Summer 2017

Intermolecular interactions that lead to the activation and inhibition of ribosome-dependent GTPases

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Intermolecular interactions that lead to the activation and inhibition of ribosome-dependent GTPases

By

Amanda J. Weis

Accepted in Partial Completion of the Requirements for the Degree Master of Science

Kathleen L. Kitto, Dean of the Graduate School

ADVISORY COMMITTEE

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Dr. Gerry Prody

Dr. John Antos
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Amanda J. Weis
July 2017
Intermolecular interactions that lead to the activation and inhibition of ribosome-dependent GTPases

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

By
Amanda J. Weis

July 2017
Abstract

Ribosomes are the macromolecular machines responsible for protein synthesis across all domains of life. Translation of genetic information into a polypeptide by ribosomes is facilitated by a multitude of proteins called translation factors, many of which belong to the guanosine 5' triphosphate hydrolase (GTPase) superfamily that utilize the hydrolysis of GTP to exert their function. Many naturally occurring antibiotics inhibit protein biosynthesis by targeting the bacterial ribosome or associated translation factors and with increasing antibiotic resistance due to bacterial evolution, the importance of studying ribosome-translation factor interactions is amplified. Determination of high-resolution structures of the ribosome has significantly bolstered our understanding of translation, yet crucial mechanisms remain poorly understood, including the mechanism of ribosome-dependent GTPase binding and activation. Several GTPases harbor conserved G domains, which bind to conserved regions of the ribosomal subunit interface. It has been observed that a conserved sequence in the C-terminal domain (CTD) of ribosomal protein L12 makes direct contacts with the G’ subdomain of the GTPase elongation factor G (EF-G), and L12-depletion studies have demonstrated that the L12 protein is required for binding and GTP hydrolysis by EF-G on the ribosome.

This work aims to identify key residues in the conserved binding region of L12 that are critical for GTPase recruitment and activation. Based on careful structural study of the L12-GTPase binding interface, single amino acid point mutations (L12-K66A, -K66D, -K82A, -K82D, -K85A, -K85D, -T77W, and -T77A) were generated
by site-directed mutagenesis to assess functionality of conserved residues. By an established L12 depletion protocol, wild-type (wt) L12 was completely removed, followed by reconstitution of L12 mutant proteins to ribosomal complexes for analysis of their ability to stimulate GTPase activity.

Here, we show that removal of the G’ subdomain reduces activity of EF-G by 60% in both the presence and absence of L12 on the ribosome while wt EF-G activity is completely abrogated in the presence of L12-depleted ribosomes. Furthermore, reconstitution of L12-depleted ribosomes with an L12 CTD mutant was insufficient for restoration of any lost activity of wt EF-G, indicating that the L12 N-terminal domain is required for proper function of L12. Finally, substitution of an aspartic acid in place of the highly conserved lysine 82 in the CTD of L12 decreased wt EF-G activity to 20%, marking the importance of charge-charge interactions at the L12-GTPase binding interface. Together, these results support and extend understanding of the essential role of L12 in recruitment and activation of ribosome-dependent GTPases, paving the way for future work on development of new antibiotic molecules targeting these interactions.
I extend my gratitude to Dr. P. Clint Spiegel for his mentorship, patience, and encouragement. His expertise and critical thinking proved indispensable for assisting me in experimental design and performance. I would also like to thank my thesis committee, Dr. Gerry Prody and Dr. John Antos, not only for their time critiquing my thesis project, but also for their educational support and excitement for science through my undergraduate and graduate studies at Western Washington University.

Many thanks are extended to Justin Walter, Michelle Wuerth, and Markus Carlson for laying the groundwork for this project, as well as past and current members of the Spiegel lab for their thoughtful discussions in and about scientific research. Importantly, I’d like to thank my fellow Spiegel lab graduate students Serena Wo and Ian Smith for their friendship, support, and scientific discussions throughout the program.

A special thanks to the Chemistry office staff, who make everything in the department run smoothly, including reserving space for numerous meetings and presentations.
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<th>Name</th>
<th>Function</th>
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<tr>
<td>30SPIC</td>
<td>30S pre-initiation complex</td>
<td>An assembly of IF1, IF2, IF3, the mRNA template, and initiator fMet-tRNA&lt;sup&gt;fMet&lt;/sup&gt;</td>
</tr>
<tr>
<td>70SIC</td>
<td>70S initiation complex</td>
<td>An assembly of the mRNA, fMet-tRNA&lt;sup&gt;fMet&lt;/sup&gt;, and the 30S and 50S subunits</td>
</tr>
<tr>
<td>A site</td>
<td>Aminoacyl site</td>
<td>Binding site for aa-tRNAs on the ribosome</td>
</tr>
<tr>
<td>aa-tRNA</td>
<td>Aminoacyl transfer ribonucleic acid</td>
<td>tRNA bound to an amino acid</td>
</tr>
<tr>
<td>ASL</td>
<td>Anticodon stem loop</td>
<td>Portion of a tRNA molecule that is complementary to the mRNA codon</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance unit</td>
<td>Measurement of the amount of light that a particular substance absorbs</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
<td>Measurement of circularly polarized light; technique to determine secondary structure and foldedness of a protein</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>Cryo-electron microscopy</td>
<td>Technique used to study protein structure</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
<td>The domain of a protein that harbors the terminal carboxyl group</td>
</tr>
<tr>
<td>CV</td>
<td>Column volume</td>
<td>Volume of packed resin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td>Storage medium for genes used by all prokaryotes and eukaryotes</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
<td>Refers to the four deoxyribonucleotides: dATP, dCTP, dGTP, and dTTP</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
<td>Gram-negative bacteria</td>
</tr>
<tr>
<td>EF-G</td>
<td>Elongation factor G</td>
<td>GTPase; involved in translocation and ribosome recycling</td>
</tr>
<tr>
<td>E site</td>
<td>Exit site</td>
<td>Site of deacylated tRNA binding before exiting the ribosome</td>
</tr>
<tr>
<td><strong>EF-Ts</strong></td>
<td>Elongation factor thermostable</td>
<td>Serves as the guanine nucleotide exchange factor for EF-Tu</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>EF-Tu</strong></td>
<td>Elongation factor thermolabile</td>
<td>GTPase; responsible for catalyzing the binding of an aa-tRNA to the ribosome</td>
</tr>
<tr>
<td><strong>fMet-tRNAfMet</strong></td>
<td>N-formylmethionine transfer RNA</td>
<td>The first amino acid in all prokaryotic proteins</td>
</tr>
<tr>
<td><strong>FPLC</strong></td>
<td>Fast protein liquid chromatography</td>
<td>Technique to analyze or purify proteins</td>
</tr>
<tr>
<td><strong>G domain</strong></td>
<td>GTP-binding domain</td>
<td>Evolutionarily conserved domain in all GTPases that imparts GTP hydrolysis functionality</td>
</tr>
<tr>
<td><strong>GAC</strong></td>
<td>GTPase associated center</td>
<td>Portion of the 50S subunit where translational GTPases primarily associate</td>
</tr>
<tr>
<td><strong>GAP</strong></td>
<td>GTPase activating protein</td>
<td>Induces a conformational change in GTPases that promotes GTP hydrolysis</td>
</tr>
<tr>
<td><strong>GDP</strong></td>
<td>Guanosine 5′-diphosphate</td>
<td>Product of GTP cleavage by GTPases</td>
</tr>
<tr>
<td><strong>GDPNP</strong></td>
<td>Guanosine 5′-([β,γ-imido]triphosphate)</td>
<td>Non-hydrolyzable analog of GTP</td>
</tr>
<tr>
<td><strong>GEF</strong></td>
<td>Guanine nucleotide exchange factor</td>
<td>Facilitates the exchange of GTP for GDP to regenerate the active form of GTPases</td>
</tr>
<tr>
<td><strong>GTP</strong></td>
<td>Guanosine 5′-triphosphate</td>
<td>Substrate for GTPases that is hydrolyzed to induce a conformational change that provides energy for GTPase function</td>
</tr>
<tr>
<td><strong>GTPase</strong></td>
<td>Guanosine 5′-triphosphate hydrolase</td>
<td>Superfamily of protein enzymes that hydrolyze GTP</td>
</tr>
<tr>
<td><strong>IF1</strong></td>
<td>Initiation factor 1</td>
<td>Involved in translation initiation</td>
</tr>
<tr>
<td><strong>IF2</strong></td>
<td>Initiation factor 2</td>
<td>GTPase; involved in translation initiation</td>
</tr>
<tr>
<td><strong>IF3</strong></td>
<td>Initiation factor 3</td>
<td>Involved in translation initiation</td>
</tr>
<tr>
<td><strong>IMAC</strong></td>
<td>Immobilized metal affinity chromatography</td>
<td>Method of purifying proteins that contain a (His)_6-tag</td>
</tr>
<tr>
<td><strong>IPTG</strong></td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
<td>Allolactose mimic, induces overexpression of proteins on vectors</td>
</tr>
<tr>
<td><strong>LB</strong></td>
<td>Lysogeny broth</td>
<td>Liquid media used to grow bacterial cells</td>
</tr>
<tr>
<td><strong>LepA</strong></td>
<td><strong>Leader Peptidase A</strong></td>
<td><strong>GTPase; involved in 30S biogenesis</strong></td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td><strong>mRNA</strong></td>
<td><strong>Messenger RNA</strong></td>
<td><strong>Molecules that convey genetic information from DNA to the ribosome; dictates the order of amino acid addition</strong></td>
</tr>
<tr>
<td><strong>MW</strong></td>
<td><strong>Molecular weight</strong></td>
<td><strong>The mass of one mole of a substance</strong></td>
</tr>
<tr>
<td><strong>MWCO</strong></td>
<td><strong>Molecular weight cut off</strong></td>
<td><strong>The lowest molecular weight (in daltons) in which 90% of the protein is retained by the membrane</strong></td>
</tr>
<tr>
<td><strong>Ni-NTA</strong></td>
<td><strong>Nickel-nitrilotriacetic acid</strong></td>
<td><strong>Substrate used to bind molecules containing a (His)$_6$-tag</strong></td>
</tr>
<tr>
<td><strong>NTD</strong></td>
<td><strong>N-terminal domain</strong></td>
<td><strong>The domain of a protein that contains the terminal α-amino group</strong></td>
</tr>
<tr>
<td><strong>OD</strong></td>
<td><strong>Optical density</strong></td>
<td><strong>The degree to which a refractive medium retards transmitted rays of light; measured as a monitor of bacterial growth rate</strong></td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td><strong>Polymerase chain reaction</strong></td>
<td><strong>Method to amplify DNA</strong></td>
</tr>
<tr>
<td><strong>PDBid</strong></td>
<td><strong>Protein data bank identification number</strong></td>
<td><strong>Code used to identify molecular structures that have been deposited onto <a href="http://www.rcsb.org">www.rcsb.org</a></strong></td>
</tr>
<tr>
<td><strong>P site</strong></td>
<td><strong>Peptidyl site</strong></td>
<td><strong>Binding site for peptidyl- and deacylated tRNA molecules on the ribosome</strong></td>
</tr>
<tr>
<td><strong>P$_i$</strong></td>
<td><strong>Inorganic phosphate</strong></td>
<td><strong>Product of cleavage of GTP by GTPases</strong></td>
</tr>
<tr>
<td><strong>PMSF</strong></td>
<td><strong>Phenylmethane sulfonyl fluoride</strong></td>
<td><strong>Serine protease inhibitor, used to prevent degradation of overexpressed proteins prior to purification</strong></td>
</tr>
<tr>
<td><strong>PostTC</strong></td>
<td><strong>Post-termination ribosomal complex</strong></td>
<td><strong>The 70S ribosomal complex after peptide cleavage</strong></td>
</tr>
<tr>
<td><strong>PTC</strong></td>
<td><strong>Peptidyl transferase center</strong></td>
<td><strong>The site of peptide bond formation on the 50S subunit</strong></td>
</tr>
<tr>
<td><strong>r proteins</strong></td>
<td><strong>Ribosomal proteins</strong></td>
<td><strong>Proteins, in conjunction with rRNA, that make up the ribosomal subunits</strong></td>
</tr>
<tr>
<td><strong>RF1</strong></td>
<td><strong>Release factor 1</strong></td>
<td><strong>Involved in translation termination</strong></td>
</tr>
<tr>
<td>RF2</td>
<td>Release factor 2</td>
<td>Involved in translation termination</td>
</tr>
<tr>
<td>-------</td>
<td>------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>RF3</td>
<td>Release factor 3</td>
<td>GTPase; involved in translation termination</td>
</tr>
<tr>
<td>RRF</td>
<td>Ribosome recycling factor</td>
<td>Involved in ribosome recycling</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
<td>Nucleic acid present in all living cells; many varied cellular functions</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
<td>Forms part of the ribosome</td>
</tr>
<tr>
<td>S</td>
<td>Svedberg unit</td>
<td>Sedimentation coefficient that reflects the rate at which a molecule sediments in a given solvent</td>
</tr>
<tr>
<td>SDM</td>
<td>Site-directed mutagenesis</td>
<td>Method to incorporate mutations into a gene of interest</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
<td>Method of separating proteins based on molecular weight</td>
</tr>
<tr>
<td>SRL</td>
<td>Sarcin ricin loop</td>
<td>Portion of the 23S rRNA essential for GTP hydrolysis catalyzed steps of translation</td>
</tr>
<tr>
<td>T. thermophilus</td>
<td>Thermus thermophilus</td>
<td>Gram-negative bacteria</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
<td>Binds amino acids and associates with the ribosome</td>
</tr>
<tr>
<td>UV/Vis spectroscopy</td>
<td>Ultraviolet-visible spectroscopy</td>
<td>Technique for quantification of ribosomes</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
<td>Reagent used to reduce disulfide bonds caused by proximal cysteine residues in a protein</td>
</tr>
</tbody>
</table>
Chapter 1 - Ribosomes: structure and function

Ribosomes are universally conserved, large ribonucleoprotein particles that catalyze protein synthesis across all domains of life. Prokaryotic and eukaryotic ribosomes contain an evolutionarily conserved core and have evolved through the addition of proteins and RNA (Melnikov et al., 2012). Eukaryotic ribosomes are more complicated than their evolutionary counterpart in bacteria, with expansion segments, variable regions in the ribosomal RNA (rRNA), and additional ribosomal proteins (r proteins), all of which confer unique functionalities to ribosomes across domains (Figure 1-1; Armache et al., 2010).

Figure 1-1. Comparison of 70S and 80S ribosomes. (A) Surface representation of the 70S prokaryotic ribosome from *Thermus thermophilus* (PDBid 4Z8C). (B) Surface representation of the 80S eukaryotic ribosome from *Saccharomyces cerevisiae* (PDBid 4V7R). Ribosomal RNA constituting the large and small subunit are colored magenta and green, respectively, with ribosomal proteins depicted in blue.
**Prokaryotic ribosome structure**

The prokaryotic ribosome consists of a large and small subunit, termed 50S and 30S, respectively, that assemble to form the 70S ribosomal complex (S stands for Svedberg unit, which is a sedimentation coefficient that reflects the rate at which a molecule sediments in a given solvent, Figure 1-2; Tissieres et al., 1959). The basic internal structure of the ribosome is defined predominantly by rRNA, which also carries out the enzymatic activity of the ribosome, whereas proteins are found mostly on the exterior and are largely nonenzymatic (Yusupov et al., 2001). The 50S subunit is comprised of 23S rRNA, 5S rRNA, and 31 r proteins named L1-31 (Ban et al., 2000). The 23S rRNA contains universally conserved sequences essential for subunit assembly (Lancaster et al., 2008), substrate binding, factor binding, and catalysis on the ribosome (Ban et al., 2000), all of which will be described in detail below. The 30S subunit consists of 16S rRNA and 21 r proteins termed S1-21 (Wimberly et al., 2000). The 16S rRNA allows for spontaneous association of the ribosome with mRNA in the initial phase of protein synthesis through complementary base-pairing (Gualerzi and Pon, 1990). The most functionally important region of the 30S subunit is the geometric center of the subunit, where helical elements of the 16S rRNA create three distinct binding sites for transfer RNA (tRNA): aminoacyl (A site), peptidyl (P site), and exit (E site) (Wimberly et al., 2000).
Transfer RNA

The functional role of tRNA is to carry amino acids to the ribosome for sequential addition to a growing polypeptide chain. The secondary structure of tRNA forms a cloverleaf shape with Watson-Crick base pairs stabilizing each distinct stem
The anticodon stem loop (ASL) is complementary to the messenger RNA (mRNA) codon in the empty A site of the 30S subunit and the acceptor stem consists of the conserved 3’ terminal CCA sequence to which an amino acid has been linked by aminoacyl tRNA synthetase (Ruff et al., 1991). These stems, together with the D stem loop, the T-arm, and an extra loop of variable size, fold into an L-shaped tertiary configuration (Figure 1-3B; Agirrezabala and Valle, 2015). Three tRNAs can be bound simultaneously to the ribosome (Rheinberger et al., 1981). The classical A, P, and E sites of the 70S ribosome bind tRNAs in their A/A, P/P, and E/E states, respectively (where the first and second letters indicate tRNA contacts on the 30S and 50S subunit, respectively). In each of these binding sites a tRNA contacts both the 30S subunit (with its ASL) and the 50S subunit (with its acceptor arm, Figure 1-3C; Yusupov et al., 2001). In the transition between sites on the ribosome, tRNAs acquire intermediate configurations called hybrid and chimeric hybrid states (Agirrezabala et al., 2008), which have been visualized through structural studies of ribosomes trapped in intermediate states of translation. These snapshots of intermediate states have been indispensable in understanding how the ribosome interacts with its tRNA substrates, contributing to a more complete view of the mechanisms of protein synthesis.
Figure 1-3. Transfer RNA. (A) Secondary structure of tRNA. (B) Initiator tRNA from *Escherichia coli*, representing the folded, tertiary structure and indicating the anticodon region and the 3’ aminoacyl recognition site (PDBid 3CW5). (C) Depiction of tRNAs making contact with both the 50S subunit (with acceptor stem) and 30S subunit (with ASL).
Translation

As mentioned above, protein synthesis is the vital translation of ribonucleic acids of an mRNA strand into a chain of amino acids destined to be a functional protein. This intricate process has taken decades to elucidate, yet many mechanistic details remain unknown. Visualization of ribosomal complexes has been achieved by cryo-electron microscopy (cryo-EM) and X-ray crystallography studies, which has helped create a better understanding of the four discrete phases of translation (Simonetti et al., 2008; Yusupov et al., 2001). Initiation is the first phase, requiring the assembly of an initiation complex and the placement of the initiator tRNA over the start codon of the mRNA in the P site (Simonetti et al., 2008). The second phase, elongation, is an iterative, sequential addition of amino acids to the nascent polypeptide (Zhou et al., 2014). Third is termination of translation, whereby an mRNA stop codon signals the end of the coding sequence (Laurberg et al., 2008). The fourth and final phase is recycling, in which the ribosomal subunits dissociate for a new round of protein synthesis to begin (Wilson et al., 2005). Each step is regulated by proteins termed translation factors, many of which are guanosine 5’ triphosphate hydrolases (GTPases) that hydrolyze guanosine 5’-triphosphate (GTP) to guanosine 5’-diphosphate (GDP) to exert their function.

Initiation

A defined sequence of events for the mechanism of prokaryotic translation initiation remains elusive despite ongoing research. Ultimately, three initiation factors (IF1, IF2, and IF3), the mRNA, and initiator N-formylmethionine tRNA (fMet-
tRNA\textsuperscript{fMet} assemble in a 30S pre-initiation complex (30SPIC), followed by 50S subunit association to form the 70S initiation complex (70SIC) (Figure 1-4; Milon et al., 2012). The association of mRNA with the 30S subunit is spontaneous and mediated by the Shine-Dalgarno sequence, located upstream of the start codon and complementary to a purine-rich region of the 16S rRNA (Shine and Dalgarno, 1974). The mRNA and fMet-tRNA\textsuperscript{fMet} bind stochastically (Grigoriadou et al., 2007), and the order of IF2•GTP and fMet-tRNA\textsuperscript{fMet} binding to the 30S subunit does not follow a defined sequence (Tsai et al., 2012), although it has been suggested that fMet-tRNA\textsuperscript{fMet} is recruited after IF2 has associated with the complex (Milon et al., 2012), possibly due to kinetic stability induced by a favorable ribosome conformation (Milon et al., 2010). Interaction of IF2 with the formyl group of fMet-tRNA\textsuperscript{fMet} is dependent on the presence of IF1 and is pivotal both for fast formation of the fMet-tRNA\textsuperscript{fMet}-containing 30SPIC and for rapid association of the 50S subunit (Antoun et al., 2006). For subunit joining, IF3 dissociates after the canonical initiator tRNA binds in the A site (Grigoriadou et al., 2007) allowing for IF2 and fMet-tRNA\textsuperscript{fMet} to create a contact region at the subunit interface for 50S subunit docking, resulting in formation of a 70SIC (Sprink et al., 2016). This promotes a conformational change in the 30S subunit that triggers hydrolysis of GTP by IF2 and release of the factors (Sprink et al., 2016). Finally, counterclockwise rotation of the 30S subunit with respect to the 50S subunit results in an elongation-competent 70S ribosomal complex (Marshall et al., 2009).
Figure 1-4. Schematic representation of translation initiation. The mRNA template spontaneously base-pairs with the 30S subunit, with stochastic association of initiation factors IF1-3 and initiator tRNA. Hydrolysis of GTP by IF2 triggers factor dissociation and subunit joining, forming the 70S ribosomal complex (PDBid 5LMV).

**Elongation**

The elongation phase allows for the sequential addition of amino acids to the polypeptide chain. It is regulated by the two translocational GTPases, EF-Tu and EF-G, and can be broken down into three steps: decoding, peptide bond formation, and translocation.

**Decoding**

Decoding is a critical process that assures accuracy of the translation of an mRNA template into an amino acid polypeptide chain (Valle et al., 2002). This cycle
begins with an aminoacyl-tRNA (aa-tRNA) binding in the empty A site of the ribosome (Rodnina et al., 1996). The aa-tRNA is delivered to the ribosome as a ternary complex with EF-Tu bound to GTP (Hazlett et al., 1989) in a partially bound A/T state in which the ASL of the aa-tRNA binds in the A site of the 30S subunit while the acceptor arm contacts the GTPase associated center (GAC) of the 50S subunit (Sanbonmatsu et al., 2005). A labile ribosomal complex is formed through protein-protein interactions of EF-Tu with the ribosomal stalk, a multi-r-protein protrusion that is critical for the binding of multiple translation factors that will be described in detail in the following chapter (Helgstrand et al., 2007). After this initial binding, the mRNA codon and tRNA anticodon line up within the A site of the 30S subunit (Girshovich et al., 1986). Selection of the correct aa-tRNA that matches the mRNA codon occurs in two consecutive selection steps: initial selection (prior to GTP hydrolysis) and proofreading (after GTP hydrolysis but before peptide bond formation) (Pape et al., 1999). The ribosome actively selects for the correct (cognate) aa-tRNA through shape discrimination of the codon-anticodon complex (Yoshizawa et al., 1999). As proposed by an induced fit mechanism, a cognate codon-anticodon interaction induces a particular conformation of the 16S rRNA decoding center that promotes GTPase activation and A site accommodation of the aa-tRNA (Pape et al., 1999; Rodnina and Wolfgang, 2001; Schmeing et al., 2005). Upon recognition of the cognate codon-anticodon pair, the incoming complex of EF-Tu•GTP•aa-tRNA rotates toward the P site (Blanchard et al., 2004b) to allow productive interaction of EF-Tu with the decoding center of the 16S rRNA (Rodnina et al., 1995). This conformational
change triggers GTP hydrolysis by EF-Tu (Kaziro, 1978; Dell et al., 1990), inducing movement of the aa-tRNA from the partially bound A/T state to the fully bound A/A state (Sanbonmatsu et al., 2005) for accommodation in the peptidyl transferase center on the 50S subunit (Pape et al., 1998; Noel and Whitford, 2016). The rate of GTP hydrolysis by EF-Tu differs for cognate, near-cognate, and non-cognate ternary complexes, serving as the initial discrimination against non-matching tRNAs (Pape et al., 1999; Maracci and Rodnina, 2016).

Initial selection and proofreading grant high fidelity and efficiency of aa-tRNA selection by the ribosome with error frequencies observed between $10^{-5}$ and $10^{-3}$ (Wohlgemuth et al., 2011). Accuracy of aa-tRNA selection is accounted for through not only the strength of Watson-Crick pairing between the codon and anticodon, but also by selective stabilization of cognate aa-tRNAs by the ribosome (Noller, 2013). If a near- or non-cognate aa-tRNA is accommodated in the A site, it can be rejected through an induced fit mechanism, as described for initial tRNA selection (Pape et al., 1999; Thompson and Stone, 1977). Recent kinetic studies suggest that proofreading occurs through two discrete steps; first, there is proofreading of aa-tRNA in the ternary complex with EF-Tu•GDP, then EF-Tu•GDP dissociates and there is an additional proofreading of aa-tRNA in an EF-Tu independent manner, allowing for strictly cognate aa-tRNA accommodation into the A site (Figure 1-5; Leong et al., 2016).
Figure 1-5. Schematic representation of tRNA selection, proofreading, and accommodation. EF-Tu brings the correct aa-tRNA to the 70S ribosome as dictated by the mRNA, with GTP hydrolysis leading to accommodation. Two proofreading steps lead to rejection of EF-Tu•GDP•aa-tRNA or aa-tRNA (PDBid 5LMV).
**Peptide Bond Formation**

The formation of peptide bonds is catalyzed by rRNA located in the peptidyl transferase center (PTC) on the 50S subunit (Nissen et al., 2000). The peptidyl transferase reaction occurs through an induced fit mechanism in which the aa- and peptidyl-tRNA substrates and 23S rRNA active-site residues are repositioned so that the ester group of the peptidyl-tRNA is accessible for nucleophilic attack by the α-amino group of the aa-tRNA (Schmeing et al., 2005). A tetrahedral intermediate forms and undergoes proton shuttling to yield a deacylated tRNA in the P site and the nascent peptide extended by one amino acid esterified to the A-site-bound tRNA (Figure 1-6; Trobro and Aqvist, 2005). This proton shuttling mechanism for transpeptidation was established by Lang et al. (2008) in which the 2′-hydroxyl group at A2451 of 23S rRNA directly hydrogen bonds to the P-site tRNA-A76 ribose. Once peptide-bond formation has occurred, the deacylated-tRNA in the P site and peptidyl-tRNA in the A site move to the E and P sites, respectively, in the process known as translocation.
Figure 1-6. Schematic representation of the peptidyl transfer step during elongation. First, the aa-tRNA undergoes a conformational change that allows for peptidyl transfer. Then, the elongating peptide is transferred from the P site tRNA to the A site-bound tRNA via catalysis by the 23S rRNA.

Translocation

Translocation is the essential process during elongation in which tRNAs and the mRNA shift relative to the ribosome in order to clear the A site for addition of the next amino acid to the polypeptide. Briefly, the mRNA template shifts by precisely one codon and the tRNAs move from the A and P sites to the P and E sites, respectively (Schmeing and Ramakrishnan, 2009).

Chemical footprinting has revealed that translocation of tRNAs largely occurs in two separate steps (Moazed and Noller, 1989b). First, after peptide bond
formation, the acceptor end of tRNAs in the P and A sites move relative to the 50S subunit, while the anticodon ends remain bound to the P and A sites of the 30S subunit, resulting in P/E and A/P hybrid states of tRNA binding, respectively (Figure 1-7A/B; Moazed and Noller, 1989b; Spiegel et al., 2007; Zhou et al., 2014). This initial movement is facilitated by intersubunit rotation in pre-translocation ribosomes, which is spontaneous and reversible (Blanchard et al., 2004a; Cornish et al., 2008; Moazed and Noller, 1989a). The second step of translocation is catalyzed by elongation factor G (EF-G), in which the tRNA ASLs and associated mRNA codons move from the A and P sites to the P and E sites of the 30S subunit, advancing the tRNAs into the classical P/P and E/E states (Figure 1-7C; Moazed and Noller, 1989b). Binding of the EF-G•GTP complex stabilizes the hybrid state conformation of the 70S ribosome (Spiegel et al., 2007), with GTP hydrolysis and inorganic phosphate release possibly triggering another structural rearrangement that further increases rate of translocation (Peske et al., 2000; Savelsburg et al., 2003). Domain IV of EF-G then penetrates the A site and either pushes the A-tRNA into the P site and/or occupies the A site after translocation, preventing backward slippage of the tRNAs (Wilson and Noller, 1998; Connell et al., 2008).
Movement of tRNAs during translocation occurs in two separate steps. (A) First, the tRNAs are in the classical P/P and A/A states. (B) Spontaneous intersubunit rotation leads acceptor stems into the E and P sites of the 50S subunit, while ASLs remain bound in original sites of the 30S subunit. (C) tRNA ASLs move upon GTP hydrolysis by EF-G to form classical E/E and P/P binding states.

Termination

Translation is terminated when the nascent polypeptide is hydrolyzed by a release factor after recognition of an mRNA nonsense (stop) codon in the A site. Three stop codons are decoded by translational proteins termed class I release factors (RF): UAG is recognized by RF1, UGA by RF2, and UAA is recognized by both (Brenner et al., 1965; Brenner et al., 1967; Scolnick et al., 1968). These two factors are able to identify the same UAA codon but discriminate between UAG (by RF1) and UGA (by RF2) with inherent, unique tripeptide motifs (PxT in RF1 or SPF in RF2, uppercase represents highly conserved residues), equivalent to a tRNA anticodon, that discriminates between the second and third purine bases of the mRNA codon (Ito et al., 2000). Essential for ester bond hydrolysis is the GGQ motif in RF1 and RF2 (Zavialov et al., 2002). Tripeptide discriminators and the GGQ motif are close to the decoding center and PTC, respectively, as suggested.
by directed hydroxyl radical probing (Wilson et al., 2000). Upon binding to the ribosome, domain 3 of RFs, which harbors the GGQ motif, flips out from the core of the protein and contacts the PTC on the 50S subunit (Petry et al., 2005). Peptide bond cleavage is accomplished by either of the class I release factors, in which individual interaction of the factors with the PTC is promoted by an induced fit of the codon and 30S subunit that results in conformational stabilization with the GGQ motif, positioned to contribute directly to peptidyl-tRNA hydrolysis (Laurberg et al., 2008; Schmeing et al., 2005; Zavialov et al., 2001). After peptide release, the class II release factor RF3, which is also a GTPase, accelerates dissociation of RF1 and RF2 from the ribosome in a GTP-dependent manner (Freistroffer et al., 1997; Zavialov et al., 2002). Specifically, in the GDP state, RF3 binds to the ribosomal complex and exchanges GDP for GTP, leading to a conformational change that allows RF1 and RF2 to dissociate from the ribosome (Koutmou et al., 2014; Pallesen et al., 2013). Subsequent GTP hydrolysis causes RF3 to dissociate from the ribosome, leaving mRNA and a deacylated tRNA in the P site (Schmeing and Ramakrishnan, 2009). Finally, a conformational rearrangement in the ribosome results in tRNA translocation from the P site to the E site (Klaholz et al., 2004) and the post-termination ribosome must be recycled to initiate active translation again, which is accomplished by separating ribosomal subunits in a controlled manner.

**Recycling**

For a new round of protein synthesis to begin, an essential ribosome recycling factor (RRF) together with EF-G separate the post-termination ribosomal complex (PostTC) into subunits in a process known as ribosome recycling. Binding of RRF
stabilizes the ribosome in a fully rotated state with a deacylated tRNA bound in a hybrid P/E state (Dunkle et al., 2011). Dissociation of the 50S subunit from the PostTC is catalyzed by RRF and EF-G and requires GTP hydrolysis (Karimi et al., 1999; Savelsbergh et al., 2009). After the PostTC has been split into subunits, IF3 binds to the 30S subunit and catalyzes the dissociation of tRNA from the P/E site, preventing the 50S subunit from reassociating prior to the next round of translation (Karimi et al., 1999).

**Ribosomal protein L7/L12**

An essential component to translation initiation, elongation, and termination by the 70S ribosome is ribosomal protein L7/L12, a 12 kDa two domain dimeric protein (Moller et al., 1972) with a double-helical N-terminal domain (NTD) and a globular C-terminal domain (CTD) that are linked by a flexible hinge region (Figure 1-8; Gudkov et al., 1977; Wahl et al., 2000). In *Escherichia coli*, L7 is identical to L12 except for the addition of an N-terminal acetylation (Terhorst et al., 1972) and will be referred to collectively as L12 hereafter. The NTD is responsible for dimerization (Figure 1-8B) and for anchoring of the protein to the ribosome (Gudkov et al., 1995), while the CTD is highly dynamic and contains the conserved binding region for translational GTPases (Mohr et al., 2002). The dynamic nature of the hinge is important for L12 function, especially for the mobility and varied localization of the CTD during translation factor interaction (Diaconu et al., 2005). Unlike other ribosomal proteins, L12 is present in multiple copies on the ribosome.
and it is the only r protein that does not directly bind to rRNA (Deroo et al., 2012; Diaconu et al., 2005). In *E. coli*, four copies of L12 are bound as two dimers via their NTD to ribosomal protein L10, which is directly attached to the rRNA of the 50S subunit, and together with protein L11 this region is collectively known as the ribosomal stalk (Figure 1-9; Subramanian, 1975; Koteliansky et al., 1978). Also depicted in Figure 1-9 is the sarcin ricin loop (SRL) and GTPase associated center (GAC) of the 23S rRNA in the 50S subunit, regions of importance for GTPase functions during translation (Zhou et al., 2013). One L12 dimer (two individual copies of L12) is sufficient to produce active ribosomes, but multiple copies of the dimer are required for efficient initiation and elongation of protein synthesis (Mandava et al., 2012). Despite decades of research towards elucidating the role of L12 in translation, it was only recently reported that L12 is required for activation and stable binding of ribosome-dependent GTPases (Carlson et al., 2017). Importantly, this illustrates the proximity of L12 to the functional center where GTPases are known to associate and supports the role that L12 plays in GTPase recruitment and activation (Carlson et al., 2017).
Figure 1-8. (A) Crystal structure of ribosomal protein L12 from *Thermotoga maritima* (PDBid 1DD4). The N-terminal domain (red) is linked to the C-terminal domain (yellow) by a flexible hinge region (magenta). (B) Nuclear magnetic resonance solution structure of L12 dimer from *E. coli* depicts the dynamic range that L12 possesses (same color scheme with second L12 molecule shown in pale colors).

Figure 1-9. Stalk region of the 50S subunit (PDBid 4W29). Ribosomal RNA (magenta), r proteins (blue), L10 (brown), L11 (cyan), L12 (yellow), SRL (orange), GAC rRNA (green).
Chapter 2 - The GTPase superfamily

A number of accessory protein translation factors regulate the steps of protein synthesis to ensure efficiency and accuracy. At the core of the essential factors are the translational GTPases, which are some of the most conserved proteins across life (Atkinson, 2015). The structural and functional information pertaining to translational GTPases will be expanded upon here, with emphasis on the similarities and differences of the G-domain containing factors: IF2, EF-Tu, EF-G, RF3, and LepA. The research presented herein is focused on these GTPases and their interactions with the 70S ribosome and ribosomal protein L12.

Homology of translational GTPases

Striking conservation is observed among GTPases, even across species. For instance, bacterial IF2, EF-Tu, and EF-G are all very structurally similar to their eukaryotic counterparts eIF5B, eEF1A, and eEF2, respectively (Figure 2-1). By sequence analysis, the families EF1, EF2, and IF2 were found in all domains of life, alluding to their descent from the last universal common ancestor (Leipe et al., 2002), affirming the high conservation of translation. Furthermore, there is high domain conservation within the prokaryotic GTPase family (Figure 2-2), imparting analogous mechanisms of GTP hydrolysis and ribosomal binding.
Figure 2-1. Structural comparison of prokaryotic and eukaryotic GTPases. (A) IF2•GDP *Thermus thermophilus* (PDBid 4KJZ), (B) eIF5B•GDPNP *Methanothermobacter thermautotrophicus* (PDBid 1G7T), (C) EF-Tu•GDP *E. coli* (PDBid 1EFC), (D) eEF1A•GDP *Sulfolobus solfataricus* (PDBid 1SKQ), (E) EF-G•GDP *T. thermophilus* (PDBid 4M1K), (F) eEF2 (apo form) *Saccharomyces cerevisiae* (PDBid 1N0U). Domains of the same color share significant homology.
**Figure 2-2.** Prokaryotic translational GTPases. (A) IF2•GTP (PDBid 4B48), (B) RF3•GDP (PDBid 2H5E), (C) EF-G•GDP (PDBid 4M1K), and (D) LepA (PDBid 3CB4). Domains of the same color share significant homology. The conserved G domain is depicted in red and harbors GTP hydrolysis activity. Additional domains specific for each GTPase are represented in gray.
Some GTPases are universally conserved while others are taxa-specific, suggesting evolution of translational control mechanisms from a base of core factors (Atkinson, 2015). Although GTPases have unique roles during protein synthesis, homologous domains impart similar activation and binding mechanisms. Specifically, the evolutionarily conserved GTP-binding domain (G domain) that provides GTP hydrolysis functionality (Leberman and Egner, 1984) is found in each GTPase that is of interest for this work: IF2, EF-G, RF3, and LepA (Figure 2-2). Both RF3 and LepA were derived from the EF2/EF-G subfamily through lineage-specific duplication and divergence, which is apparent upon structural comparison as they share conserved domains yet have at least one unique domain important for each respective function during translation (Figure 2-2B/C/D; Leipe et al., 2002).

The most highly conserved region among the translational GTPases is the G domain, the site of GTP binding and hydrolysis (Vetter and Wittinghofer, 2001). This domain consists of five conserved motifs, G1-G5, that contact the nucleotide (Figure 2-3). The G1 motif is also known as the P-loop, which holds the α- and β-phosphates of GTP. The G2 and G3 motifs, commonly known as switch I and switch II, respectively, contact the gamma phosphate and the Mg$^{2+}$ cofactor, and are flexible regions that “switch” their conformations during the GTP-to-GDP transition, a mechanism known as molecular switching (Connell et al., 2007). Finally, substrate specificity is attributed to the G4 and G5 motifs, which exclusively bind the guanine base (Maracci and Rodnina, 2016; Vetter and Wittinghofer, 2001). These highly conserved structural elements in the G domain lend
translational GTPases a common mechanism of action to execute their unique regulatory functions in protein synthesis.

**Figure 2-3.** The highly conserved G domain depicting interactions between conserved motifs and the bound guanine nucleotide (PDBid 3K8Y). The GTP analog GDPNP and the Mg$^{2+}$ ion are represented in yellow and cyan, respectively.
Translational GTPase activation

GTPases facilitate protein synthesis on the ribosome by lowering translation kinetic barriers through GTP hydrolysis and subsequent inorganic phosphate release (Figure 2-4A; Maracci and Rodnina, 2016). Acting as molecular switches, GTPases alternate between two distinct conformations: active in the GTP-bound form and inactive when bound to GDP (Wittinghofer and Vetter, 2011). Analogs of GTP, such as guanosine 5'-[β,γ-imido]triphosphate (GDPNP), have the ability to lock GTPases into their GTP bound form due to their inability to hydrolyze the phosphoramidate bond between the β and γ phosphates, which has proved indispensable for structural study of the ribosome trapped in intermediate states of translation (Figure 2-4B; Scheffzek and Ahmadian, 2005).
Figure 2-4. Structures of GTPase nucleotide substrates. (A) Scheme for hydrolysis of GTP to GDP and P$_i$ with alpha, beta, and gamma phosphates labeled. (B) Structure of GDPNP, a GTP analog with a nitrogen atom in place of the oxygen as seen in GTP, rendering it non-hydrolyzable.
**GTPases are regulated by guanine exchange factors and GTPase activating proteins**

Guanine nucleotide exchange factors (GEFs) regenerate the active form of a GTPase by facilitating the exchange of GDP for GTP, while GTPase activating proteins (GAPs) induce a conformational change in the factor that promotes GTP hydrolysis (Figure 2-5; Maracci and Rodnina, 2016). Translational GTPases tend to bind GTP with a similar or higher affinity than GDP, with high cellular GTP concentration inducing spontaneous nucleotide exchange (Maracci and Rodnina, 2016). An exception is observed with EF-Tu, which has a higher affinity for GDP than GTP and therefore, the nucleotide exchange factor elongation factor thermostable (EF-Ts) is required for conversion to the active EF-Tu•GTP form (Fasano et al., 1978; Gromadski et al., 2002). The GEF introduces a conformational change in the nucleotide pocket, promoting both the dissociation of GDP and rapid binding of GTP to EF-Tu (Andersen et al., 2000; Wang et al., 1997). Similar to EF-Tu, free RF3 binds GDP very strongly, although the mechanism of nucleotide exchange remains elusive. It is postulated that a post-termination ribosomal complex (bound with RF1 or RF2) acts as a GEF for RF3, although there is a lack of structural evidence to support it (Gao et al., 2007; Koutouv et al., 2014; Zavialov et al., 2001; Zhou et al., 2012).
Figure 2-5. The GTPase cycle. GTP hydrolysis is stimulated by GAPs, while GEFs promote the exchange of GDP for GTP.

The ribosome is a GTPase activating protein

The ribosome functions as a GAP by dramatically accelerating the GTP hydrolysis reaction of translational GTPases. A universal mechanism for GTPase activation has been proposed in which the ribosome induces a conformational change in the conserved switch I and II regions of the GTPase G domain that promotes GTP hydrolysis (Voorhees et al., 2010). The activation of translational GTPases has been actively debated, and although mechanistic differences are observed across prokaryotic GTPases, a common theme involves contacts between the SRL of the 50S subunit and the GTPase nucleotide pocket, stabilizing the active conformation (Clementi et al., 2010; Daviter et al., 2003; Fischer et al., 2016; Maracci et al., 2014; Voorhees et al., 2010). Many of the translational GTPases bind to the same region of the ribosome, reinforcing the concept that there is a common mechanism by which ribosomes activate GTPases (Voorhees et al., 2010).
Ribosome-GTPase binding region

Interactions between translational GTPases and the 70S ribosome have been studied intensely throughout the previous decades, resulting in identification of transient interactions between the 23S rRNA of the 50S subunit and the G domain of each GTPase (Moazed et al., 1988). Two portions of the 23S rRNA have been observed in binding GTPases: the SRL and the GAC. The SRL consists of 12 highly conserved residues that are critical to ribosome function and suggested to be involved in factor binding based on structural examination and mutagenesis studies (Clementi et al., 2010; Koch et al., 2015; Shi et al., 2012). The GAC is comprised of highly conserved residues 1030-1124 of the 23S rRNA as well as the ribosomal proteins L10, L11, and L12 (Diaconu et al., 2005). As mentioned in the previous chapter, ribosomal protein L12 is required for binding of GTPases to the ribosome and for stimulation of factor-dependent GTP hydrolysis (Carlson et al., 2017; Diaconu et al., 2005; Wahl and Moller, 2002). Direct contacts have been observed between the L12 CTD and the G’ domains of EF-G (Figure 2-6; Zhou et al., 2014) and RF3 (Pallesen et al., 2013), as well as the NTD of IF2 (Simonetti et al., 2013). Despite structural and mutagenesis studies, the specific residues of the L12 CTD that are essential for the function of L12 need to be further investigated (Helgstrand et al., 2007; Kothe et al., 2004; Savelbergh et al., 2005). Noteworthy are the multiple conserved lysines in the CTD, which provide a positively charged binding surface (Figure 2-7).
Figure 2-6. X-ray crystal structure of the 70S ribosome from *T. thermophila*
us trapped in an intermediate state of translocation with EF-G (PDBid 4W29). (A) Ribbon diagram representation of the 70S ribosomal subunit interface depicting 23S rRNA (magenta), 5S rRNA (orange), 16S rRNA (green), EF-G (black), L11 (cyan), L10 (sand), L12 (yellow), and r proteins (blue). (B) Representation of direct interactions between EF-G and the GTPase-associated center, illustrating direct contacts between the G' subdomain of EF-G with the CTD of L12.
Figure 2-7. Binding interface of EF-G G’ subdomain and CTD of L12 (PDBid 4V9J). Conserved residues are shown in stick representation with positive and negative charges shown in blue and red, respectively.
Thesis Statement

The objective of this research was to extend understanding into the function of the conserved GTPase G' subdomain and the L12 CTD in ribosome-dependent GTPase activation. This was accomplished through generation and purification of two domain deletion mutants and eight L12 point mutants: EF-GΔG', L12ΔNTD, L12-K66A, L12-K66D, L12-K82A, L12-K82D, L12-K85A, L12-K85D, L12-T77A, and L12-T77W. By a recently developed two step L12 depletion protocol, we were able to ensure complete removal of wt L12 from 70S ribosomes. Utilizing an inorganic phosphate detection system, the activity of EF-GΔG’ was examined in the presence of 70S and L12-depleted ribosomes. Finally, L12-depleted ribosomes were reconstituted with purified L12 mutants and tested for their ability to stimulate GTP hydrolysis by translational GTPases.
Chapter 3 - Materials and Methods

Buffers

GTPase Lysis Buffer: 50 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 60 mM NH₄Cl, 15 mM imidazole, 10% (v/v) glycerol, 6 mM β-mercaptoethanol

GTPase Wash Buffer: 50 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 60 mM NH₄Cl, 50 mM KCl, 15 mM imidazole, 10% (v/v) glycerol, 6 mM β-mercaptoethanol

GTPase Elution Buffer: 50 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 60 mM NH₄Cl, 250 mM imidazole, 10% (v/v) glycerol, 6 mM β-mercaptoethanol

GTPase Storage Buffer: 50 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 60 mM NH₄Cl, 10% (v/v) glycerol, 1 mM dithiothreitol

JE28 Lysis Buffer: 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 150 mM KCl, 30 mM NH₄Cl, 5 mM imidazole

JE28 Wash Buffer: 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 150 mM KCl, 500 mM NH₄Cl, 5 mM imidazole

JE28 Elution Buffer: 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 150 mM KCl, 30 mM NH₄Cl, 150 mM imidazole

JE28 Salt Wash Buffer: 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 150 mM KCl, 500 mM NH₄Cl
**Ribosome Storage Buffer:** 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 30 mM NH₄Cl, 25% (v/v) glycerol

**L12 Depletion Buffer:** 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 1 M NH₄Cl, 50% (v/v) glycerol, 5 mM β-mercaptoethanol

**1X Tris-Glycine Buffer:** 25 mM Trizma™, 250 mM glycine, 0.1% (w/v) SDS

**1X TAE Buffer:** 40 mM Trizma™ (pH 8.0), 0.1% (v/v) glacial acetic acid, 1 mM EDTA

**5X GTPase Reaction Buffer:** 400 mM Tris-HCl (pH 7.5), 500 mM NH₄Cl, 100 mM MgCl₂

**4X Protein load dye:** 8% (w/v) SDS, 240 mM Tris-HCl (pH 6.8), 40% (v/v) glycerol, 0.04% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol

**Coomassie gel stain:** 0.3% (w/v) Coomassie Brilliant Blue G-250, 50% (v/v) methanol, 10% (v/v) glacial acetic acid

**Gel destain solution:** 30% (v/v) methanol, 10% (v/v) glacial acetic acid
Overexpression and purification of translational GTPases

The *E. coli* genes *FusA*, *lepA*, *infB*, and *prfC* encoding EF-G, LepA, IF2, and RF3, respectively, were previously cloned into a pSV281 overexpression vector using BamHI (5’) and XhoI (3’) restriction sites, introducing an N-terminal (His)_{6}-tag, and transformed into *E. coli* BL21(DE3) cells (Walter, 2010). From glycerol stocks, cells were transferred into ten mL Lysogeny broth (LB: 1% (w/v) Bacto™ Tryptone (BD Biosciences), 0.5% (w/v) Bacto™ yeast extract (BD Biosciences), 1% (w/v) sodium chloride) containing 35 µg/mL kanamycin and were incubated at 37°C overnight at 180 rpm at a 45° angle for proper mixing. After at least eight hours of growth, one liter portions of LB with 35 µg/mL kanamycin were inoculated from the starter cultures and allowed to grow at 37°C with shaking at 180 rpm until the optical density (OD) at 600 nm was approximately 0.5 absorbance units (AU) as monitored by spectroscopy (Hewlett-Packard 8453 spectrophotometer). Induction with 400 µM isopropyl-β-D-thiogalactopyranoside (IPTG) at a temperature of 15°C with shaking overnight lead to overexpression of the protein contained on the plasmid. Cells were pelleted by centrifugation at 6,128 x g and 4°C for ten minutes in an F12-6x500 LEX rotor (Fiberlite™ fixed angle rotor from Thermo Scientific). Once resuspended in 40 mL of GTPase lysis buffer, cells were incubated in 1 mM phenylmethane sulfonyl fluoride (PMSF, Amresco) and 1 mg/mL lysozyme (MP Biomedicals) with gentle shaking at 4°C for 15 minutes. Cells were then lysed through sonication (Branson Sonifier 450, 50% duty cycle, 5 output, 3 x 30 seconds) on ice, and centrifuged at 39,375 x g and 4°C for 45 minutes in an F20-12x50 LEX rotor (Fiberlite™ fixed angle rotor from Thermo Scientific) to remove
cell debris. The soluble fraction was filtered through a 5-µm syringe filter, followed by a 0.45-µm sterile syringe filter for subsequent purification of the (His)\textsubscript{6}-tagged protein via immobilized metal affinity chromatography (IMAC). The filtered lysate was added to nickel-nitrilotriacetic acid (Ni-NTA) metal affinity resin (Qiagen) conditioned with GTPase lysis buffer and gently stirred for at least 30 minutes at 4°C. In a gravity column (Kimble-Chase), the lysate/resin mixture was washed with at least five column volumes (CV) of GTPase lysis buffer, followed by five CVs of GTPase wash buffer, then three CVs of GTPase lysis buffer. GTPases were eluted on ice with GTPase elution buffer until the eluent tested negative for protein using Bradford reagent (Coomassie Plus\textsuperscript{TM} Protein Assay Reagent, Thermo Scientific). Imidazole was removed through dialysis of the purified protein into GTPase storage buffer for at least four hours at 4°C in 12 kDa weight cut off (MWCO) membrane tubing (Spectrum Laboratories, Inc). GTPases were concentrated through a 30 kDa MWCO centrifugal filter (Millipore) and quantified through their molar extinction coefficients (calculated using ExPASy ProtParam) and the Beer-Lambert law at a wavelength of 280 nm on a BioTek\textsuperscript{®} Epoch plate reader. Appropriate size and purity of GTPases was assessed through electrophoresis. GTPases were flash frozen in liquid nitrogen and stored at -80°C.
Electrophoresis

For size and purity analysis of isolated, recombinantly expressed proteins, discontinuous sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Cleveland et al., 1977). The percentage of acrylamide (19:1 by weight acrylamide:bisacrylamide) ranged from 10% (w/v) to 17% (w/v) and is indicated in figure legends. Protein samples were diluted into 4X Protein load dye to a final 1X concentration and were thermally denatured at 95°C for five minutes immediately prior to loading into wells. Gels were subjected to 120 volts in 1X Tris-glycine buffer until the desired separation was observed between molecular weight markers (Fisher BioReagents). Gentle shaking of gels with Coomassie gel stain for at least four hours was followed by incubation with gel destain solution to remove unbound stain and visualize bound, stained proteins. A Bio-Rad Gel Doc™ EZ Imager was implemented to digitize gels and quantify protein band intensities.

To determine the size of genes or plasmids of interest, agarose gel electrophoresis was performed as previously described (Hayward and Smith, 1972). Agarose was dissolved in 1X TAE buffer with heating, followed by the addition of GelRed™ 10,000X Nucleic Acid Gel Stain (Biotium) to a 1X concentration. The DNA samples were diluted into a Blue/Orange 6X DNA loading dye (Promega), loaded into wells, and electrophoresed at 95 volts until the desired separation was reached as visualized by ultraviolet light.
Overexpression and purification of (His)$_6$-tagged 70S ribosomes

A strain of *Escherichia coli* (JE28) with an engineered (His)$_6$-tag at the 3’ end of the single copy *rplL* gene (encoding the ribosomal protein L12) was used. Cells were grown in sterile LB with kanamycin at a final concentration of 35 µg/mL at 37°C until mid-log phase was reached (OD$_{600 \text{ nm}} \sim 1.0$), at which time the cell culture flasks were immediately placed in an ice bath until the temperature reached 4°C. Ribosome extraction and purification was performed in an identical manner to the protocol described above for GTPases, with the exception of JE28 buffers in the place of GTPase buffers. Additionally, IMAC eluted ribosomes were dialyzed into JE28 salt wash buffer prior to two rounds of pelleting by ultracentrifugation in 60Ti polycarbonate tubes (Beckman Coulter) at 150,000 x $g$ for four hours at 4°C. The supernatant was discarded and the final ribosome pellet was resuspended in a small volume of ribosome storage buffer. UV/Vis spectroscopy was implemented for accurate quantification of 70S ribosomes, whereby 1.0 AU at 260 nm of a 1:1000 dilution of purified ribosomes is equal to 26.69 pmol of 70S ribosomes.

Depletion of L12 from 70S ribosomes

To completely deplete 70S ribosomes of L12, an initial salt wash and ethanol precipitation is followed by an IMAC step that removes residual full ribosomal complexes. In a 4°C room, 450 pmol of 70S ribosomes are incubated in L12 depletion buffer in a final volume of 450 µL in S140-AT polycarbonate ultracentrifuge tubes on ice. After five minutes, 225 µL of pre-chilled (-20°C) 99.5% ethanol was added to the reaction and allowed to shake at 800 rpm on a tabletop
pulsing vortex mixer (Fisher Scientific) for five additional minutes. This step was repeated, followed by immediate ultracentrifugation at 150,000 x g for 30 minutes at 4°C to pellet partially depleted ribosomes. The supernatant was incubated with a five-fold excess volume of acetone at -20°C for at least one hour to precipitate depleted proteins for SDS-PAGE analysis. Ribosome pellets were rinsed with JE28 Lysis buffer and allowed to resuspend in JE28 Lysis buffer at 4°C for at least four hours.

Following resuspension, partially depleted ribosomes were 0.22-µm filtered and applied to a five mL Ni-NTA HisTrap™ HP column (GE Healthcare) connected to an AKTA Prime fast protein liquid chromatography (FPLC) instrument (GE Healthcare). Completely L12-depleted ribosomes were collected in the initial flow through in JE28 Lysis buffer, while full ribosome particles were eluted with JE28 Elution buffer and discarded.

Recombinantly expressed, purified (His)₆-tagged L12 (described below) was reintroduced in a fivefold molar excess to L12-depleted ribosomes through incubation at 37°C for 30 minutes in ribosome storage buffer.

**Overexpression and purification of ribosomal protein L12**

The *rplL* gene encoding ribosomal protein L12 had previously been cloned into the pSV281 vector using *Bam*HI and *Xho*I restriction sites, introducing an N-terminal (His)₆-tag (Walter, 2010). The L12 protein was expressed and purified identically to the GTPases described above, with the addition of seven molar urea to each buffer after the protein was immobilized on the resin. To remove any
contaminating endogenous ribosomes, the purified L12 elution was ultracentrifuged at 150,000 x g for two hours at 4°C. The supernatant containing L12 was refolded slowly through dialysis in two separate one liter aliquots of GTPase storage buffer for 24 hours each. Refolded proteins were concentrated with a ten kDa MWCO centrifugal filter (Millipore) and quantified by Bradford assay (Bradford, 1976).

**Generation of L12 mutants**

Genetic mutations were made to the plasmid encoding wild type L12 through site-directed mutagenesis (SDM) (Agilent Technologies, QuikChange Lightning Site-Directed Mutagenesis Kit) with oligonucleotides designed via Agilent Technologies website and purchased from Integrated DNA Technologies (IDT) (Table 3-1). As suggested by the SDM kit, reactions contained 125 ng forward primer, 125 ng reverse primer, 50 ng double stranded DNA template, one µL dNTP mix, five µL 10X Quikchange Lightning Buffer, and 1.5 µL QuikSolution reagent in a final volume of 50 µL. Immediately before thermocycling, one µL of QuikChange Lightning Enzyme was added to each reaction. Reactions were incubated at an initial denaturation temperature of 95°C for two minutes, followed by 18 cycles of 95°C denaturation (20 seconds), 60°C annealing (ten seconds), and 68°C extension (3.5 minutes). After a final 68°C extension for five minutes, two µL of \textit{Dpn} I restriction enzyme was added to each reaction and allowed to incubate for five minutes at 37°C to digest the parental methylated and hemimethylated DNA. Reactions were stored on ice until transformation. Plasmid sequences were confirmed by Nevada Genomics.
Table 3-1. DNA primer sequences for the generation of L12 point mutations. Red text corresponds to mutant codons. All primers purchased from Integrated DNA Technologies.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L12-K66A Forward</td>
<td>5'-GAAAGCTGCTGGCGCTAACGCAGTTGCTGTTATCAAAGCA-3'</td>
</tr>
<tr>
<td>L12-K66A Reverse</td>
<td>5'-TGCTTTGATAACAAGCAACTGCCTGTTATCAAAGCAG-3'</td>
</tr>
<tr>
<td>L12-K66D Forward</td>
<td>5'-GAAAGCTGCTGGCGCTAACGATGTTGCTGTTATCAAAGCAG-3'</td>
</tr>
<tr>
<td>L12-K66D Reverse</td>
<td>5'-CTGCTTTGATAACAAGCAACTGCCTGTTATCAAAGCAG-3'</td>
</tr>
<tr>
<td>L12-K82A Forward</td>
<td>5'-CAACTGGCCTGGGCTCTGGCAGAAGCTAAAGACCTGG-3'</td>
</tr>
<tr>
<td>L12-K82A Reverse</td>
<td>5'-CCAGGTCTTTAGCTTTCTCCAGACCCCAGGCCGAGTTG-3'</td>
</tr>
<tr>
<td>L12-K82D Forward</td>
<td>5'-CAACTGGCCTGGGCTCTGGCAGAAGCTAAAGACCTGG-3'</td>
</tr>
<tr>
<td>L12-K82D Reverse</td>
<td>5'-ACCAGGTCTTTAGCTTTCTCCAGACCCCAGGCCGAGTTG-3'</td>
</tr>
<tr>
<td>L12-K85A Forward</td>
<td>5'-TGCGGCTGGGCTTGAAAGAAGCTGACCTGGTAGAATTGT-3'</td>
</tr>
<tr>
<td>L12-K85A Reverse</td>
<td>5'-GATTCTACAGGTCTGCGGTCTTTCCAGACCCCAGGCC-3'</td>
</tr>
<tr>
<td>L12-K85D Forward</td>
<td>5'-CCTGGGCTGAAAGAAGCTGACCTGGTAGAATTGTGAC-3'</td>
</tr>
<tr>
<td>L12-K85D Reverse</td>
<td>5'-GTCGAGATTCTACAGGTCTGCGGTCTTTCCAGACCCCAGG-3'</td>
</tr>
<tr>
<td>L12-T77W Forward</td>
<td>5'-TTATCAAGCGAGTCGCTGCGGCGACGCTGGGTATCT-3'</td>
</tr>
<tr>
<td>L12-T77W Reverse</td>
<td>5'-CACAGCCAGGCCCTACGCGCCACTGCTTTGATAA-3'</td>
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<tr>
<td>L12-T77A Forward</td>
<td>5'-GTCGCTGGGCAGCTGGGCTTGGGTC-3'</td>
</tr>
<tr>
<td>L12-T77A Reverse</td>
<td>5'-GACCAGGCGCCAGCTGCGGCACGTAC-3'</td>
</tr>
</tbody>
</table>

**Generation of L12 CTD mutant**

Polymerase chain reaction (PCR) was employed to amplify the CTD of L12. The forward primer was designed with a *Bam*HI restriction site while the reverse primer contained a *XhoI* restriction site to allow for subcloning into the pSV281 expression vector (Table 3-2). Reactions were assembled on ice to final concentrations of 1X DreamTaq Buffer, 0.6 µM forward and reverse primers, and 250 µM dNTPs with
addition of one µL of template DNA in a final volume of 50 µL in thin-walled PCR tubes. Immediately before thermocycling, one µL of DreamTaq DNA Polymerase (ThermoFisher Scientific) was added. The reaction was initially denatured at 95°C for 30 seconds, followed by 30 cycles of 95°C denaturation (30 seconds), 55°C annealing (30 seconds), and 72°C extension (30 seconds). The desired L12 CTD PCR product was then purified with a QIAquick PCR Clean-Up kit (Qiagen). The amplicon was digested in 1X CutSmart buffer with 20 units of both BamHI and XhoI restriction enzymes (New England Biolabs) and ligated into the pSV281 expression vector in a reaction containing 60 ng insert, 50 ng vector, 1X Rapid Ligation buffer, 1X T4 DNA Ligase Buffer, and one µL T4 DNA Ligase in 20 µL total. The reaction was incubated at room temperature for ten minutes, heat inactivated at 65°C for ten minutes, then stored at -20°C.

Table 3-2. DNA primer sequences for the amplification of the L12 CTD. Primers purchased from Integrated DNA Technologies.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L12 CTD Forward</td>
<td>5'-GGTGGTGTTGATCCACTGAATTCGACGTAATTCTG-3'</td>
</tr>
<tr>
<td>L12 CTD Reverse</td>
<td>5'-GGTGGTGCTCGACTTATTTAATTTCAACTTCAGC-3'</td>
</tr>
</tbody>
</table>
Transformation of expression vectors into *E. coli*

Expression vectors containing L12 point mutations were initially transformed into the XL10-Gold® Ultracompetent (Agilent) *E. coli* cell line as suggested by the SDM protocol. Chemically competent XL10-Gold® *E. coli* cells were gently thawed on ice immediately prior to transforming and 45 µL was aliquoted into a prechilled 14-mL polypropylene round-bottom tube (BD Falcon). Addition of two µL β-ME mix (Agilent) and two µL *DpnI*-treated DNA from the mutagenesis reaction was followed by gentle swirling and incubation on ice for 30 minutes. Tubes were heat pulsed in a 42°C water bath for 30 seconds, then immediately placed on ice for two minutes. To each reaction, 500 µL of prewarmed (37°C) LB was added and cells were allowed to recover at 37°C for one hour with gentle shaking prior to plating. Cells were evenly distributed onto LB with agar (LB + 1.5% (w/v) agar (Fisher Scientific)) in polystyrene Petri dishes (VWR) containing 35 µg/mL kanamycin (LB+Agar+K35) and were incubated at 37°C for 12-18 hours. Plasmids from each transformation reaction were isolated (Qiaprep Spin Miniprep Kit, Qiagen) and sequenced by the Nevada Genomics Center at the University of Nevada, Reno.

The expression vector containing the L12 CTD was initially transformed into the DH5α *E. coli* cell line. After gentle thawing on ice, five µL of the ligated plasmid was added to 100 µL of chemically competent DH5α cells in a prechilled 14-mL polypropylene round-bottom tube (BD Falcon) and incubated on ice for 30 minutes. Tubes were heat pulsed in a 42°C water bath for 60 seconds and immediately chilled on ice for three minutes. To the reaction, 900 µL of pre-warmed (37°C) LB
was added and cells were allowed to recover at 37°C for one hour with gentle shaking prior to plating on LB+Agar+K\textsuperscript{35}. Plates were incubated at 37°C for 12-18 hours and DNA was extracted from colonies for sequencing as described for the L12 point mutants.

Once DNA sequences were confirmed, plasmids were transformed into a BL21(DE3) chemically competent \textit{E. coli} cell line. Cells and plasmids were gently thawed on ice immediately prior to transformation. Into pre-chilled 14-mL polystyrene tubes, one µL DNA was added to 50 µL chemically competent BL21(DE3) cells and gently mixed before incubating on ice for 30 minutes. Mixtures were then heat-shocked at 42°C for 90 seconds followed by incubation on ice for three minutes. Reactions were mixed with 950 µL of pre-warmed (37°C) LB and allowed to shake at 250 rpm and 37°C for one hour before plating on LB+Agar+K\textsuperscript{35} as described above.

**Circular dichroism measurements**

Protein folding was examined through circular dichroism (CD) with an Olis DSM 20 CD instrument. Protein samples were diluted to 0.5 mg/mL in GTPase storage buffer and ellipticity was monitored from 200 to 270 nm in 1 nm increments at 20°C. Spectra presented represent the average of three scans for each protein sample.
GTPase activity assay

To test the GTP hydrolysis activity of both purified 70S ribosomes and GTPases, an assay to detect the presence of inorganic phosphate was performed, employing the PiColorLock™ Gold Phosphate Detection System (Innova Biosciences). Reactions were carried out in triplicate at room temperature and a final reaction volume of 50 µL. To a 96-well, flat bottom microplate, GTPase (5 µM), 70S (0.2 µM), 70SΔL12 (0.2 µM), 70SΔL12+L12 (wt/mutant) (0.2 µM), and GTPase reaction buffer (to 1X) were combined as appropriate and incubated for ten minutes. To applicable reactions wells, GTP (25 µM; Sigma Aldrich) was added and allowed to react for ten minutes. Reactions were quenched by addition of 12.5 µL of Gold Mix (1:100 ratio of Accelerator:PiColorLock™ Gold reagent) followed by one hour of color development. Absorbance was measured at 635 nm on a BioTek® Epoch plate reader.
Chapter 4 - Effect of domain deletions and L12 point mutations on GTPase activation

Results

GTPase Expression and Purification

All of the GTPase genes were originally cloned from genomic E. coli DNA into the pSV281 expression vector. This vector contains gene sequences encoding the T7lac promoter, kanamycin resistance, and an N-terminal (His)$_6$-tag, allowing for overexpression of the desired protein followed by purification through a well-established method. After purification, the concentrated proteins were quantified and determined to have greater than 95% purity as well as correct size before being tested in any biochemical assays.

Purification of 70S ribosomes

Ribosomes were grown and purified from E. coli JE28 cells as previously described (Ederth et al., 2009). Briefly, this engineered strain of E. coli produces endogenous ribosomes that carry a (His)$_6$-tag at the N-terminus of each copy of ribosomal protein L12, facilitating a simple means of isolating highly active ribosomes. This allows for an analogous affinity purification scheme to that of (His)$_6$-tagged proteins. Ribosomes were quantified by UV/Vis spectroscopy, whereby absorbance at 260 nm of a 1:1000 dilution of purified ribosomes was measured,
with a strong absorbance at 260 nm and a ~2:1 ratio of $A_{260}:A_{280}$ being characteristic of pure ribosomes (Figure 4-1A). Purity was confirmed through SDS-PAGE analysis (Figure 4-1B). Although rRNA cannot be visualized by SDS-PAGE, the profile of ribosomal proteins suggests that ribosomal subunits are intact and present in purified JE28 ribosomes, based on comparison with the protein profile of full 70S particles (Bickle and Traut, 1971). Typical yields ranged from 500-800 pmol of ribosomes per liter of cell culture.

![Graph and Image](image)

**Figure 4-1.** Purification of JE28 70S ribosomes. (A) Typical 70S absorbance spectrum, indicating an $A_{260}:A_{280}$ ratio of ~2.0, and showing no aberrant peaks. (B) Coomassie stained 12.5% SDS-PAGE gel. (1) Clarified lysate, (2) Ni-NTA flowthrough, (3) wash, (4) precipitated protein from first round of ultracentrifugation, (5) elution, (6) precipitated protein from second round of ultracentrifugation, (7) concentrated 70S sample, (8) Spectra™ BR Protein Ladder (Thermo Scientific).
**Depletion of L12 from JE28 70S ribosomes**

Ribosomal protein L12 was completely removed from (His)$_6$-tagged ribosomes through a two-step purification scheme. Depletion of L12 only, without disrupting other protein and rRNA constituents, is a time and temperature sensitive procedure, as previously described (Wuerth, 2013). Loss of other ribosomal proteins, including L10 and L11 of the stalk complex, was observed with reaction temperatures above 4°C or when mixing times exceeded ten minutes. The initial NH$_4$Cl-ethanol treatment served to remove a large fraction of L12, producing a heterogeneous population of ribosomes. Therefore, a second purification step was employed to isolate completely L12-depleted 70S particles from the portion of ribosomes still harboring L12. This was accomplished by flowing initially depleted ribosomes over an Ni-NTA column and collecting the initial flowthrough fraction that was assumed to be completely depleted 70S, referred to as 70SΔL12 (Figure 4-2).
Figure 4-2. Depletion of L12 from 70S ribosomes. FPLC chromatogram of Ni-NTA secondary purification procedure for L12-depleted 70S ribosomes. Fully depleted ribosomes do not bind to the Ni-NTA resin (first peak from 2-6 mL) while ribosomes still containing (His)$_6$-tagged L12 remain attached to the column and must be eluted with an imidazole containing buffer (second peak from 17-20 mL).

**Purification of ribosomal protein L12**

Since the folded, functional form of L12 has an inherent affinity for endogenous ribosomes, additional steps were required to isolate the pure protein. This included purification under denaturing conditions, followed by ultracentrifugation and subsequent refolding with native buffer conditions. Proper folding of L12 after purification was confirmed by CD, which verified strong α-helical character under both native and refolding buffer conditions (Figure 4-3). A Bradford assay was
implemented for quantification of L12, since its sequence lacks tryptophan or any other aromatic residue that enables protein quantification by Beer’s law (Bradford, 1976). Recombinant L12 was incubated with 70SΔL12 to fully reconstitute ribosomes for analysis in GTPase activity assays.

**Figure 4-3.** Circular dichroism spectra of purified L12. (Blue) GTPase Storage Buffer, (black) L12 purified under native conditions, (red) L12 purified under denaturing conditions in 7 M urea.
**L12 point mutants generated via site-directed mutagenesis**

In order to study the importance of conserved L12 residues at the binding interface with the G domain of GTPases, amino acid point mutations were made to the *rplL* gene encoding *E. coli* ribosomal protein L12. Primers to introduce a single amino acid mutation were rationally designed through careful interpretation of the X-ray crystal structure of the 70S ribosome from *T. thermophilus* complexed with EF-G•GDPNP in an intermediate state of translocation. High sequence conservation of the L12 CTD provided the opportunity to make mutations to the L12 *E. coli* gene based on the structure from *T. thermophilus*. Mutant DNA was generated via SDM. To confirm that the desired sequence was generated, plasmids were sequenced at the Nevada Genomics Center (University of Nevada) and aligned via Clustal Omega software (Figure 4-4; Sievers et al., 2011).
Figure 4-4. Clustal Omega multiple sequence alignment of L12<sub>wt</sub> and L12 point mutants.
The C terminal coding region of the rplL gene can be successfully isolated from the wild type full length L12 gene

Primers to clone the CTD of L12 were designed based on the domain structure of L12, with residues 53-121 marked for amplification. As described in the Materials and Methods, PCR was performed and the resultant plasmid was transformed into chemically competent *E. coli* cells. Restriction digestion of this plasmid determined that the generated mutant genes were the correct size (Figure 4-5). Lanes 8 and 9 indicate the size of the pSV281 vector (~5600 bp) and L12 CTD amplicon (~210 bp), respectively. The successfully ligated and transformed plasmid is visualized in lane 4. The lower bp bands in lanes 2, 3, and 5, however, are the remnants of the gene that was cloned out of the pSV vector prior to ligation of L12 CTD, in which the pSV sample that was employed for cloning procedures was a mixture of double- and single-cut vectors that allowed the single-cut vectors to re-anneal during the ligation reaction with the L12 CTD. Therefore, only the plasmid from lane 4 was used in subsequent experiments pertaining to the L12 CTD. To confirm that the desired sequence was generated, the plasmid was sequenced and aligned with L12<sub>wt</sub> as described above for the L12 point mutations (Figure 4-6).
Figure 4-5. Restriction digestion of L12 CTD plasmid. GelRed stained 2% agarose gel of (1) 100 bp ladder, (2-5) L12 CTD plasmid preparations cut with BamHI and XhoI, (6) 1 kilobase DNA ladder (Promega), (7) empty, (8) pSV281 vector, and (9) L12 CTD PCR amplicon. Lane 4 contained the correct plasmid whereas lanes 2, 3, and 5 were re-ligation products from singly cut plasmids that contain a gene for a different protein studied in the Spiegel lab.

Figure 4-6. Clustal Omega multiple sequence alignment of L12 wt and L12 CTD.
Overexpression and purification of L12 mutants

L12 mutants were expressed and purified in an identical manner to L12\text{wt}. Analysis of refolded proteins by SDS-PAGE confirmed that mutants had the appropriate molecular weight (Table 4-1) and were >95% pure (Figure 4-7). CD spectroscopy was performed to evaluate whether the secondary structure of the protein was altered by either the introduction of a given amino acid substitution or an entire domain deletion (Figure 4-8). The L12 CTD showed a decrease in the magnitude of the CD signal compared to wild type at the same concentration, indicating a lesser degree of folding, which was expected after removal of the highly structured NTD. All of the L12 point mutants showed similar spectra to wild-type L12, indicating proper folding.

Table 4-1. Characteristics of L12 mutant proteins.

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<thead>
<tr>
<th>Mutation type</th>
<th>Name</th>
<th>Molecular Weight (kDa)</th>
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</thead>
<tbody>
<tr>
<td>None – wild type</td>
<td>L12\text{wt}</td>
<td>14551</td>
</tr>
<tr>
<td>Alanine scanning mutation</td>
<td>L12-K66A</td>
<td>14494</td>
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<tr>
<td></td>
<td>L12-K82A</td>
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<td></td>
<td>L12-K85A</td>
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<td>L12-T77A</td>
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<td>Aspartic acid mutation</td>
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<td>Domain truncation</td>
<td>L12 CTD</td>
<td>9157</td>
</tr>
</tbody>
</table>

Figure 4-8. Circular dichroism spectra of L12 proteins.
Effect of L12 depletion on GTP hydrolysis activity of EF-G

Upon complete removal of L12 from 70S ribosomes, followed by purification of L12 for reconstitution studies, the effect of L12 depletion on ribosome-dependent GTPase activity was determined through a simple inorganic phosphate detection system. For simplicity, mRNA and tRNA were omitted from reactions, as ribosomes have been previously demonstrated to stimulate GTP hydrolysis in the absence of these reagents (Rodnina et al., 1999). GTPase reactions were performed in vitro utilizing a malachite green colorimetric assay. As expected, the GTPase activity of EF-G increased substantially in the presence of 70S ribosomes (Figure 4-9). Upon complete removal of L12 from 70S ribosomes, the GTPase activity of EF-G was of comparable magnitude to that of EF-G alone, indicating that L12-depleted ribosomes were unable to stimulate GTP hydrolysis by EF-G, as previously reported (Carlson, 2015; Carlson et al., 2017). Reconstitution of recombinant L12 to completely depleted ribosomes restored activity of EF-G to levels similar to full 70S particles. These data suggest that ribosomal protein L12 is required for ribosome-dependent GTPase activation of EF-G.
Figure 4-9. Effect of L12 depletion on ribosome dependent GTPase activity of EF-G. All experiments were performed in triplicate and data were normalized to EF-G + GTP + 70S reactions. Error bars represent standard deviation from the mean.
Effect of the G’ subdomain deletion from EF-G on ribosome-dependent GTP hydrolysis

To further investigate the function of the G’ subdomain on GTP hydrolysis activity, a G’ domain truncation mutant of EF-G (EF-GΔG’) was cloned by Markus Carlson (Carlson, 2015) and purified as described for GTPases. The EF-GΔG’ protein was found to have the appropriate molecular weight by SDS-PAGE assessment (Figure 4-10A) and was folded correctly, based on CD analysis (Figure 4-10B). In the presence of 70S ribosomes, EF-GΔG’ exhibited approximately 40% activity relative to wild-type EF-G (Figure 4-10C), similar to previous observations (Carlson, 2015; Carlson et al., 2017; Nechifor et al., 2007). A comparable level of activity was measured upon incubation of EF-GΔG’ with L12-depleted ribosomes, suggesting that the ribosome-dependent GTPase activity of EF-GΔG’ is unaffected by the absence of L12 (Carlson et al., 2017).

To extend understanding of the role of the G’ domain in GTP hydrolysis activity, GTPase titration experiments were performed (Figure 4-10D; Carlson et al., 2017). With the malachite green colorimetric assay, increasing amounts of EF-G and LepA (from 0.05 µM to 5 µM) were added to a constant concentration of 0.2 µM 70S ribosomes. Upon incubation with L12-depleted ribosomes, EF-G reached approximately 20% activity while LepA maintained 80% GTP hydrolysis activity compared to that observed with wild-type 70S complexes. This reinforces previous reports that LepA maintains GTPase activity on the ribosome in the absence of L12 (Carlson, 2015; Carlson et al., 2017).
Figure 4-10. GTP hydrolysis activity for EF-GΔG’ in the presence and absence of ribosomal protein L12. (A) Coomassie stained 12.5% SDS-PAGE gel. Lanes: (1) EF-G, (2) MW standards, (3) EF-GΔG’. (B) Far UV CD spectra for EF-G (red solid line), EF-GΔG’ (blue dashed line), and buffer (green dotted line). (C) Malachite green GTP hydrolysis activity measured relative to 70S/EF-G/GTP. Red: EF-G, Blue: EF-GΔG’. Single time points were recorded at 60 minutes. Each complex was measured in triplicate and represented as the mean. Error bars represent the standard deviation from the mean. Experiment C performed by Colby Blackwood. (D) Malachite green GTPase activity for titrations of EF-G and LepA relative to 0.2 µM 70S or 70SΔL12. Open circles (blue): EF-G, open triangles (red): LepA, solid lines: 70S ribosomes, dashed lines: 70SΔL12 ribosomes. Each data point measured in triplicate and error bars represent standard deviations from the mean. B, C, and D from Carlson et al., 2017.
**Effect of L12 point mutations on GTPase activity of EF-G**

Each L12 mutant was reconstituted in five-fold molar excess of L12<sub>wt</sub>-depleted ribosomes and tested for effects on the ribosome-dependent activation of EF-G (Figure 4-11). The alanine scanning mutations showed a small deviation from wild type, with K66A and K82A lowering EF-G activity to roughly 70% while K85A fully restored activity. The mutation of lysines to aspartic acid had a more dramatic effect on EF-G activity. Specifically, all lysine to aspartic acid point mutations decreased EF-G activity to at least 50%, with K82D imparting the most dramatic decrease to roughly 20% compared to wild-type L12. This finding suggests that K82 has an important electrostatic interaction at the binding interface with the G’ subdomain of EF-G. At 15-fold molar excess, the L12 CTD-reconstituted ribosomes were unable to restore any activity that was lost, suggesting that the NTD is critical for the function of L12 in the recruitment of GTPases to the ribosome for proper activation. Finally, the attempt to disrupt binding, and ultimately GTPase activation, by introducing the bulky residue tryptophan in the place of a conserved threonine was unfruitful; no difference in EF-G activity was observed upon reconstitution of L12<sub>wt</sub>-depleted ribosomes with L12-T77W. In fact, mutating the conserved threonine 77 to an alanine did not disturb EF-G activity either, suggesting that function of residue 77 in the CTD of L12 is not critical to activation of EF-G.
**Figure 4-11.** GTPase activity assay of EF-G with ribosomes reconstituted with L12 samples. All experiments were performed in triplicate and data were normalized to EF-G + GTP + 70S reactions. Error bars represent standard deviation with \( n \geq 4 \).
Discussion

Ribosomal protein L12 plays an important part in the function of GTPase translation factors on the ribosome. The dynamic CTDs of the L12 dimer reach out from the ribosome to recruit factors and stimulate GTP hydrolysis through stabilization of the active GTPase conformation (Diaconu et al., 2005). Recent efforts in the Spiegel lab have determined that L12 is required for ribosome-dependent GTPase activation of EF-G, RF3, and IF2 (Carlson et al., 2017). Furthermore, the GTPase LepA was previously found to harbor some baseline level of GTP hydrolysis activity independent of L12 (Carlson, 2015, Walter 2010). This thesis work aimed to extend understanding of intermolecular interactions essential to GTPase activation by the ribosome.

**L12 is required for ribosome-dependent GTPase activity**

Although L12 has been studied since the 1970s, major findings in the last two decades have largely increased understanding of its indispensable role in ribosome-dependent GTPase activation (Carlson et al., 2017; Diaconu et al., 2005, Helgstrand et al., 2007). Our hypothesis was that initial claims pertaining to the role of L12 in GTPase activation were made on the basis of incomplete purification schemes. For example, Savelsburg et al. reported that L12 strongly stimulated GTPase activity of EF-G in the absence of ribosomes (Savelsburg et al., 2000). Whereas, previous work in the Spiegel lab by Michelle Wuerth and Justin Walter revealed that L12 purified through chemical denaturation followed by slow refolding does not independently stimulate GTP hydrolysis (Walter, 2010). It is possible that
endogenous ribosomes were co-purified with L12 under the native purification conditions in experiments by Savelsburg et al. due to the inherent affinity of these particles in vivo. Furthermore, although partial removal of L12 from 70S ribosomes results in a significant decrease in GTP hydrolysis by translational GTPases (Walter, 2010), full removal of L12 through the two-step method described (Materials and Methods) completely abrogates GTPase activity of EF-G, RF3, and IF2 (Carlson et al., 2017).

**Deletion of the G’ subdomain abolishes the stimulatory effect of L12 in ribosome-dependent GTPase activity of EF-G**

X-ray crystallography structures have illustrated direct contacts between the G’ subdomain of EF-G with L12 on the ribosome (Gao et al., 2009). Similar observations have been made of RF3 with the ribosome through cryo-EM reconstructions, emphasizing the significance of the G’ domain in ribosome-GTPase interactions (Pallesen et al., 2013). In this way, just as L12 is important for GTPase activation on the ribosome, it is not surprising that removal of the G’ subdomain from EF-G impedes its ability to hydrolyze GTP.

While cloning of the domain truncation mutant EF-GΔG’ was accomplished previously by Markus Carlson in the Spiegel lab, expression and purification of the protein for experimentation presented here was not without difficulties. Multiple purification steps including IMAC and anion exchange chromatography along with careful concentration to avoid precipitation produced a properly folded protein sample suitable for biochemical analysis. The GTPase activity of EF-GΔG’ in the
presence of 70S ribosomes was roughly 40% compared to wild-type EF-G. Although protein contaminants in the sample of EF-GΔG’ are evident by SDS-PAGE analysis (Figure 4-10A), and while this level of impurity is not favorable, the results are similar to previous reports that demonstrate up to a ten-fold reduction in EF-G activity upon deletion of the G’ subdomain (Carlson, 2015; Mikolajka et al., 2011; Nechifor et al., 2007). The higher level of EF-GΔG’ activity reported here could be an artifact of the timing of procedures during the malachite green assay. It is well established that EF-G approaches maximal GTP hydrolysis activity within ten minutes (Carlson et al., 2017; Rodnina et al., 1997). Therefore, if reactions were allowed to incubate for more than ten minutes before quenching, late rounds of GTP hydrolysis events by EF-GΔG’ could be captured, elevating its apparent activity compared to wild-type EF-G. Similarly, a shorter extent of time for color development after reaction quenching is often accompanied by high background signal and low resolution between samples. Nevertheless, upon incubation with ribosomes depleted of L12, EF-GΔG’ activity remained near 40%, indicating an insensitivity to the presence or absence of L12. This finding suggests that the ribosome exploits another one of its many components besides ribosomal protein L12 to fully stimulate GTP hydrolysis by EF-G. Perhaps the low level of EF-GΔG’ activity is due to binding of domain IV of EF-G in the A site of the ribosome with only a fraction of those binding events resulting in GTP hydrolysis due to a lack of L12 acting as a buttress to hold EF-G on the ribosome.

To further study implications of the G’ subdomain, wild-type 70S ribosomes and L12-depleted ribosomes were titrated with EF-G and LepA. These two GTPases
share strong structural homology with the exception that the G’ subdomain is absent from the conserved GTP-binding domain of LepA. In the presence of 0.2 µM full ribosomal complexes, both EF-G and LepA approach maximal activity at 2.5 µM GTPase. Whereas in the absence of L12, five µM EF-G displayed low levels of activity while five µM LepA maintained close to 80% activity compared to wild-type (Figure 4-10D). This provides another line of evidence that the G’ subdomain plays an important role in GTPase activation on the ribosome.

**Single conserved lysine surface residues 66, 82, and 85 at the L12 CTD binding interface are not individually responsible for activation of EF-G**

In an effort to develop a better understanding of the mechanism by which L12 stimulates GTPase activity on the ribosome, several single amino acid exchanges were made through site-directed mutagenesis to the highly conserved surface of the CTD. Structural investigation of the binding interface between the L12 CTD and the G’ subdomain of EF-G reveals a largely positive charged surface on the L12 CTD due to multiple conserved lysine residues (Zhou et al., 2013). A common approach to study single residue functionality is by mutation of the amino acid of interest to an alanine residue, otherwise known as alanine scanning mutagenesis (Morrison and Weiss, 2001). Alanine is a favorable substitute due to its non-bulky, chemically inert, methyl functional group that adopts the secondary structure of most other amino acids. In the case of L12, three highly conserved lysines were selected for mutagenesis studies: K66, K82, and K85 (numbering from *E. coli*). Although mutations K66A and K82A lowered EF-G activity by approximately 25%, the K85A mutant restored complete activity. Together, this suggests that any
single positively charged residue on the binding surface of L12 is not independently responsible for the function of L12 in stimulating GTPase activity. By contrast, mutation of each of these lysines individually to a negatively charged aspartic acid dramatically decreased stimulation of EF-G activity. The mutants K85D, K66D, and K82D effectively decreased EF-G activity to approximately 55%, 50%, and 20%, respectively. Collectively, these findings support the hypothesis that double and triple point mutations of these surface exposed lysines to negatively charged aspartic or glutamic acids will significantly or completely abrogate GTPase activity of EF-G on the ribosome.

**Mutagenesis of a conserved threonine on the L12 CTD binding surface did not perturb EF-G GTPase activity**

Structural study of the binding interface between L12 and EF-G gave rise to the notion that a highly conserved threonine makes important hydrogen bonds with an aspartic acid (D222) of the G’ domain of EF-G. Mutation of this threonine to an alanine did not disrupt the ability of L12 to stimulate EF-G activity. This is not too surprising because the CTD binding surface is made up of multiple residues with positively charged side chains. In the event of substituting a hydrogen bond donor (in the form of the threonine hydroxyl side chain) with an inert methyl group, it is more likely that L12-T77A would bind more tightly to its EF-G target and effectively stimulate GTP hydrolysis to the same or even higher level compared to wild-type L12. Furthermore, the attempt to disrupt binding by introducing a bulky tryptophan in the place of T77 was ultimately futile as well, with L12-T77W restoring full activity of EF-G in the presence of L12\textsubscript{wt}-depleted ribosomes. Upon structural modeling,
T77W was predicted to induce the most steric clash with the G’ subdomain of EF-G, compared to other conserved surface exposed residues on the CTD of L12. The crystal structure utilized for modeling was resolved to 3.86 Å, which is incredible for resolution of 2.5 megaDalton prokaryotic ribosome (Zhou et al., 2013). However, it gives rise to measurement error in the distances between atoms at the binding interface, alluding to an imprecise rational design of this point mutant that ultimately showed identical GTPase stimulatory effects as wild-type L12.

Reconstitution of L12-depleted ribosomes with the L12 CTD alone is not sufficient to restore GTP hydrolysis activity of EF-G

A previous mutagenesis study reported that removal of the L12 CTD decreased the rate of EF-Tu•GTP•Phe-tRNA^{Phe} binding to the ribosome by a magnitude of ten compared to wild type ribosomes, alluding to the significance of the L12 CTD in factor binding (Diaconu et al., 2005). GTPase activity experiments bolstered the importance of the L12 CTD, with strongly impaired rates of GTP hydrolysis by EF-Tu (Diaconu et al., 2005; Pape et al., 1998) and EF-G (Diaconu et al., 2005; Savelsburgh et al., 2003). Since the CTD has proved necessary for proper activation of GTPases by the ribosome, our objective was to investigate the ability of the CTD alone to restore GTPase activity with L12-depleted ribosomes. Upon removal of the L12 NTD, it is apparent that the CTD alone cannot restore GTPase activity of EF-G. The most overt rationale for this outcome can be described by the structural evidence supporting the role of the NTD in dimerization and anchoring of L12 to the 70S ribosome (Diaconu et al., 2005; Gao et al., 2009;
Gudkov et al., 1995). It is conceivable that the L12 CTD associates with free EF-G in solution, and even with EF-G on the ribosome, due to their inherent binding affinity. However, without the NTD to affix the L12 CTD to the ribosome, it appears that the CTD is incapable of escorting EF-G to its binding site on the 30S subunit nor stabilizing EF-G in the conformation favorable for GTP hydrolysis. Furthermore, without dimerization, there is no way for isolated CTDs to group together for efficient recruitment of GTPases to the ribosome. In fact, ribosomes with single L12 dimers are less competent in stimulation of GTPase activity of EF-G, with a two-fold slower rate of GTP hydrolysis compared to ribosomes with two L12 dimers (Mandava et al., 2012). Altogether, it is not surprising that the L12 CTD alone is unable to stimulate EF-G activity due to its inability to dimerize and bind to the ribosome.
Conclusions and future work

The work presented here confirms the role of ribosomal protein L12 in GTPase activation of EF-G and extends understanding into the functional importance of the G’ subdomain of EF-G, the CTD of L12, and conserved residues at the binding surface of the L12 CTD. As anticipated, the EF-GΔG’ mutant showed the same low level of activity with full 70S complexes as with L12-depleted ribosomes, confirming that it contains the major contacts responsible for binding to 70S ribosomes. By site-directed mutagenesis, this binding interaction could be explored further by making additional residue substitutions or domain deletions along with removal of the G’ subdomain. For example, since domain IV of EF-G is implicated in tRNA translocation and makes contacts with the 30S subunit, it is imaginable that simultaneous deletion of both domain IV and subdomain G’ would expunge all critical residues for EF-G to associate with the 70S ribosome.

Titration of wt EF-G and LepA to 70S ribosomes and L12-depleted ribosomes provided support for previous findings that LepA displays high levels of activity in the absence of L12 while EF-G does not, prompting further probing of domains of LepA to identify critical residues for interaction with the ribosome. Upon examination of the conserved L12 CTD binding surface through mutagenesis, our suspicion that the sizable positively charged surface area would overcome any single point mutation was validated and heralds further investigation through double and even triple point mutations for potential synergistic inhibition of GTPase activation. Specifically, an L12-K66A/K82A double point mutant might effectively double the loss in EF-G activity observed through the single alanine scanning
mutations and we would expect to see complete loss of EF-G activity by double and triple aspartic acid substitutions such as L12-K66D/K82D, L12-K66D/K82D/K85D, and other variations. Stimulation of GTPase activity is largely dependent on binding of the GTPase to the ribosome, which induces a conformational change that favors GTP hydrolysis. Therefore, to strengthen the results presented herein, it is necessary to study the effects of L12 mutants on GTPase binding to 70S ribosomes, which is feasible through established methods (Carlson et al., 2017).

Altogether, this work demonstrates the enormity of research questions to be pursued as well as the elusiveness of critical aspects of protein synthesis, a vital process conserved across all domains of life. Due to the increasing resistance of bacterial species to the limited effective antibiotics, it is imperative that research in this field continue so as to elucidate novel methods of inhibiting bacterial protein synthesis.
References


