed of several microscopic rate constants obtained from enzyme kinetics experiments and not always reflect the corresponding equilibrium dissociation constant. In this work we measured the binding kinetics of GDP and GTP to EFL1 alone and in the presence of SBDS. The affinity of EFL1 for GDP is an order of magnitude greater than for GTP, however, in complex with SBDS the corresponding dissociation constant decreased 60-fold. These results established that SBDS is a guanine nucleotide exchange factor for EFL1 that favors the release of GDP. We found that the SBDS S143L mutation present in patients with Shwachman-Diamond syndrome disrupted the binding to EFL1 and thus prevented the regulation SBDS exerts on the affinity of EFL1 toward guanine nucleotides. Additionally, the use of pre-steady state kinetics allowed us to obtain mechanistic information on the binding process.

Experimental Procedures

Chemicals—The fluorescent mant-derivatives of GDP, GTP, dGDP, dGTP, and GppNHp were obtained from Jena Biosciences (Jena, Germany). All other chemicals, including GDP and GTP sodium salts were purchased from Sigma. Purity of GDP and GTP was assessed using the method described in Ref. 17 and was found to be ~98.5% for both nucleotides.

Protein Expression and Purification—Human and Saccharomyces cerevisiae SBDS recombinant proteins were expressed in Escherichia coli C41 and purified as described in Ref. 18. Human and S. cerevisiae recombinant EFL1 proteins were expressed in S. cerevisiae BCY123 and purified as described in Ref. 6. The yeast EFL1 protein was also expressed in bacteria and purified as described in Ref. 14. After purification, the yield of the proteins corresponded to 10–12 mg of protein/liter of culture for the SBDS proteins, 7 mg of protein/liter of culture for the S. cerevisiae EFL1 produced in yeast, 0.5 mg of protein/liter of culture for the S. cerevisiae EFL1 produced in bacteria, and 4 mg of protein/liter of culture for the human EFL1 produced in yeast. The EFL1 protein used for the experiments presented in this work corresponds to that expressed in yeast except when specifically stated. For the anisotropy experiments, a FlAsH tag corresponding to the sequence Cys-Cys-Pro-Gly-Cys-Cys (19) was added to the C terminus of the human SBDS coding sequence by PCR. The disease mutation S143L was introduced using the QuickChange Site-directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s specification. The human SBDS protein was labeled with the chemical Lumio™ Green (4’,5’-bis(1,3,2-dithiaro-solan-2-yI)fluorescein) (Invitrogen). Briefly, 3 nmol of Lumio™ Green were mixed with 3 nmol of protein at 4 °C for 8 h. Subsequently the sample was dialyzed against buffer C containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5 mM mercaptoethanol to remove the free dye. The amount of labeled protein was calculated spectrophotometrically using the molar extinction coefficients of 11,460 M\(^{-1}\) cm\(^{-1}\) for SBDS at 280 nm, and 41,000 M\(^{-1}\) cm\(^{-1}\) and 12,500 M\(^{-1}\) cm\(^{-1}\) for the Lumio™ Green dye at 508 and 280 nm, respectively.

FRET Stopped-flow Kinetic Measurements—The experiments were carried out at 28 °C in buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM MgCl\(_2\), 10% glycerol), except for the experiments in the absence of Mg\(^{2+}\) ions that used buffer B (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA, 10% glycerol). Fluorescence stopped-flow experiments were done using an sx.18MV stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, UK). The mant-nucleotides used in this study were excited via FRET from the tryptophan residues in the protein at 290 nm and measured after passing a 400-nm cut-off filter. Data were evaluated by fitting to a double-exponential function. The values of the individual rate constants, \(k_{+1}\), \(k_{-1}\), \(k_{+2}\), and \(k_{-2}\) were calculated from the \(k_{app,1}\) and \(k_{pp,2}\) values by plotting their concentration dependences as described in Ref. 20. The slope of the straight line fitted to the data points defines the association rate constants, \(k_{+1}\) and \(k_{+2}\), and the intercept with the y axis yields the dissociation rate constants, \(k_{-1}\) and \(k_{-2}\). Dissociation rate constants were more accurately determined by a displacement experiment in which a 20-fold molar excess of unlabeled GDP was used to displace the mant-GDP from the complex with EFL1. Displacement experiments were not done for mant-GTP. The overall dissociation constants were calculated as \(K_d = (k_{-1},k_{-2})/(k_{+1},k_{+2})\). Competition binding kinetics were done in buffer A at 28 °C by mixing 10 μM EFL1 with a solution containing both 80 μM mant-GDP and increasing concentrations of unmodified GDP.

Fluorescence Anisotropy Measurements—Human EFL1 (30 μM) was titrated into 15 nM FlAsH-labeled SBDS wild-type or SBDS S143L mutant in buffer C. Measurements were made at 25 °C in a OLIS SLM8000 spectrofluorimeter (Olis, Inc.) by excitation of the FlAsH tag at 495 nm and the anisotropy was measured through a filter with a cut-off of 535 nm. Slits for the excitation corresponded to 16 nm. For each data point, the mixture was stirred for 30 s and incubated for a further 5 min before measuring the fluorescence signal.

Results

Binding Kinetics of EFL1 to GDP and GTP—The interaction of EFL1 with GDP and GTP was studied at pre-steady state kinetics by stopped-flow FRET. Fluorescence changes of the mant nucleotides upon binding to EFL1 were monitored via indirect excitation of the tryptophan residues close to the
nucleotide-binding site that acted as fluorescence donors and the mant groups as fluorescent acceptors. Upon excitation at 290 nm, this energy transfer was evidenced as a simultaneous decrease in the tryptophan emission and an increase in mant fluorescence compared with that of the protein alone and the free mant nucleotide, respectively (data not shown). Yeast EFL1 contains a total of 9 tryptophans with 4 of them contained in the G-domain, however, it is difficult to establish which tryptophan residues are involved in the fluorescence energy transfer process. Binding kinetics was measured under pseudo-first order conditions at $4\mu M$ EFL1 and 20 to 125 $\mu M$ mant nucleotides.

Time courses could be fitted by a double-exponential term, yielding two pseudo-first order rate constants, $k_{app,1}$ and $k_{app,2}$, which increased linearly on the mant nucleotide concentration (Fig. 1 and Fig. 2). This behavior was observed for all the transients obtained irrespective of the mant nucleotide studied or the presence of the SBDS protein. Additionally, in displacement experiments, the dissociation of mant-GDP was also biphasic. These two-exponential time courses could result from either a two-step binding mechanism or heterogeneity of the mant nucleotides or protein sample. The EFL1 protein used in this study was expressed in yeast, so it is conceivable that protein heterogeneity arising from post-translational modifications may account for the double exponential observed. To rule out

**FIGURE 1. Binding of mant-GDP to EFL1 alone or in complex with SBDS.** Mant fluorescence was excited by FRET from the intrinsic tryptophan residues in EFL1. Concentrations after mixing consisted of $4\mu M$ EFL1, 10 $\mu M$ SBDS, 50 $\mu M$ mant-GDP, and 5 mM Mg$^{2+}$ as relevant for the corresponding experiment. Gray traces correspond to the fit to a double exponential equation. A, association of mant-GDP to EFL1. B, dissociation of mant-GDP from EFL1 with 500 $\mu M$ GDP. C, concentration dependence of $k_{app}$ values for mant-GDP association to EFL1; circles, $k_{app,1}$; squares, $k_{app,2}$. D, association of mant-GDP to EFL1 in the absence of Mg$^{2+}$. E, dissociation of mant-GDP from EFL1 with 500 $\mu M$ GDP in the absence of Mg$^{2+}$. F, concentration dependence of $k_{app}$ values for mant-GDP association to EFL1 in the absence of Mg$^{2+}$; circles, $k_{app,1}$; squares, $k_{app,2}$. G, association of mant-GDP to the EFL1-SBDS complex. H, dissociation of mant-GDP from the EFL1-SBDS complex with 500 $\mu M$ GDP. I, concentration dependence of $k_{app}$ values for mant-GDP association to the EFL1-SBDS complex; circles, $k_{app,1}$; squares, $k_{app,2}$.
FIGURE 2. Binding of mant-GTP to EFL1 alone or in complex with SBDS. Mant fluorescence was excited by FRET from the intrinsic tryptophan residues in EFL1. Concentrations after mixing consisted of 4 μM EFL1, 10 μM SBDS, 50 μM mant-GTP/Gpp(NH)p, and 5 mM Mg²⁺ as relevant for the corresponding experiment. Gray traces correspond to the fit to a double exponential equation. A, association of mant-GTP to EFL1. B, concentration dependence of $k_{\text{app}}$ values for mant-GTP association to EFL1; circles, $k_{\text{app},1}$; squares, $k_{\text{app},2}$. C, association of mant-GTP to EFL1 in the absence of Mg²⁺. D, concentration dependence of $k_{\text{app}}$ values for mant-GTP association to EFL1 in the absence of Mg²⁺; circles, $k_{\text{app},1}$; squares, $k_{\text{app},2}$. E, association of mant-GTP to the EFL1-SBDS complex. F, concentration dependence of $k_{\text{app}}$ values for mant-GTP association to the EFL1-SBDS complex; circles, $k_{\text{app},1}$; squares, $k_{\text{app},2}$. G, association of mant-Gpp(NH)p to EFL1. H, concentration dependence of $k_{\text{app}}$ values for mant-Gpp(NH)p association to EFL1; circles, $k_{\text{app},1}$; squares, $k_{\text{app},2}$. 

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this possibility, we also expressed the protein in bacteria and obtained the corresponding transient for binding to mant-GDP (Fig. 3). Although a single exponential model did fit the binding data reasonably well over a wide range of time, the residuals of the fit (the difference between the model and the data) were appreciable and nonrandom. In contrast, the fit to a double exponential model showed no systematic deviation of the residuals supporting a two-step association model. Additionally, mant nucleotides are a 1:1 mixture of the 2’/H11032 and 3’/H11032 isomers, however, the double exponential kinetics could not be explained by preferential binding to one of the isomers because double exponential transients were also observed for the mant deoxy nucleotides that consist only of the 3’ isomer (Fig. 4).

Therefore, the double exponential transients most likely reflect a two-step binding mechanism consisting of an initial second-order binding event followed by a first-order isomerization of the EFL1/H18528 nucleotide complex. This mechanism has two kinetic phases with the fast binding event changing linearly with the total concentration of ligand and the slow isomerization process varying hyperbolically (20). Our results are in agreement with this description except that we could only observe the initial linear region of the hyperbola because experimentally it was not possible to reach a wider range of ligand concentration (Fig. 1, traces C, F, and I, and Fig. 2, traces B, D, F, and H).

The binding of EFL1 to mant-GDP was tested under different conditions (Fig. 1). In the presence of Mg2+/H11001 ions it has a Kd of 10 μM with an association rate constant for the first step of 0.9 μM−1 s−1, the rate constant of the following rearrangement was k+2 = 0.2 s−1, and the dissociation rate constants were 3.7 and 0.4 s−1, respectively (Table 1). Previous results in our group suggested that the SBDS protein increased the affinity of EFL1 for GTP as evidenced by a decrease on its Km value (16). If SBDS acted as GTP-stabilizing factor or GSF for EFL1 it would only modify the binding to GTP but not to GDP. To further elucidate the role of SBDS, we investigated the binding kinetics of guanine nucleotides to EFL1 in complex to SBDS. Binding of EFL1 to mant-GDP in complex with SBDS resulted in a dissociation constant of 620 μM, 60-fold higher than that observed for EFL1 alone. Thus, the interaction of EFL1 with SBDS greatly decreased the binding to mant-GDP mostly due to an increase of both dissociation rate constants that favor the release of the mant-GDP. The dissociation rate constants increased almost 4-
and 8-fold in the presence of SBDS compared with the values of \( k_{-1} \) and \( k_{-2} \) in its absence, respectively (Table 1). Competition experiments were carried out to exclude the influence of the fluorescent moiety of the mant-GDP in the aforementioned result (Fig. 5). In these experiments, EFL1 was mixed with a solution containing both mant-GDP and unmodified GDP. As previously shown, the binding to mant-GDP occurs in two steps with \( K_1 \) representing the equilibrium constant of the first step and \( k_2 \) the rate constant of the subsequent conformational change (\( k_{-2} \) can be neglected). Thus the apparent rate constant is defined as,

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k_{app} = k_2 \cdot K_1 \cdot [\text{mant-GDP}] + k_{GDPP} \cdot [\text{GDP}]
\]

with \( k_{GDPP} \) being the association rate constant of unmodified GDP (21).

In the competition experiments, the binding kinetics of mant-GDP is governed by the disappearance of the free binding site on EFL1 as it is occupied by the unmodified GDP. Thus, increasing the amount of unmodified GDP results in a decrease in the observed fluorescence signal because only mant-GDP binding contributes to the amplitude of the signal (Fig. 5A). The apparent rate constant follows the predicted linear increase and the slope value of \( 0.63 \pm 0.03 \text{ } \mu M^{-1} \text{ s}^{-1} \), corresponding to the bimolecular-binding rate constant for GDP, agrees well within experimental error to the \( 0.9 \pm 0.05 \text{ } \mu M^{-1} \text{ s}^{-1} \) value obtained for the association rate constant of mant-GDP (Fig. 5B). This suggests that the fluorescent modification of GDP does not modify the binding to EFL1. Furthermore, studies of the binding of EFL1 to unmodified guanine nucleotides obtained by isothermal titration calorimetry showed similar results to those presented here using a fluorescent approach.4

Binding of EFL1 to mant-GDP in the absence of \( \text{Mg}^{2+} \) ions showed that they are not necessary for the interaction as we could obtain transients different from a flat line. The traces could be fitted by a double exponential term (Fig. 1, traces D and E) with a calculated \( K_d \) value of 2 \( \mu M \) (Fig. 1F). This value indicates that in the absence of \( \text{Mg}^{2+} \) the affinity of mant-GDP to EFL1 was increased nearly five times because of both a small increase in the association rate constants and a small decrease in the dissociation rate constants (Table 1). The requirement of \( \text{Mg}^{2+} \) for the binding of GDP varies among G proteins, however, higher affinity for GDP in the absence of this ion has been observed for translation G proteins such as EF-Tu and eRF3.

The interaction of EFL1 to mant-GTP was also tested under different conditions (Fig. 2). The affinity of EFL1 to mant-GTP was almost 20 times lower than that for mant-GDP with a dissociation constant of 171 \( \mu M \). The lower affinity for mant-GTP resulted mainly from a slower association to the nucleotide although the rearrangement for the dissociation occurred slightly faster compared with the values observed for mant-GDP (Table 1). In the presence of SBDS, the binding of EFL1 for GTP was not altered and the corresponding microscopic rate constants were the same within the experimental error limits, suggesting that SBDS has no effect on the binding of GTP to EFL1. The corresponding dissociation constants were 171 and 231 \( \mu M \) in the absence and presence of SBDS, respectively.

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**TABLE 1**

Rate and dissociation constants of the interaction between mant-guanine nucleotides and EFL1 alone or in complex with SBDS

| Complex       | Nucleotide   | \( k_{+1} \) \( \mu M^{-1} \text{ s}^{-1} \) | \( k_{+2} \) \( \mu M^{-1} \text{ s}^{-1} \) | \( K_{d} \) \( \mu M \) |
|---------------|--------------|---------------------------------------------|---------------------------------------------|----------------------|
| EFL1          | Mant-GDP     | 0.9 ± 0.05 \( (0.63 ± 0.03)^{a} \)          | 0.17 ± 0.04                                | 0.4 ± 0.025          | 9.7 ± 3 |
| EFL1          | Mant-GTP     | 0.85 ± 0.04                                | 0.08 ± 0.005                              | 14 ± 3               | 618 ± 136 |
| EFL1          | Mant-GTP     | 0.42 ± 0.01                                | 0.02 ± 0.004                              | 2.4 ± 0.7            | 0.6 ± 0.1 | 171 ± 93 |
| EFL1          | Mant-GTP     | 0.36 ± 0.021                               | 0.05 ± 0.002                              | 5.2 ± 1.3            | 0.8 ± 0.2 | 231 ± 93 |
| EFL1          | Mant-GTP     | 0.85 ± 0.12                                | 0.5 ± 0.03                                | 45 ± 7               | 6.4 ± 2  | 677 ± 151 |
| EFL1(-Mg\(^{2+}\)) | Mant-GTP     | 1.3 ± 0.01                                | 0.4 ± 0.05                                | 3.1 ± 0.04           | 0.32 ± 0.04 | 1.9 ± 0.3 |
| EFL1(-Mg\(^{2+}\)) | Mant-GTP     | 0.25 ± 0.002                               | 0.026 ± 0.0003                           | 0.34 ± 0.2           | 4.6 ± 0.04 | 240 ± 67 |

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\(^{a}\) Bimolecular association rate constant for unmodified GDP obtained from competition experiments.
Although the GTP- and Mg\(^{2+}\)-binding sites are tightly coupled, in the absence of Mg\(^{2+}\) the affinity of EFL1 for GTP was only slightly altered with a dissociation constant of 240 \(\mu M\) compared with that of 171 \(\mu M\) in the presence of these ions. This discrete change resulted from a combination of a slower association rate compensated by a slower dissociation rate for the binding event (Table 1). The effect observed for the binding of GTP in the absence of Mg\(^{2+}\) was specific and not caused by the disruption of EFL1-fold. The far UV-CD spectra of the native protein purified in the presence of Mg\(^{2+}\) and that obtained after treatment with EDTA are identical suggesting that no changes in the EFL1 secondary structure content occurred (data not shown). Gpp(NH)p is commonly used as a non-hydrolysable analogue of GTP in functional and structural studies of GTPases. The binding of Gpp(NH)p to EFL1 was compared with that of GTP. The dissociation constant for Gpp(NH)p was 3.6-fold higher than that of GTP suggesting that binding was weaker for the analogue compared with that of the physiological substrate. Although these values may not be significantly different, the dissociation rate constants for both limiting steps and the rearrangement are almost 10 and 20 times greater for Gpp(NH)p than for GTP, respectively. This suggests that the weaker affinity of EFL1 for the substrate analogue results from a faster release. By inference, GTP and Gpp(NH)p most likely bind in different manners and the latter may be not such a good analogue of GTP. In fact, the \(k_d\) for each GTP analogue resembles that obtained for GDP in the presence of SBDS, 677 and 618 \(\mu M\), respectively, with the corresponding dissociation rate constants varying only 3-fold. The dissociation rate constants for the different GTP binding experiments were calculated from the y intercept of the apparent rate constant dependence on the nucleotide concentration and not directly from a dissociation experiment to avoid ambiguous results arising from the catalysis (\(k_{cat}\), 0.4 min\(^{-1}\)) (16). This had an impact on the fitting uncertainties because the \(k_{app}\) for the rearrangement were almost independent of the nucleotide concentration at the attainable experimental conditions (20).

Interaction of Human SBDS Disease Mutant and Human EFL1—The binding of human EFL1 to SBDS and disease mutant SBDS S143L was measured using fluorescence anisotropy. The SBDS constructs were labeled with the Lumio\(^{TM}\) Green dye and the experiments were set up to detect the fluorescent probe. The interaction between human EFL1 and wild-type SBDS is described by a typical binding curve that reaches saturation close to 6 \(\mu M\) EFL1 concentration. Interestingly, testing the interaction of EFL1 with the disease mutant SBDS S143L resulted in a steady linear increase of the fluorescence anisotropy signal. Addition of 12 \(\mu M\) EFL1 final concentration resulted in a very small but positive slope with an increase of 0.02 fluorescence anisotropy units enough to be detected above the limit of detection of the equipment. This suggested that some binding was occurring but the interaction between the proteins was very weak (Fig. 6A). It was not possible to obtain a proper binding curve because experimentally the EFL1 protein aggregated at higher concentrations. To exclude the possibility that the binding was disrupted due to the loss of structure of the SBDS mutant, we performed a far-UV CD analysis to assess its secondary structure content. The CD spectra of SBDS S143L is almost identical to that of the wild-type protein consisting of minima at 208 nm and a plateau centered at 218 nm consistent with a mixture of \(\alpha\)-helices and \(\beta\)-sheets as observed in the NMR structure (6) (Fig. 6B). Furthermore, the intensity of such minima is similar in magnitude demonstrating that both proteins have the same secondary structure content. This agrees with previous reports that suggested the mutation S143L does not disrupt the overall fold of the protein (6). Thus, the loss of binding with EFL1 resulted specifically from the presence of the disease mutation on SBDS. Other disease-associated mutations also disrupted the binding with EFL1 to different extents (data not shown).

Discussion

In this report we describe a kinetic analysis of the interaction of EFL1 with guanine nucleotides in the presence and absence of SBDS, the protein mutated in the Shwachman-Diamond syndrome. The association and dissociation rate constants of guanine nucleotide binding to EFL1 were measured using GDP and GTP fluorescent analogues.

Our results showed that the binding of EFL1 to guanine nucleotides is a complex process consisting of an initial association followed by a structural rearrangement for all the complexes, the GTPase bound to GDP and GTP, alone and in the
presence of SBDS (Fig. 7). However, in the absence of structural information it is not possible to discern the differences or similarities of the aforementioned complexes. A two-step binding mechanism for guanine nucleotides is common for GTPases alone or in complex with their effector biomolecules. Different situations have been described for the binding of guanine nucleotide to GTPases. The eukaryotic release factor 3 displays a two-step binding mechanism only for GDP and a single one for GTP irrespective if it is bound to the effector biomolecules eRF1 or the ribosome (23). On the contrary, binding of bacterial GTPase HflX to GTP consists of a two-step binding mechanism but a single-step when bound to GDP and the ribosome (24). In contrast, the elongation factor SelB exists only in one conformation when bound to both GDP and GTP alone and in the presence of its cognate tRNA (25). Further work will establish whether this two-step binding mechanism to guanine nucleotides of EFL1 also happens in the presence of the 60S ribosomal subunits or whether the ribosomal subunit can lock EFL1 in a single conformation.

At physiological Mg\textsuperscript{2+} concentrations, the dissociation constants for GDP and GTP to EFL1 are nearly an order of magnitude different with values of 10 and 171 \mu M, respectively. Both mant-guanine nucleotides dissociate rapidly from EFL1 with rates of 3.7 and 2.4 s\textsuperscript{-1} for the limiting step of GDP and GTP dissociation, respectively. In mammalian tissues, the average concentration of GTP is 3 \mu M, whereas that of GDP is 10-fold lower (26). Thus, in the cytoplasm, free EFL1 will exist in a mixed population consisting of twice the amount of EFL1 bound to GDP compared with that bound to GTP. This scenario may occur under certain pathological conditions such as the Shwachman-Diamond syndrome (see discussion below). However, EFL1 and SBDS form a tight complex both in the presence and absence of guanine nucleotides (6, 14). In the absence of nucleotides, the dissociation constant for the interaction between EFL1 and SBDS is 80 nm for the yeast orthologue (14). Although the levels of expression of these proteins have not been established, under normal physiological conditions, EFL1 most likely will not exist free in the cytoplasm and will be present in a complex with SBDS. Our results showed that SBDS did not influence the binding of EFL1 to GTP such that the corresponding dissociation constants were similar within the experimental error. In contrast, SBDS did have a profound effect on the binding of EFL1 to GDP. The affinity to GDP was depleted 60-fold suggesting that SBDS acts as a guanine nucleotide exchange factor (GEF) that facilitates the release of GDP. The dissociation rate of GDP from the EFL1-SBDS complex is much faster than that of GTP suggesting that EFL1 will quickly release GDP and will subsequently bind GTP yielding EFL1 on its active conformation. Furthermore, the affinity of the EFL1-SBDS complex to GDP was disrupted to such an extent that became even weaker than to GTP. Taking into account the cellular concentrations of the guanine nucleotides, and the observation that the EFL1-SBDS complex has a \approx 3-fold higher affinity (K_d) to GTP than to GDP, in the cytoplasm, the EFL1 associated with SBDS will exist predominantly in the GTP-bound form. There will be 50 times more EFL1-GTP-SBDS complex than EFL1-GDP-SBDS. EFL1 can bind independently or in complex with SBDS to the 60S ribosomal subunit that in turn functions as the GTPase activating protein or GAP modifying the k_{cat} of EFL1 (6). All this information suggests that EFL1 most likely binds the 60S ribosomal subunit as a ternary complex with SBDS and GTP, confirming the proposed model that EFL1 and SBDS collaborate to couple GTP hydrolysis to release eIF6 from the 60S surface (6, 7). Binding of this ternary complex to the 60S subunit will accelerate the catalysis and once GTP hydrolysis had occurred the resulting EFL1-GDP-SBDS complex will release from the 60S particle and will exchange the GDP for GTP. Release of EFL1 and SBDS is a prerequisite to trigger the final maturation step of the 60S ribosomal subunit elicited by the GTPase Lsg1 (27).

Most GTPases bind guanine nucleotides through their G-domain with the switch 1 and switch 2 contacting the \gamma-phosphate of GTP. In addition, switch 2 contains a conserved threonine that coordinates a Mg\textsuperscript{2+} ion that stabilizes both the \gamma- and \beta-phosphate of the nucleotide (28). This may explain the slight decrease in the affinity of EFL1 for GTP in the absence of Mg\textsuperscript{2+}, however, is difficult to address the opposing effect observed for GDP. Finally, the experiments done with the non-hydrolysable GTP analogue demonstrated that the affinity is weaker compared with that for the substrate suggesting it might not be a reliable substrate substitute. Several results have demonstrated functional differences between non-hydrolysable GTP analogues for translation GTPases such as EF-G (29), SelB (30), and eRF3 (31). The crystal structures of these GTPases are almost
identical in the apo, GDP, and Gpp(NH)p-bound forms with the residues of the switch 2 not being able to contact the γ-phosphate of the Gpp(NH)p. In fact, the dissociation constant of EFL1 for Gpp(NH)p is very similar to that for GDP in the presence of SBDS. It may be possible that EFL1 bound to Gpp(NH)p adopts a conformation similar to that of EFL1 in complex with GDP and SBDS, or as observed for some translational GTPases, Gpp(NH)p may not be able to fully trigger the concomitant conformational change elicited by GTP.

The mechanism of guanine nucleotide exchange on GTPases by their cognate GEFs varies greatly, but mainly involves either fully obstructing the nucleotide-binding site or partial hamper of the Mg2⁺ - or γ/β-phosphate binding sites (15). Most GEFs constitute extrinsic biomolecules, however, some GTPases have built-in intrinsic exchange factors as described for the N terminus of the bacterial signal recognition particle FtsY (32). Structurally, the GEFs for small GTPases such as Ran, Ras, Rhino, and Rab vary greatly even among specific subfamilies. However, the GEFs for translational GTPases seem to emulate the shape of a tRNA in an example of molecular mimicry between nucleic acid and protein (33). For example, translation termination requires the GTPase eRF3 and its cognate GEF, eRF1, whose shape resembles a tRNA. On the other hand, EF-G, a translocation case involved in the elongation phase of translation, contains a C-terminal portion (domains III-V) that mimics the shapes of the acceptor stem, anticodon helix, and the T stem of the tRNA (34). Because of the large sequence identity, the structure of EFL1 is expected to be very similar to that of EF-G except for an additional unstructured region in domain II. As we have shown in this work, SBDS constitute the GEF for EFL1 and coincidentally its shape also resembles a tRNA. Together, these evidences suggest that this molecular mimicry is not only limited to translational GTPases and their effector molecules, but also to GTPases involved in ribosome biogenesis. Furthermore, EF-G and eRF3-eRF1 occupy similar physical space in the ribosome making contacts with the tRNA present in the P site. The C-terminal portion of EF-G and eRF1 bind in the ribosomal A site, whereas the N terminus of EF-G and eRF3 occupy the GTPase-associated center and they undergo conformational changes during their course of action (35, 36). EFL1 interacts with the GTPase-associated center on the 60S subunit (37) most likely through its G-domain and it is tempting to speculate that its C terminus may protrude toward the A-site. However, it is difficult to visualize the SBDS sitting close to the P site on the 60S subunit if regions I and II of EFL1 involved in the interaction point toward the opposite side of the molecule. In the absence of structural information regarding the complex EFL1-SBDS and/or EFL1-SBDS-60S with different guanine nucleotides it is difficult to explain the molecular bases SBDS stimulation of GDP release from EFL1.

Shwachman-Diamond syndrome is a rare autosomal recessive disease caused by mutations in the gene SBDS (OMIM607444) (38). Most of the patients have mutations in at least one allele that prematurely truncates the protein, while 25% of the patients have mutations corresponding to missense, splice, nonsense, and indels mutations located anywhere in the coding region of the gene. These rare mutations result in loss or reduction of the protein stability, or are predicted to affect the surface electrostatic potential or to locally alter surface epitopes (18). So far a specific molecular function to any of these rare mutations has not been established. Here, we demonstrated that at least one such missense mutation in the SBDS protein is directly involved in the interaction with EFL1. The effect of such mutation is to disrupt the interaction between the proteins without altering the overall fold of the SBDS protein. Thus, we have addressed a specific function to a missense mutation present in the Shwachman-Diamond syndrome that for mutation S143L corresponds to a binding epitope for EFL1. In turn, the disruption of the interaction between both proteins abrogates the regulation that SBDS exerts on the binding of EFL1 for GDP. Under pathological conditions arising from SBDS missense mutations that abolish binding to EFL1 such as mutation S143L, the GTPase will exist free in the inactive form bound to GDP with a very low catalytic turnover. Although, the exact mechanism relating GTP hydrolysis by EFL1 and release of eIF6 from the 60S subunit is not known, this will be inefficient if EFL1 cannot release GDP, exchange for GTP, and engage in a new catalytic cycle. These findings, however, do not exclude the possibility that other SBDS mutations may have different roles. For example, domain I of SBSD binds RNA and mutations in this domain may alter the binding to the ribosomal subunit.

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Note Added in Proof—The solid red trace corresponding to the fit of the data to a one-binding site model has been removed from Fig. 6A from the version of this article that was published as a Paper in Press on May 19, 2015. This change does not affect the interpretation of the results or the conclusions, as the aim of Fig. 6A was to show qualitatively that binding to EFL1 is abrogated for a disease mutant compared to wild type SBDS. In addition, a one-binding site model does not adequately describe the association between the two proteins.

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