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A Novel Depletion Technique for Studying the Role of Protein L12 in the Activation of Ribosome-Dependent GTPases

Michelle E. Wuerth
Western Washington University

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WASHINGTON UNIVERSITY

Honors Program
516 High Street
Bellingham, Washington 98225-9112

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A Novel Depletion Technique for Studying the Role of Protein L12 in the Activation of Ribosome-Dependent GTPases

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Abstract

The ribosome is a complex macromolecular machine that is responsible for the synthesis of proteins from a nucleic acid template. This process is largely regulated by various protein translation factors, many of which are GTPases. Ribosome-dependent GTPase activity has been observed to be coincident with the presence of ribosomal protein L12. Of current interest is to understand how L12 interacts with the GTPase factors on the 70S ribosome. A key to the investigation of these interactions is to produce ribosomes fully depleted of L12 for comparisons of factor activity and binding in the presence and absence of this protein. Here, we present a novel two-step depletion protocol that takes advantage of the JE28 ribosomes' engineered C-terminal $(His)_6$-tag chromosomally encoded on protein L12. Fully depleted ribosomes were shown to be absent of L12 in Western blotting studies. Furthermore, these 70S ribosomes were shown not to stimulate ribosome-dependent GTP hydrolysis by translation factor EF-G in malachite green GTP hydrolysis assays. This population of ribosomes purified in the complete absence of protein L12 will make possible investigations of factor binding and ribosome-dependent GTP hydrolysis to further elucidate the role of L12 in translation.

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Table of Contents

Abstract iii
Acknowledgements iv
List of Figures vi
Introduction 1
Materials and Methods 13
Results 18
Discussion 26
References 28

List of Figures

Figure 1: Crystal Structure of the Ribosome 2
Figure 2: Initiation Overview 4
Figure 3: Accommodation and Peptidyl Transferase Reaction 5
Figure 4: Translocation Overview 6
Figure 5: Termination Overview 7
Figure 6: Ribosome Recycling Overview 8
Figure 7: Translational GTPase Structures 9
Figure 8: Structure of the Ribosomal Stalk 10
Figure 9: SDS-PAGE of Purified GTPases 18
Figure 10: FPLC Chromatogram of GTPase SEC 19
Figure 11: Ribosome Purification 20
Figure 12: Ribosome-Dependent GTP Hydrolysis 20
Figure 13: SDS-PAGE of Depleted Proteins 22
Figure 14: Post-Depletion Malachite Green Assay 22
Figure 15: Depleted Ribosomes Elution Profile 23
Figure 16: Western Blot of Depleted Ribosomes 23
Figure 17: Effects of Depletion on GTPase Activity 24
Figure 18: Post-Depletion GTP Hydrolysis Activity 25

Introduction:

Across all domains of life, a complex macromolecular machine called the ribosome is responsible for synthesizing proteins from a nucleic acid template. The process by which the ribosome translates this nucleic acid message into functional units of protein is called translation. In translation, an mRNA message directs the formation of a polypeptide chain, aided by tRNA molecules (which serve as adaptors between the mRNA and amino acids) and catalyzed by the ribosome.[1] The ribosome itself is a 2.5 MDa complex of proteins and RNA that catalyzes the formation of peptide bonds.[2] It is comprised of two unequal subunits, which, in prokaryotic systems, are referred to as the 30S and 50S subunits. Together, these subunits form the 70S ribosome (Figure 1).

Due to its vast size and complexity, detailed structural understandings of the ribosome have only become possible relatively recently.[3-5] With advancements in techniques such as x-ray crystallography, cryo-electron microscopy (Cryo-EM), single molecule FRET (smFRET), and chemical footprinting, there have been great strides made in elucidating the step-by-step process of how a ribosome functions in protein synthesis.[6] However, while many aspects of translation have been illuminated over the past several decades, many of the details of this process remain poorly understood. There is a need to fill these gaps in our comprehension for both practical and academic reasons.

From a medical perspective, the study of the ribosome has important practical applications, as the ribosome is a key target of a number of important antibiotic compounds.[7] In the face of growing drug resistance among many pathogenic organisms, developing a greater understanding of antibiotics and the processes they target serves a vital role. At an essential

level, however, there is an interest in understanding the mechanism of translation because of its fundamental role throughout life. In studying the ribosome, we seek to understand one of the most key biological processes in nature.

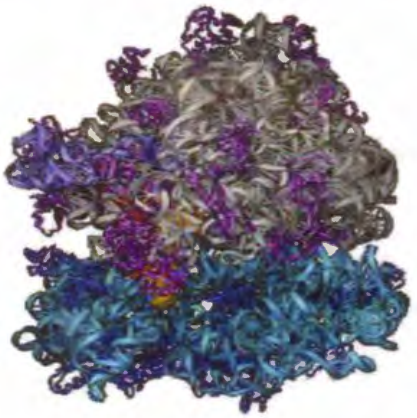

Figure 1. X-ray crystal structure of the 70S ribosome.[3]

Ribosomal Structure

As mentioned previously, the prokaryotic ribosome is comprised of a complex of ribosomal RNA (rRNA) and accessory proteins. The 50S subunit contains two RNA strands, the large 23S and smaller 5S strands, while the 30S subunit has one rRNA strand designated the 16S.[8] Importantly, it is the RNA that catalyzes the formation of peptide bonds.[9-11] Together, both subunits contain approximately 50 proteins that serve mainly structural roles. The 50S subunit contains the peptidyl transferase center (PTC) that catalyzes the formation of peptide bonds, while the 30S subunit binds the messenger RNA (mRNA) through contacts with the 16S RNA and is largely responsible for decoding mRNA.[12] Each ribosomal subunit has three binding

sites for transfer RNA (tRNA) molecules called the A (aminoacyl), P (peptidyl), and E (exit) sites respectively.[13, 14]

Translation

In the process of translation, the ribosome catalyzes the formation of peptide bonds between amino acid residues transported to the ribosome via aminoacylated tRNA molecules in a specific sequence determined by the mRNA molecule bound to the 30S subunit. Translation occurs in several steps, which will be described separately for clarity.

Initiation

The first step of translation is initiation. Although the exact sequence of events remains subject to debate, prokaryotic initiation involves the formation of an "initiation complex" consisting of the 70S ribosome, an mRNA molecule, a specific initiator tRNA known as fMet-$tRNA^{fMet}$, and three protein initiation factors designated IF-1, IF-2, and IF-3 (Figure 2).[15] Initiation is launched by the binding of the mRNA to the 30S subunit, which involves contacts between the 16S RNA of the 30S subunit and a specific sequence on the mRNA known as the Shine-Dalgarno sequence.[16] The first of the three protein factors to be involved is believed to be IF-3.[6] IF-3 has multiple roles; it both prevents the premature association of the 30S and 50S subunits to form the 70S ribosome and prevents tRNA molecules aside from the fMet-$tRNA^{fMet}$ from binding to the P site.[17] Secondly, IF-1 binds in the A site and prevents the entry of tRNA to this site.[18] In a final step, IF-2, a ribosome-dependent GTPase (see page 8), is believed to be involved in binding the fMet-$tRNA^{fMet}$ and transferring it to the P site.[19] After the dissociation of

the initiation factors from the initiation complex, what remains is a 70S ribosome bound to an mRNA molecule and containing fMet-tRNA^{fMet} in the P site.

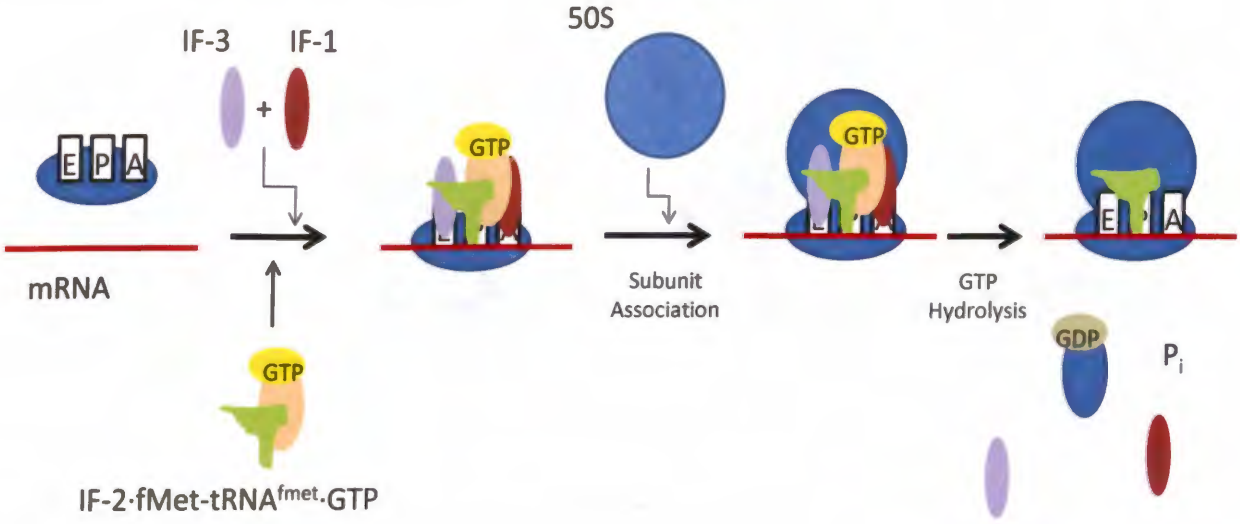

Figure 2. Overview of the formation of the initiation complex.

Elongation

Elongation is not a single step, but rather an iterative process that is repeated as amino acids are added one-by-one to the growing polypeptide chain. In elongation, aminoacylated tRNA molecules are delivered to the A site of the ribosome by GTP-bound elongation factor Tu (EF-Tu), a translational GTPase. The anticodon stem loop of each aminoacyl-tRNA molecule base pairs with each mRNA codon through codon-anticodon base pairing interactions.[20] If the match is correct, EF-Tu hydrolyzes its bound GTP molecule to GDP with the release of inorganic phosphate and dissociates from the ribosome.[21] The selectivity of the ribosome is remarkable; its error rate is reported as 10^{-3} to 10^{-4} mutations per nucleotide.[2] Once the tRNA has been accommodated (fully bound) into the A site, the ribosome catalyzes the peptidyltransferase reaction. In this reaction, the amino acid bound to the P site tRNA molecule is transferred-

through the formation of a peptide bond with the amino acid bound to the A site tRNA- to the A site tRNA molecule (Figure 3).

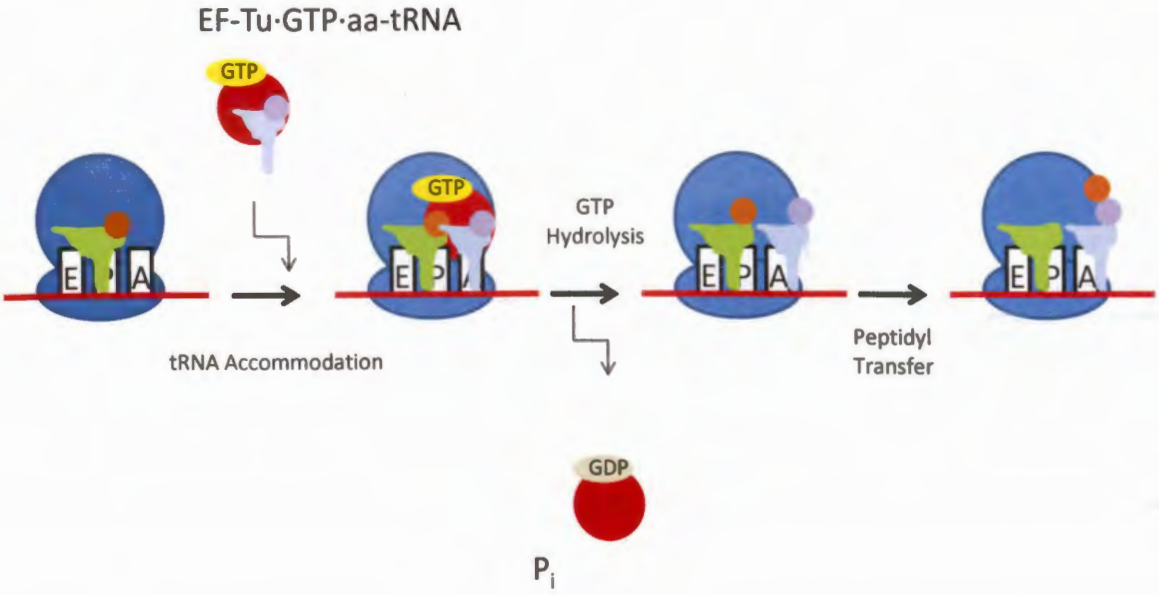

Figure 3. The accommodation of the aminoacyl tRNA and subsequent peptidyltransferase reaction.

This tRNA accommodation is followed by a step called translocation, in which the tRNA molecules in the P and A sites move into the E and P sites, respectively (Figure 4).[22-23] The mechanism of translocation occurs by the formation of so-called hybrid states, produced simultaneously with the counterclockwise rotation of the 30S ribosomal subunit with respect to the 50S.[24] For clarity in describing the hybrid states, we can divide each site into its 30S and 50S component, denoted A/A, P/P, and E/E, respectively. After the peptidyl transfer reaction has taken place, the A and P site tRNA molecules spontaneously move between the "classical" A/A and P/P state and the "hybrid" A/P and P/E state.[25-26] Elongation factor G (EF-G) in its GTP-bound form stabilizes the hybrid state conformation.[27] Upon hydrolysis of GTP by EF-G, the

ribosome once again returns to its classical state, with the tRNA molecules now in the P/P and E/E states.[28-29] At this point, the ribosome is ready to repeat the cycle of elongation.

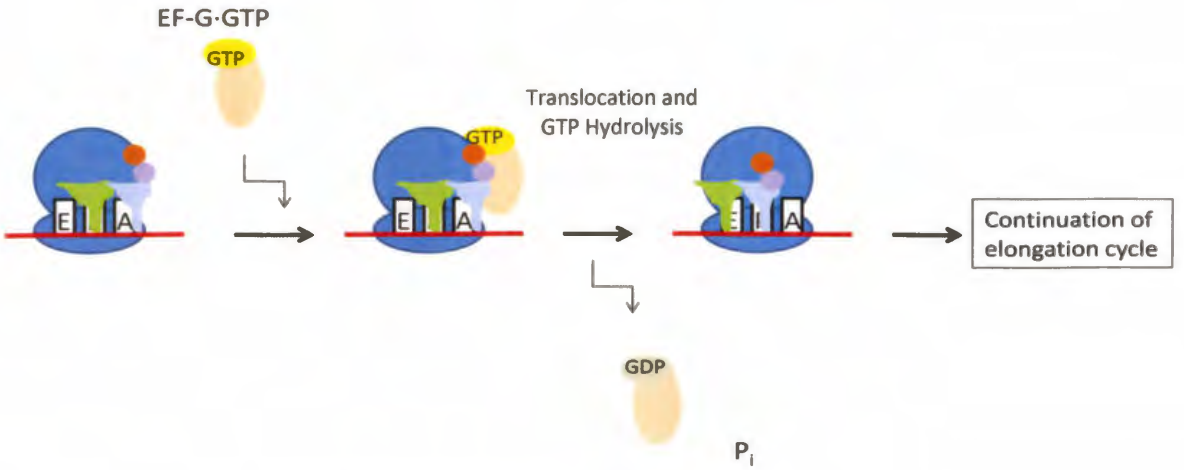

Figure 4. Depiction of the translocation of the ribosome.

Termination

Translation is terminated by recognition of a stop codon in the mRNA message. This step also involves the help of an accessory protein called a release factor. These factors come in two flavors, designated class I and class II release factors. The class I release factors are RF-1 and RF-2, each of which recognizes two of the three possible stop codons found in mRNA.[30] RF-1 recognizes the codons UAG and UAA, whereas RF-2 recognizes UGA and UAA.[31] After the binding of a class I release factor, the polypeptide chain is released from the ribosome, and the class II release factor RF-3 binds.[32] RF-3 facilitates the dissociation of the class I release factor from the ribosome. While RF-3 initially binds to the ribosome in its GDP-bound form, upon association with the ribosome it exchanges GDP for GTP. It then hydrolyzes GTP to GDP and

inorganic phosphate, causing the release of RF-3, the class I release factor (RF1 or RF2), and the E-site deacyl tRNA to be released from the ribosome (Figure 5).[33]

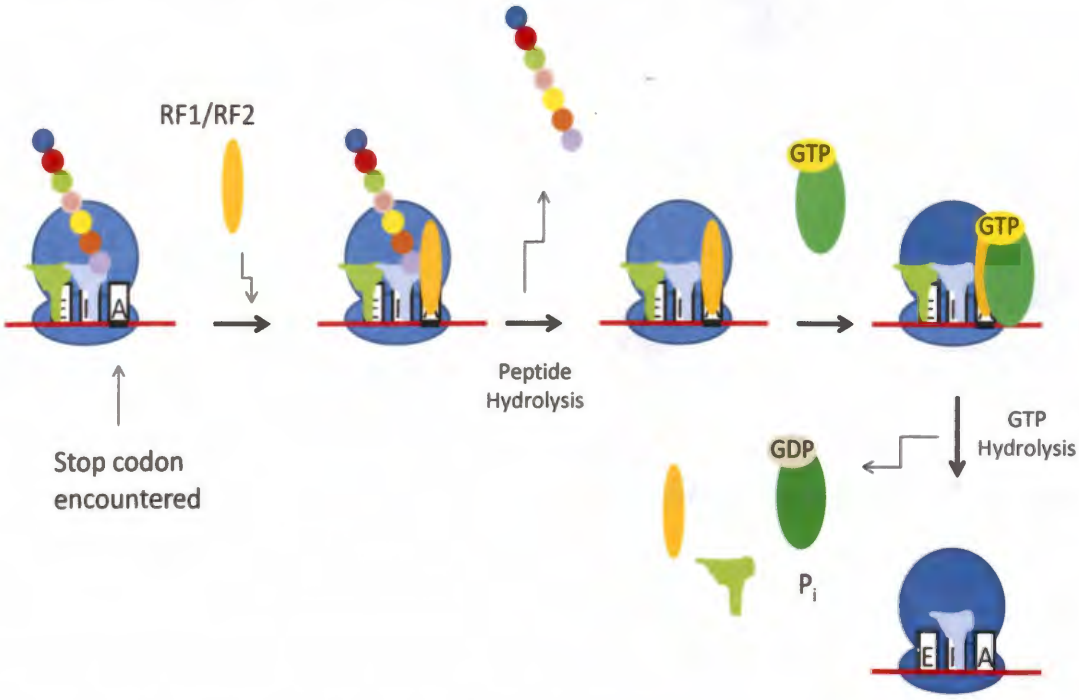

Figure 5. An overview of the steps of translation termination.

Recycling

Following termination, the ribosome remains bound to the mRNA with a single deacylated tRNA still bound in the P site. In order to translate other mRNA molecules, the ribosome must be disassembled into its subunits. It does this through the combined help of a protein called ribosome recycling factor (RRF) and EF-G.[34] Although the exact mechanism and steps involved remain indistinct, both factors bind to the ribosome, and, upon GTP hydrolysis by EF-G, the two ribosomal subunits dissociate.[35] The 30S subunit remains bound to its mRNA and P site tRNA until the binding of IF-3, which causes the dissociation of these molecules, thus preparing the subunit to begin the translation process once again (Figure 6).[36]

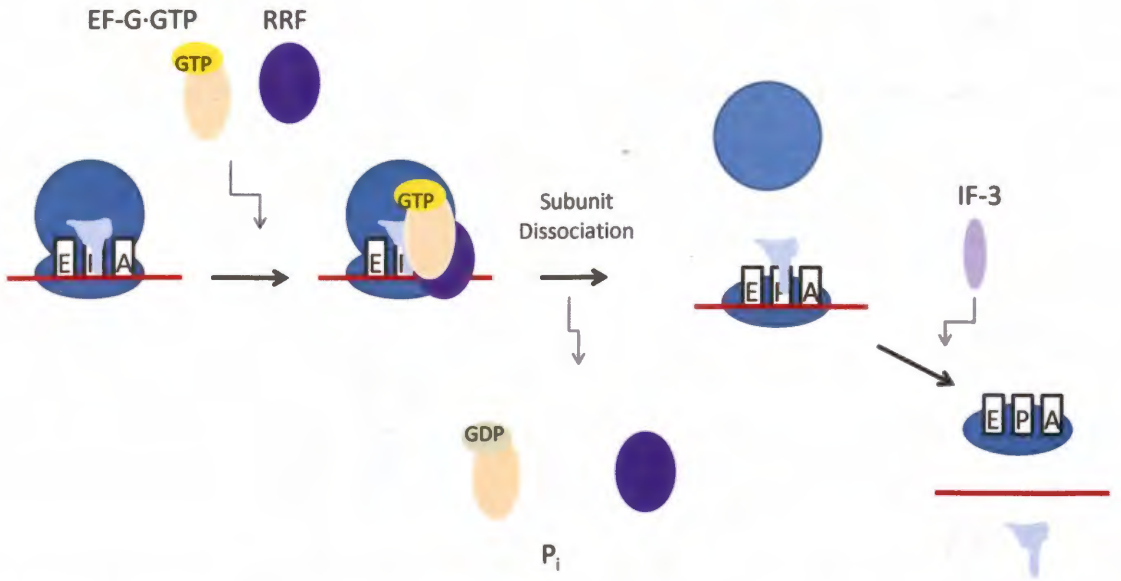

Figure 6. Schematic depiction of the steps of ribosome recycling, which are necessary for additional rounds of translation to take place.

GTPases

Throughout the process of translation, ribosome activity is regulated by a number of protein factors. As described previously, many of these proteins are GTPases (Figure 7). GTPases hydrolyze GTP to GDP, accompanied by the release of inorganic phosphate. This hydrolysis reaction causes a conformational change in the protein that allows it to carry out its unique function. In general, the GTP-bound form is the active form of the protein, while the GDP-bound form is inactive. While GTPases fill diverse roles, from involvement in signal transduction pathways to cellular transport functions to translation, they share a conserved region of structure called the G-domain.[37] This domain is responsible for the binding and hydrolysis of guanine nucleotides. GTPases, however, have external regulatory factors that determine when GTP is hydrolyzed. GTPase activating proteins (GAPs) are responsible for stimulating the GTP hydrolysis activity of GTPases, while additional factors called guanine-

exchange factors (GEFs) stimulate the release of GDP by the GTPase, allowing it to once again be prepared to bind GTP.[38] For the translational GTPases, the ribosome itself serves as the GAP, which will be discussed further below.

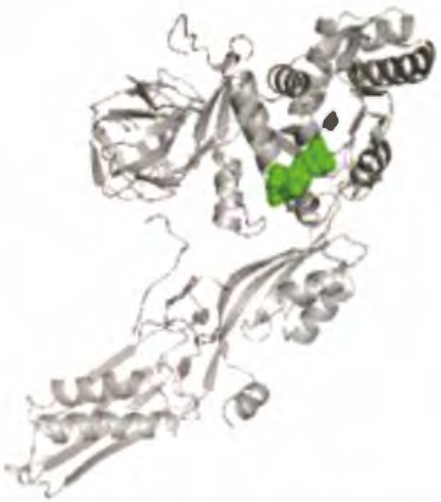

Figure 7. Example of a translational GTPase. Shown is the x-ray crystal structure of EF-G complexed with GDP (in green).[39]

As seen in the above overview of translation, factors IF-2, EF-Tu, EF-G, and RF-3 are all translational GTPases. Additionally, there are two other translational GTPases whose exact roles remain under investigation. Elongation factor 4 (EF-4, also known as LepA) is a protein that shows a high degree of structural homology to EF-G but appears to have a function somewhat opposite in nature.[40] This protein is responsible for the reverse translocation of the ribosome, though what exact role this serves in vivo has not been concretely determined.[41] Finally, a novel protein factor called BipA, which shows structural homology to the family of elongation factors (which includes EF-Tu, EF-G, and EF-4) has recently been characterized and shown to interact with the 70S ribosome.[42] While it has been established that BipA serves a role

in stress and virulence processes in multiple bacterial species, what the exact function of this protein is remains under investigation.[43]

The GTPase Associated Center and Protein L7/L12

The translational GTPases interact with the ribosome at a region of the 50S subunit called the GTPase associated center (GAC), which consists of both protein and RNA elements. The GAC is composed of a region of 23S RNA called the sarcin-ricin loop (SRL) and the ribosomal stalk, which protrudes from the 50S subunit (Figure 8).[44] The stalk consists of an approximately 100 nucleotide sequence of 23S RNA, ribosomal proteins L11 and L10, and multiple copies of protein L7/L12.[44]

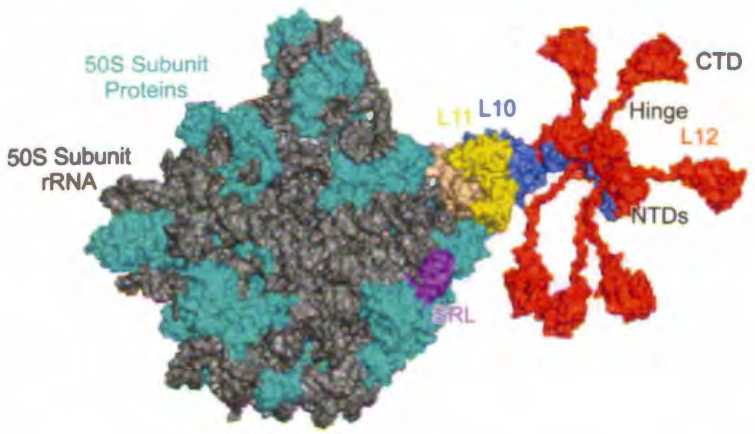

Figure 8. The 50S subunit and ribosomal stalk.[44]

Proteins L7 and L12 differ only by N-acetylation (L7 is the acetylated form of L12) and will be referred to as L12 henceforth for clarity. Depending on the species of organism, this

protein is present in a different number of copies on the ribosome; in *E. coli*, four copies of L12 are found. Together, these elements that compose the GAC interact with the translational GTPases.[45] However, the details of these interactions remain subject to debate, with particular interest surrounding protein L12. L12 is necessary for stimulating the GTP hydrolysis activity of the translational GTPases, but the extent to which it is necessary and its effects on different factors remains under debate.[45-48] Additionally, this protein has been implicated in factor recruitment and binding,[45] but difficulties in obtaining precise structural data have hindered efforts to resolve these questions. The basis for this difficulty is likely in the dynamic nature of L12 itself; the protein consists of globular N and C-terminal domains that are connected by a flexible hinge region.[49] Furthermore, studies involving the selective removal of L12 from the ribosome have often produced inconclusive results thanks to the difficulty in fully and selectively removing the protein from the ribosome. Thus, there remains an interest in definitively establishing the role of L12 in translation.

Research Aims

In order to investigate the role of protein L12 in both factor binding and stimulating GTPase activity, it is necessary to develop a novel method for fully removing L12 from the ribosome. Thus, the initial goal of this project was to prepare a population of so-called "fully-depleted" ribosomes for factor binding and GTP hydrolysis assays. Subsequently, we intend to use these ribosomes to carry out binding and hydrolysis assays on GTPases EF-G, IF-2, EF-4, RF-3, and BipA to compare how removal of L12 differentially affects these factors.

Materials and Methods

Buffers

GTPase lysis buffer: 50 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 7 mM $MgCl_2$, 15 mM imidazole, 25% (v/v) glycerol, 6 mM β-mercaptoethanol.

GTPase wash buffer: 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 60 mM NH_4Cl, 7 mM $MgCl_2$, 15 mM imidazole, 25% (v/v) glycerol, 6 mM β-mercaptoethanol.

GTPase elution buffer: 50 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 7 mM $MgCl_2$, 250 mM imidazole, 25% (v/v) glycerol, 6 mM β-mercaptoethanol.

GTPase storage buffer: 50 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 7 mM $MgCl_2$, 25% (v/v) glycerol, 1 mM dithiothreitol (DTT).

JE28 lysis buffer: 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 30 mM NH_4Cl, 5 mM imidazole, 1 mM DTT.

JE28 wash buffer: 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 500 mM NH_4Cl, 5 mM imidazole, 1 mM DTT.

JE28 SW buffer: 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 500 mM NH_4Cl, 1 mM DTT.

JE28 elution buffer: 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 30 mM NH_4Cl, 150 mM imidazole, 1 mM DTT

Ribosome storage buffer: 50 mM Tris-HCl (pH 7.5), 30 mM NH_4Cl, 7 mM $MgCl_2$, 25 % (v/v) glycerol

L12 Initial Depletion Buffer: 20 mM Tris-HCl (pH 7.5), 1.0 M NH_4Cl, 20 mM $MgCl_2$, 50% v/v) glycerol, 5 mM β-mercaptoethanol.

Malachite Green Reaction Buffer: 90 mM K-Hepes (pH 7.5), 100 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT

Fluorescence Assay Buffer: 50 mM Tris (pH 7.5), 100 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM GDPNP

Protein Expression and Purification

Expression of GTPases:

E. coli translation factors engineered with an N-terminal $(His)_6$- tag in pSV expression vectors were transformed into the BL21 *E. coli* cell line. Selected colonies were used to inoculate 10-mL Luria Bertani (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) broth cultures containing 35 μg/mL Kanamycin (LB-Kan). These cultures were grown to saturation overnight at 37° C and used to inoculate 1-L LB-Kan cultures. Cell growth was supervised by monitoring the optical density at 600 nm by UV-vis spectroscopy until an OD_{600} of 0.5 was reached. Subsequently, the cultures were induced with 1 mM isopropyl-β D-1-thiogalactopyranoside (IPTG) and allowed to shake overnight at 15° C. Cells were pelleted via centrifugation at 6000 rpm for 10 minutes at 4° C in a Sorvall GS-3 rotor. Pellets were resuspended in 30 mL GTPase lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mg/mL lysozyme. Following a 30 minute incubation period accompanied by gentle rocking, the cells were sonicated for 2 minutes (Branson Sonifier 450, 50% duty cycle, output of 5) before centrifugation at 19,000 rpm for 45 minutes in a Sorvall SS34 rotor to separate the insoluble cell remains from the lysate. The lysate was then filtered with 5.0 and 0.45 micron filters prior to affinity chromatography.

Purification of GTPases:

The filtered lysate was incubated with approximately 4 mL HisPur™ nickel-nitriloacetic acid (Ni-NTA) resin (Thermo Scientific) for approximately 2 hours at 4° C with gentle stirring before application to a gravity-flow column. The resin was washed with 3 column volumes of lysis buffer, followed by 10 column volumes of GTPase wash buffer. The protein was eluted

with GTPase elution buffer and monitored with Bradford reagent. Samples were then dialyzed overnight into GTPase storage buffer, concentrated with Amicon Ultra Centrifugal Filters, and quantified by A_{280} UV-vis spectroscopy. The purity of the samples was assessed by SDS-PAGE. Size-exclusion chromatography with a GE HiLoad™ 16/60 Superdex™ column was then employed to remove additional contaminating proteins.

Expression and Purification of Ribosomes

Ribosomes containing an engineered C-terminal $(His)_6$- tag on each of the four copies of protein L12 were expressed in the *E. coli* JE28 line[50]. JE28 cell stocks were used to inoculate 10-mL LB-Kan cultures, which were grown overnight to saturation and then utilized to seed 1-L LB-Kan cultures. Cultures were grown to an OD_{600} of 1.0 and then immediately placed on ice. The temperature was monitored until 4° C was reached, at which point the cells were pelleted via centrifugation for 10 minutes at 6,000 rpm at 4° C in a Sorvall GS-3 rotor. The cell resuspension, lysis, and clarification procedure described above for the GTPases was employed with the substitution of JE28 buffers. The $(His)_6$-tagged ribosomes were then purified with TALON™ resin (Clontech) immobilized metal affinity chromatography (IMAC) via the procedure described above for Ni-NTA resin. Eluted ribosomes were dialyzed overnight into JE28 SW buffer and then pelleted by two successive rounds of ultracentrifugation at 150,000 x g for 2.5 hours. Pellets were finally resuspended in JE28 storage buffer, quantified by A_{280} UV-vis spectroscopy, and assessed for purity via SDS-PAGE. Ribosomes were flash frozen and stored at -80° C.

L12 Depletion Protocol: a Two Step Process

Step 1:

The initial step involved a standard depletion procedure in which 70S ribosomes were treated with NH_4Cl and ethanol. A 450 pmol sample of ribosomes was incubated in 450 µL of L12 depletion buffer for 5 minutes on ice. A 250 µL portion of pre-chilled absolute EtOH was added to the ribosomes, which were then stirred at 4° C for 5 minutes. After the addition of a second 250 µL portion of EtOH, the ribosomes were stirred for an additional 5 minutes before centrifugation at 150,000 g for 30 minutes. The supernatant was then removed and saved for analysis. The pellet was gently rinsed with JE38 storage buffer and then resuspended in that buffer. The supernatant was treated with a 5X excess of chilled acetone and allowed to incubate at -20° C for an hour. Following centrifugation at 3,500 rpm for 10 minutes, the supernatant was removed by decanting and the pellet of precipitated proteins allowed to air dry overnight. After resuspension in GTPase storage buffer, the proteins were analyzed via SDS-PAGE.

Step 2:

The second step of the L12 depletion protocol took advantage of the engineered C-terminal $(His)_6$-tag present on every copy of L12 on JE28 ribosomes. Following the initial depletion step described above, ribosomes were injected onto a 10-mL Ni-NTA column connected to a ÄKTAprime™ Plus (GE) FPLC system equilibrated in JE28 lysis buffer. After washing the column with 15 mL of lysis buffer, an imidazole gradient of 0-150 mM was employed to elute bound 70S ribosomes. Fractions were collected and analyzed by SDS-PAGE and western blotting.

Western Blots

Following the depletion protocol, the extent of L12 removal was assessed via western blotting. Samples were run on a 17% w/v SDS-PAGE gel and then transferred to a nitrocellulose membrane (8 hours at a constant 15 volts). After transfer, the membrane was subjected to the HisDetector AP-Ni Western Blot (KPL). Samples were incubated in 20 mL 1X Detector Block solution for 1 hour with gentle rocking prior to addition of the HisDetector™ Nickel-AP Conjugate. Following 1 hour of additional incubation time, the membrane was subjected to three 5 minute washing steps with Tris-buffered saline-tween 20 (TBST). The blot was then placed in 10 mL 5-bromo-4-chloro-3'-indolyphosphate p-toluidine-nitro-blue tetrazolium chloride (BCIP-NBT) and monitored for the development of color for approximately 10 minutes before the reaction was quenched by rinsing the membrane in dd H_2O.

Malachite Green GTP Hydrolysis Assay

To assess the GTP hydrolysis activity of the translation factors, the malachite green assay was employed using PiColorLock Gold Phosphate Detection System (Innova Biosciences). Reactions were carried out in duplicate at a final reaction volume of 25 µL. Five µM concentrations of translation factors were incubated with 0.2 µM 70S ribosomes in malachite green assay buffer for 5 minutes at room temperature prior to the addition of 25 µM GTP. Reactions were allowed to proceed in a 96-well plate at 37°C for 15 minutes prior to quenching with the addition of 6.25 µL of Gold Mix. After 5 minutes, 2.5 µL of stabilizer was added to each reaction well. Following a 30 minute incubation period for color development, plates were read at 635 nm on a BioTek plate reader.

Results:

GTPase Expression and Purification

As detailed in Materials and Methods, GTPase translation factors were expressed and purified via a standard protocol. Affinity chromatography provided the primary purification step, utilizing the engineered N-terminal $(His)_6$-tags present on the proteins. Following this step, protein purity was assessed by SDS-PAGE (Figure 9). In general, affinity chromatography alone resulted in greater than 90% purity of the proteins. However, to ensure the removal of lingering contaminants, size-exclusion chromatography was performed on the post-affinity purified proteins. Figure 10 depicts a typical elution profile, which consisted of one large protein peak accompanied periodically by smaller impurity peaks that could be separated out by collecting peaks in fractions.

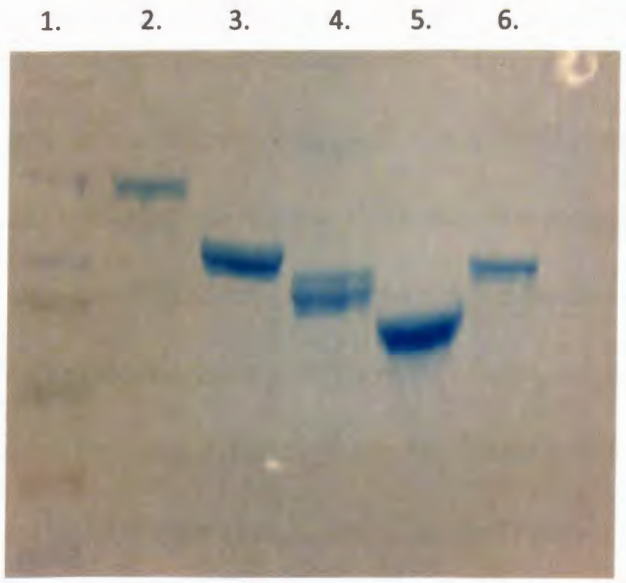

Figure 9. Coomassie-stained 10% w/v SDS-PAGE gel depicting GTPases following affinity purification; lanes: (1) MW marker, (2) IF-2, (3) EF-G, (4) EF-4, (5) RF-3, (6) BipA

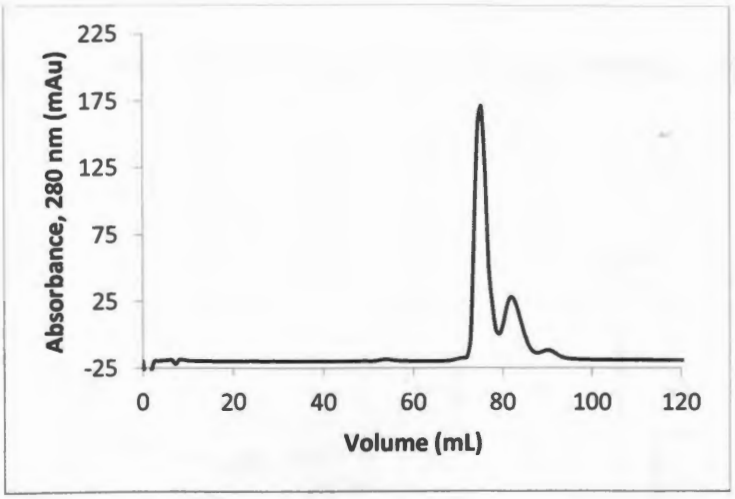

Figure 10. Example size-exclusion chromatogram from purification of EF-4.

Ribosome Purification

The engineered $(His)_6$-tag present on the C-terminus of each copy of ribosomal L12 allowed for a simple and rapid ribosomal purification scheme. Following affinity chromatography, ribosomes were immediately subjected to two rounds of ultracentrifugation prior to assessment. SDS-PAGE was utilized to examine the profile of ribosomal proteins (Figure 11A). As all *E. coli* ribosomal proteins are under 35 kD (with the exception of the 61 kD S1), an absence of bands of greater than this molecular weight cutoff was deemed necessary for acceptable purity; if such impurity bands were detected, additional ultracentrifugation steps were performed. Furthermore, the UV-vis profiles of all purified ribosomal stocks were assessed (Figure 11B). A typical profile is shown below, demonstrating a strong absorbance peak at 260 nm, typical of nucleic acids. Additionally, the malachite green GTP hydrolysis assay was utilized to check ribosomes for the presence of contaminating GTPases. Because ribosomes do not hydrolyze GTP themselves but stimulate the hydrolysis of GTP by the translational

GTPases, the concentration of inorganic phosphate following incubation of 70S particles and GTP was assessed and compared to background levels (Figure 12).

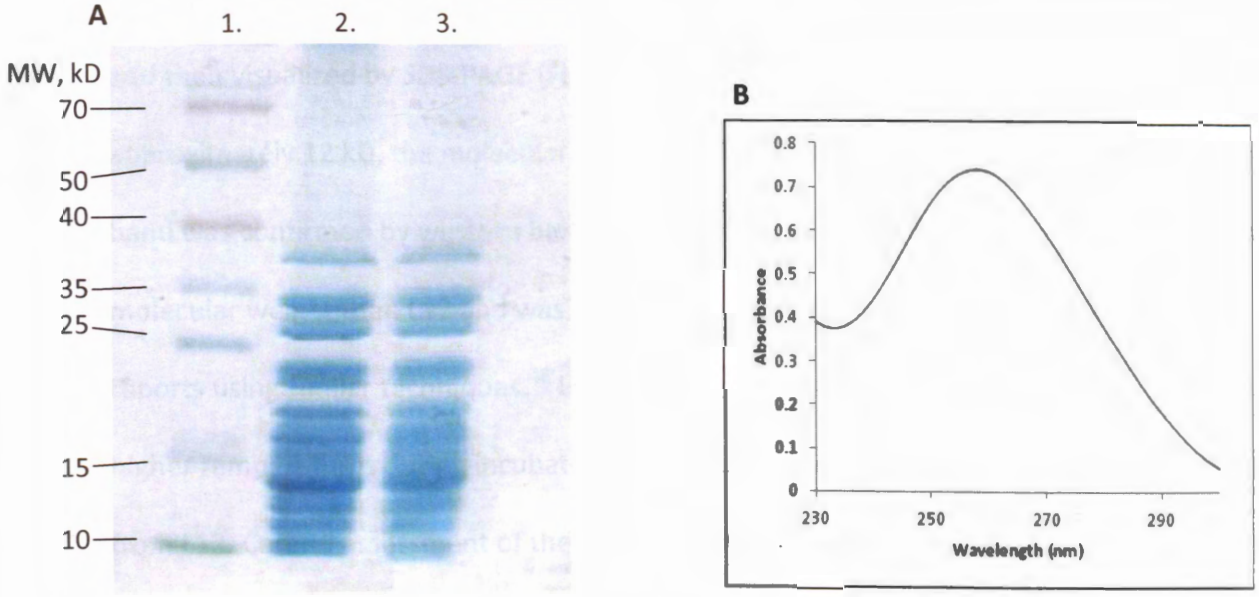

Figure 11. (A) 15% SDS-PAGE profile of 70S ribosomal proteins; Lanes: (1) Molecular weight standards, (2) and (3) 70S ribosomal proteins. (B) Typical ribosomal UV-vis absorbance spectrum.

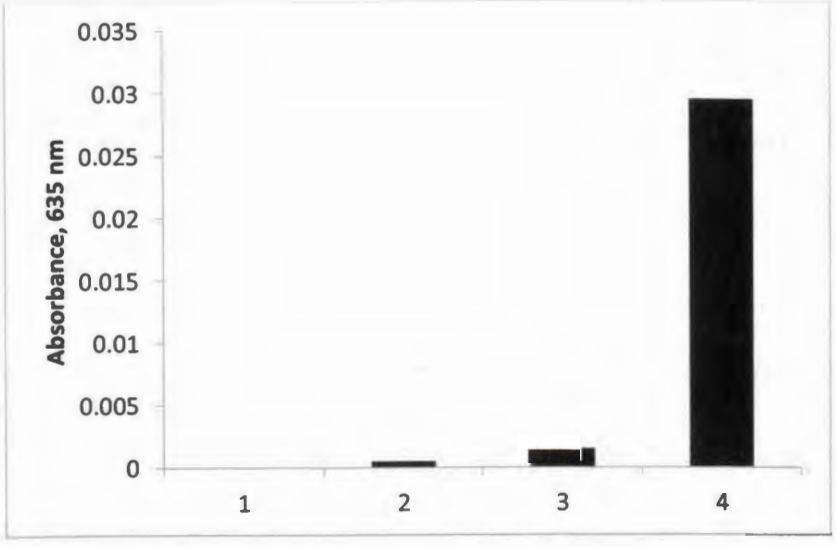

Figure 12. Malachite green assay testing purified ribosome stocks for presence of contaminating GTPases and ability to stimulate GTPase activity of factors; (1) Buffer + GTP, (2) 70S + GTP, (3) EFG + GTP, (4) 70S + EF-G + GTP.

L12 Depletion Protocol

The initial depletion step resulted in the partial removal of L12 from the 70S stalk. The supernatant proteins from the preliminary procedure were subjected to acetone precipitation and then visualized by SDS-PAGE (Figure 13). The strongest intensity band appeared at approximately 12 kD, the molecular weight of L12. As demonstrated below, the identity of this band was confirmed by western blot. Another band was occasionally seen at a slightly higher molecular weight than L12 and was reasoned to be L10 from its agreement with previous reports using similar techniques.[48] Longer incubation times in the L12 extraction buffer and higher temperatures during incubation corresponded to the nonspecific loss of proteins aside from L12. Careful assessment of the supernatant proteins was carried out; the appearance of significant quantities of additional proteins besides L12 resulted in these populations of ribosomes being excluded from additional use. Additionally, 70S samples from each depletion were reconstituted by incubation with a 5-fold excess of L12 and assessed for ability to stimulate the GTPase activity of translation factors (Figure 14). After a significant population of partially depleted 70S ribosomes was accumulated, they were subjected to the novel second depletion step, which utilizes the presence of the $(His)_6$-tag on L12 in the JE28 line. After injection of the 70S ribosomes onto the Ni-NTA column, a well-defined absorbance peak was rapidly detected prior to the addition of the high-imidazole elution buffer (Figure 15), indicating the presence of ribosomes not bound to the resin due to a lack of $(His)_6$-tagged L12 (70SΔL12). Following the transition to elution buffer, a small peak was detected, which was reasoned to belong to the small population of ribosomes not fully depleted of L12.

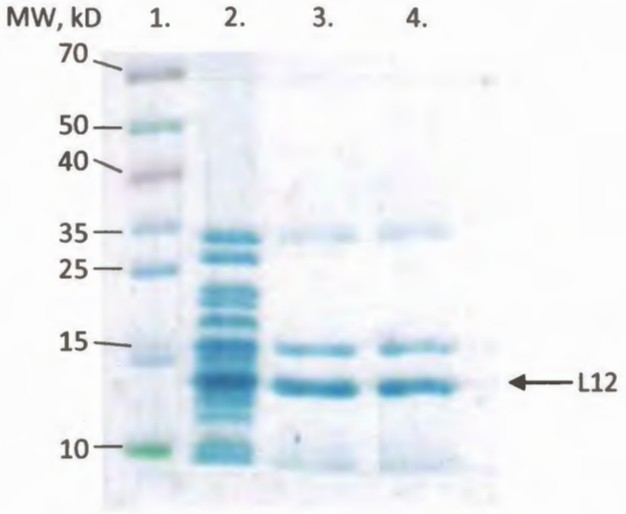

Figure 13. 15% SDS-PAGE of ribosomal proteins isolated from depletion supernatant; lanes: (1) molecular weight marker, (2) 70S ribosomal proteins, (3) and (4) L12 depletion supernatant proteins.

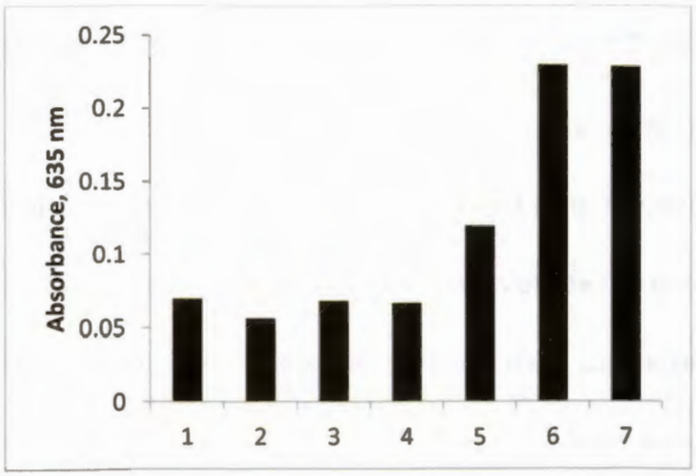

Figure 14. Malachite green assay assessing stimulation of GTPase activity by depleted and reconstituted 70S ribosomes following initial depletion step. (1) Buffer + GTP, (2) 70S + GTP, (3) EF-G + GTP, (4) L12 + GTP, (5) 70SΔL12 + EF-G + GTP, (6) 70SΔL12 + L12+ EF-G + GTP, (7) 70S + EF-G + GTP.

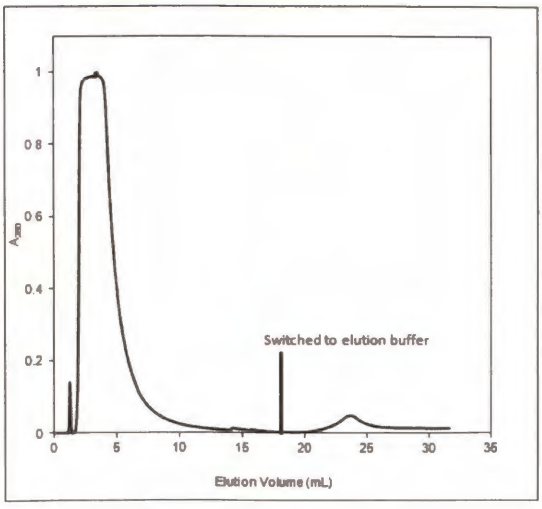

Figure 15. Elution profile of depleted 70S ribosomes over a Ni-NTA column.

Assessment of Fully Depleted 70S Ribosomes

Following the two-step depletion scheme, characterization of the fully depleted ribosomes was undertaken. Once again taking advantage of the $(His)_6$-tag on L12, the HisDetector AP-Ni Western Blot Kit was utilized to detect the presence of L12 in the partially and fully depleted ribosomes. A strong band corresponding to L12 was present in the undepleted JE28 70S ribosomes. A faint L12 band was detected in the partially depleted 70S but was absent from the fully depleted population.

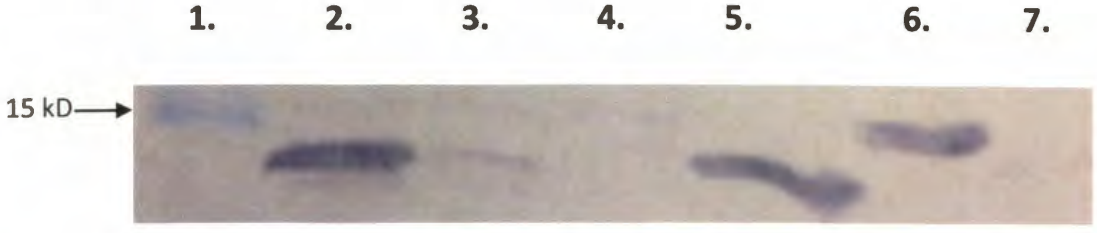

Figure 16. Western blot detecting presence of $(His)_6$-tagged L12. Lanes: (1) Molecular weight marker (15 kD), (2) 70S ribosomes, (3) Depleted 70S, (4) Fully depleted 70S, (5) Supernatant proteins, (6) L12, (7) Lysozyme.

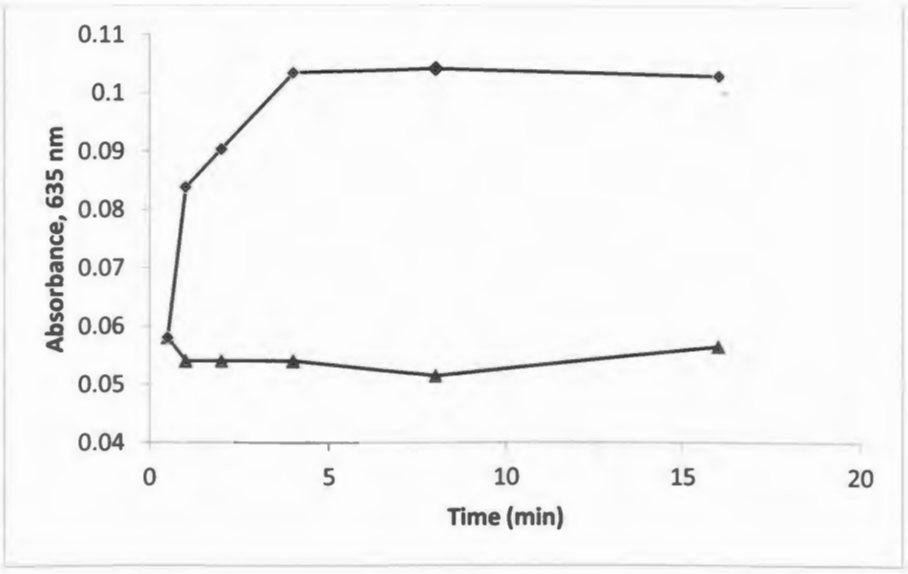

Figure 17. Effects of L12 depletion on the GTP hydrolysis activity of EF-G. (◆) Wild-type 70S + EF-G +GTP. (▲) Fully-depleted 70S + EF-G + GTP.

Preliminary GTPase Hydrolysis and Binding Studies

It has been well-documented that L12 is necessary for the ribosome-dependent GTP hydrolysis activity of EF-G.[45-47] Subsequently, to assess the extent of L12 depletion of the partially- depleted versus fully-depleted ribosomes, the malachite green assay was used to measure the ribosome-dependent GTP hydrolysis activity of EF-G. A reduction in the GTPase activity of EF-G in the presence of the fully-depleted versus partially-depleted ribosomes was seen, which was restored upon incubation of these ribosomes with a 5-fold excess of L12 for 30 minutes prior to addition of EF-G (Figure 18).

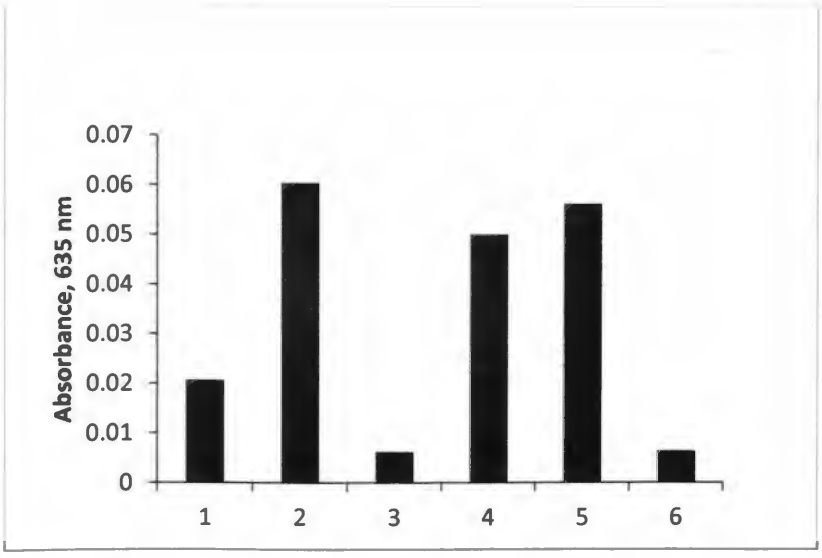

Figure 18. Effects of L12 removal on GTP hydrolysis activity of EF-G. (1) Partially-depleted 70S + EF-G + GTP, (2) Reconstituted partially-depleted 70S + EF-G + GTP, (3) Fully-depleted 70S + EF-G + GTP, (4) Reconstituted fully-depleted 70S + EF-G + GTP, (5) Wild-type 70S + EF-G + GTP, (6) EF-G + GTP

Discussion

The field of ribosome study has advanced rapidly in the past two decades. However, many aspects remain not fully understood, including the role of protein L12 in GTPase binding, recruitment, and GTP hydrolysis activity. Studying the ribosome in the complete absence of L12 is a difficult proposition. A genetically engineered line of ribosomes, JE105, which contain only one of two L12 dimers, was shown to have significantly retarded growth and reduced fitness in comparison with wild-type ribosomes.[51] Because L12 is required for efficient protein synthesis, chemical treatments to remove L12 in vitro are necessary, as opposed to engineering ribosomes lacking this protein completely. NH_4Cl-ethanol treatment, however, is an imprecise technique; it inherently creates heterogeneous populations of ribosomes.

It is not possible to quantify the number of L12 proteins present on each individual ribosome following depletion. However, the focus of this work was to optimize a novel technique for separating these populations of ribosomes by taking advantage of the engineered C-terminal $(His)_6$-tag present on each copy of L12 in JE28. In this work, we demonstrated the use of an affinity chromatography-based second depletion step to isolate a population of fully depleted ribosomes. The elution profile of the ribosomes indicated the separation of populations containing L12 versus those with this protein fully removed. Western blotting was used to further confirm this result; ribosomes following the second depletion step lacked the faint L12 band present in those ribosomes following the initial depletion. We then began to investigate the effects of fully removing L12 on the GTP hydrolysis activity of canonical GTPase EF-G. In agreement with previous reports,[43-45] we found the GTP hydrolysis activity of EF-G to

be severely reduced upon removal of L12, with a noted reduction in activity between EF-G in the presence of fully depleted ribosomes and partially depleted.

This work has laid the foundation for a comprehensive examination of the role of L12 on GTPase activity and binding for translational GTPases EF-G, IF-2, RF-3, EF-4 and BipA. While studies have previously examined some of these effects on individual GTPases, a comprehensive comparative study of the effects of L12 depletion amongst different GTPase translation factors has been lacking. Current investigations are underway comparing the GTPase activity of factors in addition to EF-G using the fully depleted 70S ribosomes. Probing the effects of removing L12 on factor binding will also be a crucial undertaking. In the case of EF-G, a fluorescence binding assay will be employed. This protocol takes advantage of a fluorescent tag engineered onto EF-G, the signal of which is quenched upon binding to the ribosome. For the remaining GTPases, sucrose gradient fractionation studies will be a valuable tool in assessing the ability of these proteins to bind to fully depleted ribosomes. Ultimately, there is hope that these studies will help elucidate the role of L12 and lead to a greater understanding of the intricacies of the ribosome and the process of translation.

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