DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS
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VIKTORIYA SHYP

G nucleotide regulation of translational GTPases and the stringent response factor RelA

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LIST OF ORIGINAL PUBLICATIONS

The current dissertation is based on the following original publications, referred to by their Roman numerals.

I. Hauryliuk, V; Mitkevich, VA; Draycheva, A; Tankov, S; **Shyp, V;** Ermakov, A; Kulikova, AA; Makarov, AA; Ehrenberg, M: Thermodynamics of GTP and GDP binding to bacterial initiation factor 2 suggests two types of structural transitions. J. Mol. Biol. 2009, 394(4):621–6.

II. Mitkevich, VA; Ermakov, A; Kulikova, AA; Tankov, S; **Shyp, V;** Soosaar, A; Tenson, T; Makarov, A; Ehrenberg, M; Hauryliuk, V: Thermodynamic Characterization of ppGpp binding to EF-G or IF2 and of initiator tRNA binding to free IF2 in the presence of GDP, GTP, or ppGpp. J. Mol. Biol. 2010, 402:838–846.

III. Chen L; Muhlard D; Hauryliuk V; Zhihong C; Lim KM; **Shyp V**; Parker R; Song H: Structure of the Dom34-Hbs1 complex and implications for no-go decay. Nat. Struct. & Mol. Biol. 2010 Oct; 17(10):1233–40.

IV. **Shyp, V**; Tankov, S; Ermakov, A; Kudrin, P; English, BP; Ehrenberg, M; Tenson, T; Elf J; Hauryliuk, V: Positive allosteric feedback regulation of the stringent response enzyme RelA by its product. EMBO reports, 2012, 13:835–839.

V. Mitkevich, V*; **Shyp, V***; Petrushanko, IYu; Soosaar, A; Atkinson, GC; Tenson, T; Makarov, AA; Hauryliuk, V: GTPases IF2 and EF-G bind GDP and the SRL RNA in a mutually exclusive manner. *Submitted*

* Equal contribution

In papers I, II and V I have purified some recombinant proteins, contributed to the preparation of initiator tRNA, prepared some of the components and performed several ITC experiments. In paper III I have performed the nitrocellulose filtration experiment for G nucleotide dissociation from Dom34-Hbs1 complex and analyzed the data.

In paper IV I have designed and performed the majority of the experiments, and contributed to writing up the paper.

LIST OF ABBREVIATIONS

A	Adenosine
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
aa-tRNA	animoacyl-tRNA
C	Cytidine
Ca^{2+}	calcium ion
CRP	cAMP Receptor Protein
CTP	Citidine Triphosphate
cryoEM	cryo-electron Microscopy
CysN	ATP sulfurylase subunit
NodQ	adenosine-5'-phosphosulfate kinase
DTT	Dithiothreitol
EF-G	Elongation Factor G
EF-Tu	Elongation Factor Tu
G	Guanosine
GDP	Guanosine Diphosphate
GDPNP	Nonhydrolyzable GTP analog
GDI	Guanine nucleotide Dissociation Inhibitor
GMP	Guanisine Monophosphate
GTP	Guanosine Triphosphate
ITC	Isothermal Titration Calorimetry
IF1	Initiation Factor 1
IF2	Initiation Factor 2
IF3	Initiation Factor 3
fMet-$tRNA_i$	N-formyl-methionyl-tRNAi, bacterial initiator tRNA
LepA	Translation elongation factor catalyzing reaction of back translocation
L10 (11, 7/12)	Large ribosomal subunit protein 10 (11, 7/12)
MCW	Monod, Wyman and Changeux model
Mg^{2+}	magnesium ion
MonoQ	ion exchange chromatography medium with strong anion exchange properties
MSI	Magic Spot I
MSII	Magic Spot II
NGD	No-Go-Decay
NMD	Nonsense-Mediated Decay
NMR	Nucleic Magnetic Resonance
RF1/2	Releasing Factor 1 or 2
RF3	Releasing Factor 3
RNAse	ribonuclease
RRF	Ribosome Recycling Factor
rRNA	ribosomal RNA
RSH	RelA/SpoT Homologue

SAXS	Small Angle X-ray Scattering
SelB	specialized elongation factor required for selenocysteine insertion
SRL	Sarcin-Ricin Loop
U	Uridine
Tet	tetracycline resistance proteins
TypA/BipA	a tyrosine-phosphorylated GTPase that mediates interactions between enteropathogenic *E. coli* and epithelial cells
trGTPase	translational GTPase

INTRODUCTION

The most multifunctional molecules in the living cells are proteins. Proteins can have numerous functions, participating in cellular enzymatic catalysis, transport, storage, protecting, building molecules etc. Cellular homeostasis as well as any kind of metabolic reconstitution directly depends on the activity of proteins. Both production of proteins and regulation of their activity are tightly controlled.

A recurring motif in cellular control systems is regulation of protein function via its interaction with a small regulatory molecule. On the following pages I shall discuss two examples of such regulation. First is regulation of the translational GTPases IF2, EF-G and Hbs1 via their interactions with G nucleotides: GDP, GDP and, in the case of IF2 and EF-G, the stringent response alarmone ppGpp. The second part of the thesis is devoted to my investigations of the *Escherichia coli* stringent response enzyme RelA regulation by its product, ppGpp.

REVIEW OF LITERATURE

1. Regulation of protein function via regulation of protein structure

Proteins are highly flexible molecules, in principle able to attain astronomical numbers of various conformations but somehow managing to fold into the functional, native structure; a phenomenon referred to as 'Levinthal's paradox' (Levinthal, 1969). The native, active state represents a sub-set of possible protein structures residing on the bottom of the 'folding tunnel' – an energy landscape relating protein conformation to its potential energy (**Fig. 1**).

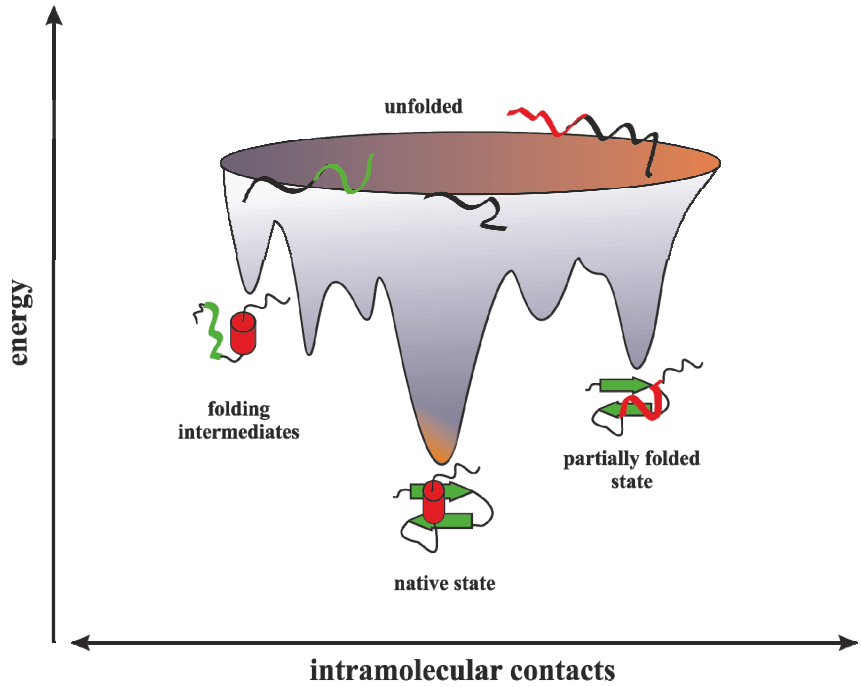

Figure 1. The protein folding energy landscape. The surface represents multitudes of conformations "funneling" towards the native state via formation of intramolecular contacts. Figure is adapted from (Jahn, 2005) with modifications.

In recent years numerous theoretical (Daily & Gray, 2007; Weinkam et al, 2012) and experimental (Popovych et al, 2006; Volkman et al, 2001) investigations have started uncovering how the geometry of the folding tunnel is exploited by the protein-binding ligands to regulate protein structure, and, therefore, activity. One of specific case of such regulation of protein function by ligands is so-called allosteric regulation, from Greek *allos* (ἄλλος), "other", and *stereos* (στερεὸς), "solid (object)". It is a mechanism of enzyme regulation

effected via binding of a ligand to a region which is reported as non-overlapping and stereochemically remote from the active site. With recent advances, several general features of ligand-mediated regulation of proteins have become increasingly evident.

First, rather than by inducing a *novel* protein conformation, absent in the structural ensemble of ligand-free proteins, protein ligands exert their regulatory role via "conformational selection" by shifting the *distribution* between several discrete *pre-existing* conformations (**Fig. 2**) (del Sol et al, 2009). Recent development of the experimental techniques able to follow protein dynamics with sufficient temporal and structural resolution, such as NMR (Kalodimos, 2011), or Small Angle X-ray Scattering (Fetler et al, 2007) was instrumental for demonstrating this mechanism.

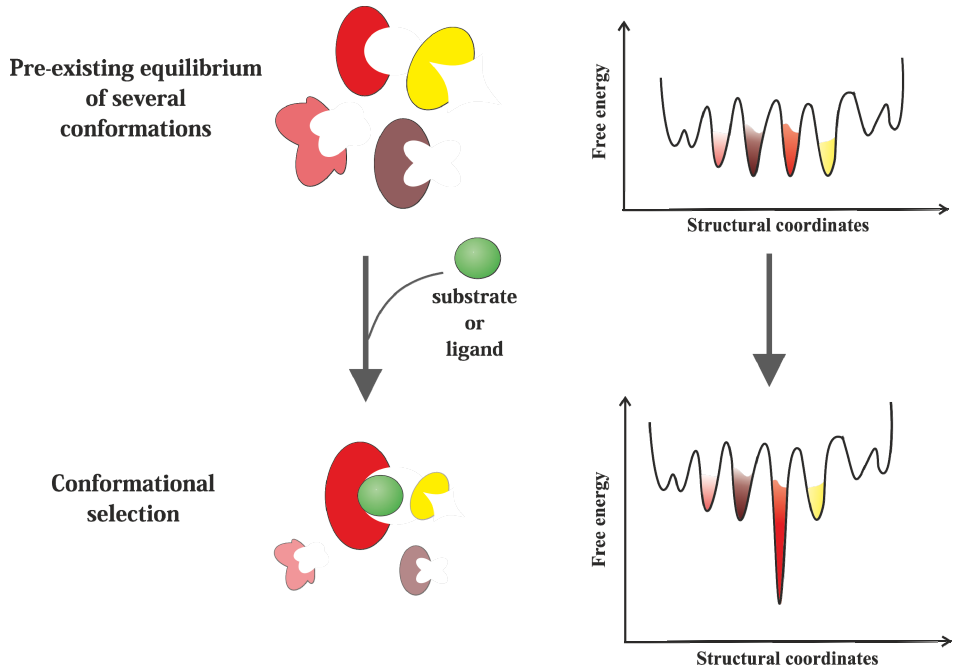

Figure 2. Mechanism of conformational selection upon the biding of ligand. Pre-existing distribution of protein conformations is altered by biding of the substrate, driving the ensemble to one predominant conformation. Since different conformations have different functional activity, conformational selection leads to change in the activity of the protein ensemble as a whole.

The conformational selection mechanism is in remarkable agreement with so called "MCW" model for allosteric regulation that was proposed in 1965 by Monod, Wyman and Changeux (thus the name MCW) (Monod et al, 1965). It described allostericaly regulated proteins as being in an equilibrium between the

two differing in activity conformations, referred as *T* and *R* (*T*, taut and *R*, relaxed). Binding of the allosteric regulator was postulated to shift the distribution between the *T* and *R*, resulting in change of the protein activity overall. Despite the simplification postulating existence of the protein in only *two* rather than *multiple* conformations, the MCW model often captures the essence of the ligand-induced regulation (Changeux, 2012).

Second, allosteric regulation does not necessarily work via changes in the protein structure *per se* – surprisingly, efficient regulation is possible without changing the *average* structure of the protein ensemble (Tsai et al, 2008). The mechanism in play relies on the ligand-induced changes in so called vibrational activity, mostly represented by protein side-chain mobility, inducing its regulatory effects via alterations in the protein's entropy (Popovych et al, 2006). Therefore one should be cautious when interpreting allosteric regulation using static snapshots of protein conformations captured by x-ray investigations.

Linking the structural information with enzyme activity assays via mutational analysis and quantum mechanics & molecular dynamics simulations brings the protein structures to life.

2. GTPases: function and structure

2.1. GTPase functional cycle and its regulation

GTPases are a large and broadly distributed group of proteins separated into three main sub-families: small GTPases involved in cellular differentiation and growth (Paduch et al, 2001), translational GTPases (trGTPases) involved in protein biosynthesis (Margus et al, 2007) and multisubunit G-proteins which are mediating signal transduction (Simon et al, 1991).

The main characteristic feature of GTPases is their ability to bind and hydrolyze the GTP nucleotide to GDP. This conversion of the bound G nucleotide in turn is translated into the conformational changes in the protein, and in the simplified, MCW-inspired scheme GTPases are assumed to toggle between the two conformations, inactive GDP- (*D*) and active GTP- (*T*) bound (Bourne et al, 1991). The apo (nucleotide unbound) state is usually treated as functionally identical with to the GDP-bound *D* state, and indeed, x-ray structures of these two states are often very similar. Transitions between the *T* and *D* conformations are regulated by several mechanisms (**Fig. 3**).

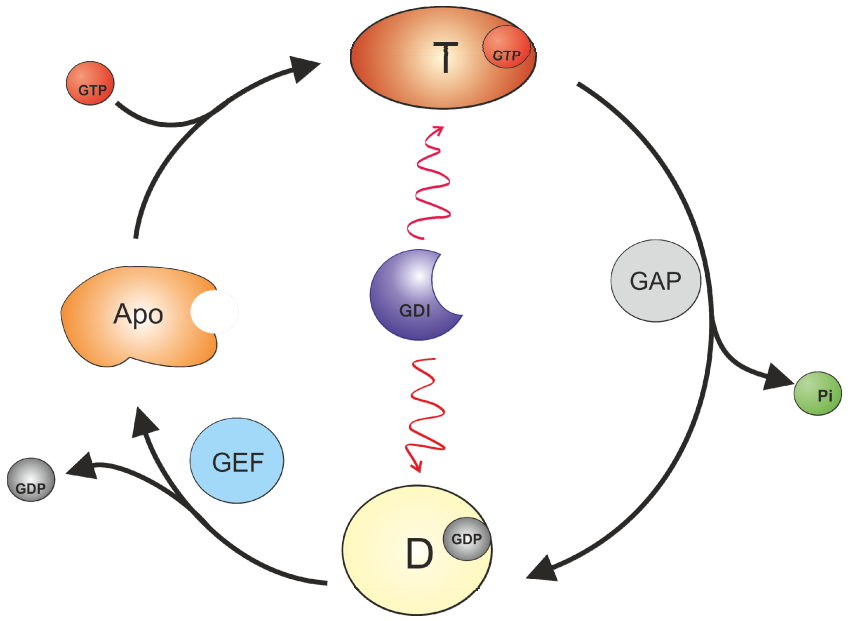

Figure 3. A GTPase's functional cycle. Stabilization effect of GDI is represented with wavy arrows.

First, the intrinsic ability of a GTPase to hydrolyze triphosphate is very low and is strongly activated by the GTPase activation proteins (GAPs) (Siderovski & Willard, 2005). Second, Guanine nucleotide exchange factors (GEFs) promote the exchange of GDP to GTP, driving a higher affinity of GTP to the nucleotide-free protein and stabilizing this transition state or increasing the rates of nucleotides dissociation and further exchange and preparing the protein for another round in active *T* form (Cherfils & Chardin, 1999). Third, guanine nucleotide dissociation inhibitors (GDI) prevents G nucleotides dissociation from GTPase, thus stabilizing both the GTPase:GTP and GTPase:GDP complexes (Siderovski & Willard, 2005).

2.2. Structure of the G domain

All members of the GTPase family share a structurally and functionally conserved G-domain, differing markedly in their repertoire of auxiliary domains. Small GTPases, such as Ras, consist of just one domain. Translational GTPases, on the other hand, contain up to 5 domains, and receptor G-proteins can be even more complex. However, despite of significant difference in secondary and tertiary structures, they share a highly conserved core – 166–168 residues nucleotide binding construct or G domain.

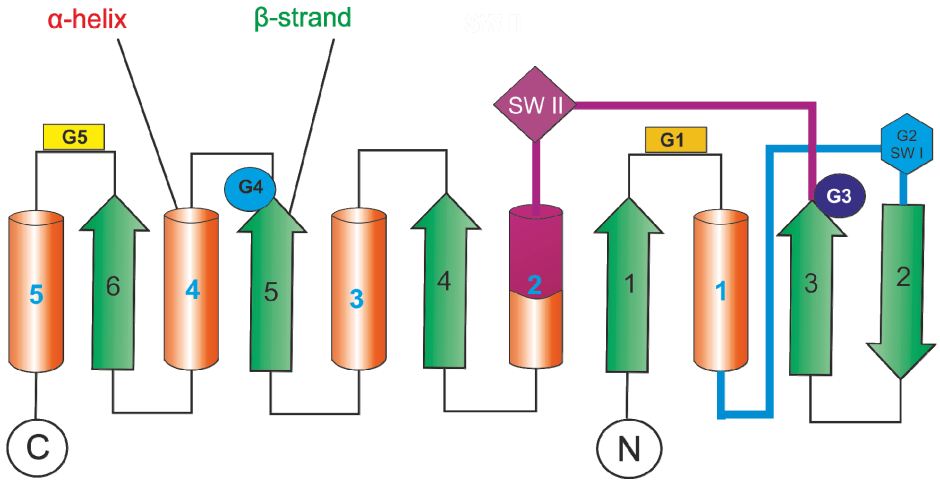

Figure 4. Topology diagram of the G domain. β-strands B1-B6 are in green, α-helices A1-A5 in red, G1-G5 motifs and N and C termini as indicated. Picture is adopted from (Wittinghofer & Vetter, 2011).

The G domain consists of α-helices (A1–A5) and β-sheets (B1–B6) interconnected with each other via highly conserved loops (Dever et al, 1987) **(Fig. 4)**. The main contribution in nucleotides recognition and binding comes from the loop elements. Non-discriminating binding to G nucleotides is mediated by G1 conserved sequence motif together with flanking regions of A1 and B1 (so called P loop) chelating the α- and β-phosphates. G4 and G5 motifs are interacting with the guanine base. Discrimination between di- and triphosphate guanine nucleotides is achieved by the G2 (or Switch I) and G3 with the part of A3 (or Switch II) motifs which Mg^{2+}-dependently bind the γ-phosphate. These two elements undergo significant conformational changes upon GTP/GDP binding, fuelling the GTPase's conformational 'switching' (Sprang, 1997).

3. Role of translational GTPases in the functional cycle of bacterial ribosome

In silico searches of bacterial genomes have identified nine subfamilies of translational GTPases in bacteria: EF-G, EF-Tu, IF2, RF3, SelB, Tet, TypA/BipA, LepA and CysN/NodQ (Margus et al, 2007). Out of these, three – elongation factors EF-Tu and EF-G, and initiation factor IF2 – are universal across all life, indicating that they are indispensable for the bacterial, eukaryotic and archaeal cell. Indeed, these factors facilitate the four basic steps of translation: initiation, elongation, termination and ribosomal recycling (**Fig. 5**).

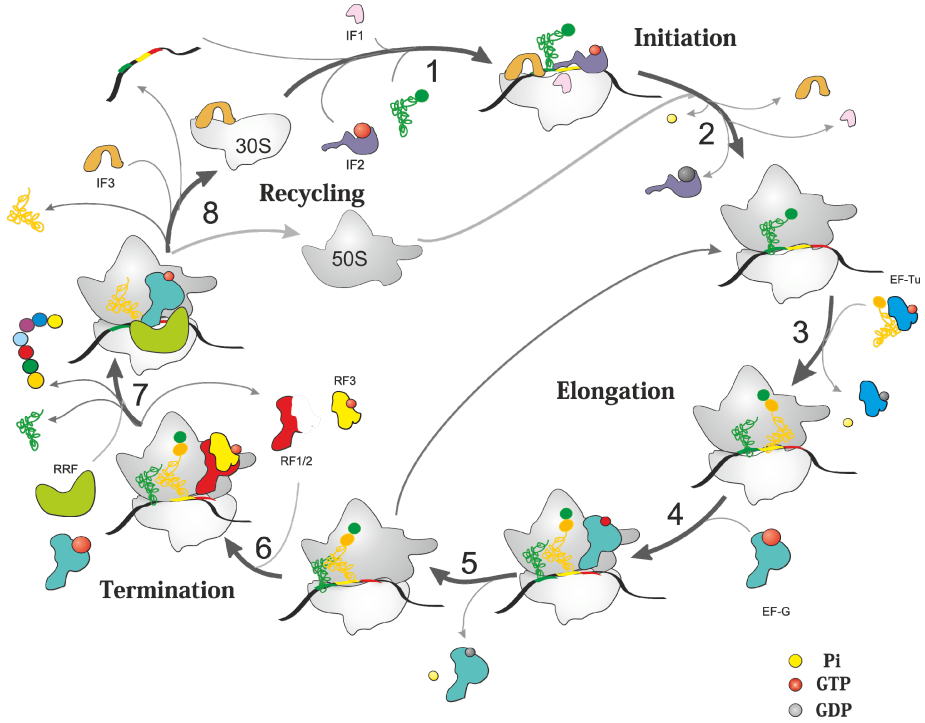

Figure 5. Schematic view of bacterial translation. Key steps of translation are shown as solid arrows and numbers from 1 to 8. *1* – formation of 30S initiation complex containing small ribosomal subunit (30S), mRNA, initiation factors IF1 (light pink), IF2 (violet) in complex with GTP and IF3 (orange) and initiator fMet-tRNA$_i$ (green). *2* – joining of 50S subunit and formation of 70S initiation complex containing mRNA and initiator fMet-tRNA$_i$ in the P-site with subsequent GTP hydrolysis by IF2 and release of all initiation factors. IF2:GDP complex is shown in violet with grey ball. Inorganic phosphate is shown as yellow ball. Elongation cycle is represented by *3* – *5* stages. *3* stands for aa-tRNA delivery by EF-Tu in complex with GTP (blue with red ball) to the ribosomal A-site and EF-Tu:GDP dissociation after codon-anticodon cognition followed by transpeptidation. *4* to *5* stages represents translocation with participation of EF-G:GTP complex (light blue) and subsequent post-translocation complex formation with peptidyl tRNA in P-site, deacylated tRNA in E-site and free A-site. *6* – association of releasing factors complex RF1/2:RF3:GTP (red and yellow) in respect to the presence of stop codon in A-site. *7* – nascent polypeptide (multicolor chain) and E-site tRNA (green) release and incorporation of RRF (light green) and EF-G:GTP complex catalyzing splitting the ribosome into two subunits – *8*.

3.1. Initiation: IF2

During the initiation step mRNA is loaded on the ribosome and the initiator codon is recognized by the P-site incorporated initiator tRNA (fMet-tRNA$_i$) (Milon & Rodnina, 2012). In bacteria this process is facilitated by several initiation factors. Initiation Factor 3 (IF3) prevents non-productive subunit joining

and contributes to selection of the correct initiator tRNA (Antoun et al, 2006a; Antoun et al, 2006b). Selection of the cognate tRNA and initiator codon is aided by another initiation factor, IF1 (Antoun et al, 2006a; Antoun et al, 2006b). Both of these factors exert their roles by interacting with translational GTPase IF2.

The GTPase cycle of IF2 drives its function during translation initiation. IF2 achieves its functionally active form via interactions with initiator tRNA, GTP, and – to a much lesser extent – GDP (Pavlov et al, 2011). Several lines of evidence suggest that this conformational change, rather than hydrolysis of GTP *per se* is necessary for IF2 to perform its function (Antoun et al, 2003; Pavlov et al, 2011), perhaps the most striking being recent a report documenting that an IF2 mutant incapable of GTP hydrolysis still supports *E. coli* viability (Fabbretti et al, 2012).

After assembly of the ribosomal initiation complex programmed with mRNA and P- (Peptidyl) site fMet-tRNAi and subsequent release of initiation factors 1 and 3, the ribosome is ready to accept an aminoacylated tRNA into its free A- (Aminoacyl) site to form first peptide bond. Recent single-molecule investigations demonstrated an overlap between IF2 dissociation and binding of the elongator tRNA in the complex with EF-Tu GTPase, somewhat blurring the linear perspective of the ribosomal cycle (Tsai et al, 2012).

3.2. Elongation: EF-G and EF-Tu

During the elongation stage the polypeptide chain grows by one amino acid at time, with amino acids being delivered in a ternary complex consisting of aminoacyl-tRNA (aa-tRNA) bound to elongation factor Tu (EF-Tu) with GTP. Complementarity between the mRNA codon triplet in the A-site and anticodon sequence in aa-tRNA acts as a signal for GTP hydrolysis by EF-Tu, leading to release of the GTPase in the GDP form. GTP hydrolysis by EF-Tu acts as an irreversible step during the decoding process, increasing the overall accuracy of the process by means of proofreading (Thompson & Stone, 1977; Wohlgemuth et al, 2011). Binding of GTP to EF-Tu is strongly stimulated by formation of the EF-Tu:aa-tRNA complex, and a specialized GEF, EF-Ts catalyzes the GDP to GTP exchange reaction (Gromadski et al, 2002; Ruusala et al, 1982). After accommodation on the ribosome, the A-site aa-tRNA engages in the trans-peptidation reaction with the P-site tRNA, resulting in a polypeptide-tRNA situated in the A-site and deacylated tRNA in the P-site.

Next, the ribosome completes the elongation cycle by moving one codon along the mRNA, relocating the peptidyl-tRNA into the P-site and deacylated tRNA into the E- (Exit) site. This process – translocation – in bacteria is catalyzed by elongation factor G (EF-G). Translocation was extensively studied over the last decades, and is in general well-understood (Rodnina & Wintermeyer, 2011). GDP purified from traces of GTP by monoQ ion exchange chromatography fails to stimulate productive EF-G-catalyzed translocation, suggesting that GTP hydrolysis but not just the conformational change is required (Ermolenko & Noller, 2011; Pan et al, 2007; Spiegel et al, 2007; Zavialov et al, 2005a).

3.3. Termination: RF3

Translation termination occurs when the ribosome arrives at a so-called stop codon (UAA, UAG or UGA). Instead of the aa-tRNA, these codons are recognized by class-1 termination factors. In bacteria UAG is recognized by release factor 1 (RF1), UGA triplet is recognized by release factor 2 (RF2) and UAA can be read by both (Scolnick et al, 1968). Upon recognizing the stop codon, class-1 factors induce hydrolysis of the peptidyl-tRNA, thus releasing the nascent polypeptide from the ribosome. Bacterial class-2 termination factor RF3 is a GTPase which accelerates removal of the class-1 factors from the ribosome (Freistroffer et al, 1997). RF3 is not essential for bacterial viability (Grentzmann et al, 1994) and is far from universal (Margus et al, 2007), suggesting that its role in translation termination is auxiliary. In line with that, recent experiments suggested that the main role of RF3 is not in translation termination, but rather in protein quality control (Zaher & Green, 2009; Zaher & Green, 2011).

In eukaryotes, translation termination is governed by a pair of factors – eRF1 and eRF3 – that are non-orthologous to the bacterial termination factors (Atkinson et al, 2008). The class-1 factor, eRF1 recognizes all the three termination codons (Frolova et al, 1994), and the class-2 factor, eRF3, is a translational GTPase assisting eRF1 (Alkalaeva et al, 2006; Zhouravleva et al, 1995). Unlike their bacterial counterparts, eRF1 and eRF3 form a tight complex off the ribosome (Zhouravleva et al, 1995), and formation of the complex promotes the GTP binding to eRF3 (Hauryliuk et al, 2006; Mitkevich et al, 2006; Pisareva et al, 2006). Detailed kinetic analysis reveals that eRF3 acts as GDI, dramatically reducing the GTP dissociation rate from eRF3 (Pisareva et al, 2006), and thus promoting formation of the eRF1:eRF3:GTP ternary complex.

In addition to termination, eRF1 and eRF3 are involved in Nonsense-Mediated mRNA Decay, NMD – degradation of the mRNAs containing in-frame premature stop codons (Kobayashi et al, 2004). Their homologues Dom34 and Hbs1 mediate another quality control mechanism, No-Go Decay (Doma & Parker, 2006). NGD degradation happens to mRNA found in complex with a ribosome stalled at structural obstructions like irresistible hairpins, stem loops or more complicated elements. Just like eRF1 and eRF3, Dom34 and Hbs1 form a ternary complex, with Dom34 promoting GTP binding to the Hbs1 GTPase (Graille et al, 2008).

3.4. Ribosome recycling: EF-G

After translation termination and polypeptide release, the ribosome is split into subunits during the so-called ribosomal recycling step (Hirokawa et al, 2006; Jackson et al, 2012). In bacteria ribosomal recycling is orchestrated by three factors: specialized ribosomal recycling factor, RRF, (Ishitsuka & Kaji, 1970) together with EF-G splits the ribosome into subunits (Zavialov et al, 2005b), and initiation factor 3 prevents subsequent re-association and induces dissociation of the mRNA (Peske et al, 2005).

3.5. Ribosomal elements regulating translational GTPases

All translational GTPases bind to the ribosome in the same region in the vicinity of the A-site. This binding region consists of part of 23S rRNA domain II, part of domain VI (the sarcin-ricin loop), proteins L10, L11 and L7/L12. Numerous structural and biochemical investigations over the years have identified the key players involved in ribosome-mediated regulation of GTPase activity.

Ribosomal protein L7/L12 is believed to act as a baseball glove catching trGTPases and delivering them to the ribosome (Diaconu et al, 2005). Over the years it was first suggested to act as a GAP for trGTPases (Mohr et al, 2002), or ribosomal element controlling phosphate release after the GTP hydrolysis (Savelsbergh et al, 2005). The ribosomal rRNA region referred to as the sarcin-ricin loop (SRL) forms extensive contacts with trGTPases (Gao et al, 2009) and was suggested to be directly involved in GTPase activation (Clementi et al, 2010). However, this point of view was recently challenged, and SRL was suggested to act merely as an anchoring point for trGTPase binding (Chan & Wool, 2008; Shi et al, 2012). *In vitro* investigations using EF-G and an RNA oligonucleotide mimicking SRL demonstrated that complex formation between these two components is strongly inhibited in the presence of GDP, suggesting that SRL could potentially play a role in discrimination by the ribosome between EF-G:GDP and EF-G:GTP (Munishkin & Wool, 1997). Ribosomal protein L11 is associated with the rRNA region called the thiostrepton loop, and the interaction between L11 and trGTPases is affected by the binding of the antibiotic thiostrepton (Harms et al, 2008). This results in destabilization of the 70S:trGTPase complex, leading to inhibition of translation (Walter et al, 2012). In addition to its importance for regulation of trGTPases, L11 is crucial for function of the stringent response factor RelA (Smith et al, 1978) (see below).
Although the exact mechanisms of GTPase activation are still unknown, it is apparent that both rRNA and proteins seem to work in collaboration to facilitate positioning of the factors relative to other ribosomal components, thereby contributing to catalysis, and stabilization of the active conformation of factors. The requirement for multiple signals for GTPase activity stimulation such as contacts with L7/L12, SRL, and L11 may help to avoid premature GTP hydrolysis during initial factor binding.

4. The stringent response

Bacterial cells sense tightly controlled intracellular nucleotide concentrations. Concentrations of 'general use' nucleotides can act as triggers of physiological responses, e.g. decrease in GTP concentration induces sporulation (Lopez et al, 1981) and genetic competence (Inaoka & Ochi, 2002) in *Bacillus subtilis*. In addition to sensing the 'general' nucleotides, several nucleotides are utilized in bacteria specifically as intracellular messengers (Pesavento & Hengge, 2009). Cyclic AMP (c-AMP), interacting with multiple target proteins possessing a cAMP Receptor Protein (CRP) domain regulates transcription of numerous

catabolic pathways, flagellum biosynthesis, biofilm formation, and virulence (McDonough & Rodriguez, 2012). Cyclic di-GMP (c-di-GMP) is bound by several different sensory domains and acts as an allosteric regulator of enzyme function (Amikam & Galperin, 2006), modulator of transcription factor activity (Sudarsan et al, 2008) and localized proteolysis (Duerig et al, 2009).

Two highly-charged G nucleotides, dubbed magic spot I and II (MSI and MSII) were identified in *E. coli* during amino acid starvation (Cashel & Gallant, 1969). Subsequent analyses showed that these compounds are produced during the idling step of protein biosynthesis (Haseltine et al, 1972) and are formed by addition of two extra phosphate groups to GDP and GTP at the 3' position, with ATP acting as a donor of the diphosphate group (Sy & Lipmann, 1973) MS1 and MS2 (or ppGpp and pppGpp) are mediators of the stringent response, a global regulatory mechanism in bacteria.

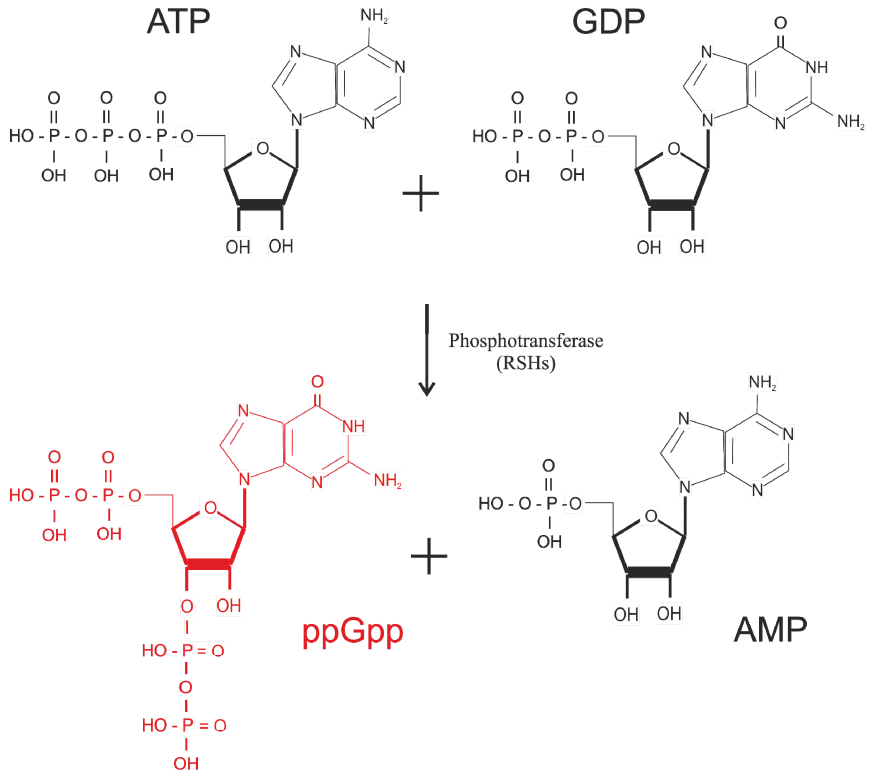

Fig. 6. Synthesis of ppGpp. Guanosine tetraphosphate (in red) is formed by phosphotransferases from ATP and GDP nucleotides. Guanosine pentaphosphate, pppGpp, is formed from ATP and GTP instead of GDP.

4.1. RSH proteins

In the broadest definition, the stringent response is a regulatory mechanism mediated by changes in the intracellular concentrations of ppGpp and pppGpp (Potrykus & Cashel, 2008). There are several proteins involved in both synthesis and degradation of these nucleotides. Historically, the first proteins to be discovered are *E. coli* RelA (Stent & Brenner, 1961) and SpoT (Laffler & Gallant, 1974). These two gave the name to the protein family, RelA-SpoT Homologue, RSH (Atkinson et al, 2011; Mittenhuber, 2001).

RelA has a strong, ribosome-dependent ppGpp synthetic activity (Haseltine et al, 1972). Its catalytic cycle is tightly regulated by the ribosome's functional state: by inspecting the CCA' end of the A-site tRNA, RelA reads the translational status of the cell and converts this signal into the rate of the production of the messenger nucleotide ppGpp (Haseltine et al, 1972; Wendrich et al, 2002). Deacylated A-site tRNA acts as a strong activator of ppGpp production, and active translation acts as a strong inhibitor of RelA (Haseltine et al, 1972).

Despite almost forty years of research, our understanding of the mechanistic details of the RelA cycle is still quite limited. First is the lack of structural information. We lack a full length x-ray structure of RelA, nor do we have a cryoEM reconstruction of the ribosome-bound protein. Our understanding of the RelA:70S topology is mainly based on papers mapping the RelA binding site using competition experiments with other translational factors (Richter et al, 1975) or investigating which of the ribosomal proteins can activate RelA *in vitro* (Richter et al, 1975). It is clear that RelA binds to the large subunit (Ramagopal & Davis, 1974), and the L11 ribosomal protein is one of the key components of the ribosome necessary for RelA activation, since it can activate RelA in the absence of the ribosome (Jenvert & Schiavone, 2007) and disruption of the L11 gene results in compromised stringent response, so-called 'relaxed phenotype' (Smith et al, 1978). Second, due to an absence of comprehensive biochemical investigations, even the basic mechanism of the RelA active cycle is a matter of debate. According to the 'hopping model' backed up by *in vitro* biochemical data, RelA synthesizes one ppGpp molecule while in complex with the ribosome, the act of ppGpp production dislodges RelA from the ribosome and then it 'hops' to the next ribosome, this way sampling the whole ribosomal population (Wendrich et al, 2002). Alternatively, according to the 'extended hopping model' based on *in vivo* single molecule investigations, multiple rounds of ppGpp production are performed by RelA off the ribosome, and only transient binding to the ribosome with deacylated tRNA in the A-site is necessary to convert RelA into the catalytically active form (English et al, 2011). Clearly, further experiments are required to reach a coherent understanding of the RelA mechanism.

SpoT has both weak synthetic and strong ppGpp hydrolytic activities (An et al, 1979; Xiao et al, 1991). The synthetic activity of SpoT is activated by numerous stresses, e.g. limitation of iron (Vinella et al, 2005) and fatty acids (Battesti & Bouveret, 2006). Detailed *in vitro* investigations of SpoT's

mechanism are largely hindered by difficulties with purification of the protein, and most of what we know about it comes from *in vivo* experiments used to identify the stress conditions inducing SpoT activity, determine the interaction partners (Battesti & Bouveret, 2006) and to map different activities on the protein's primary sequence (Angelini et al, 2012; Xiao et al, 1991).

Both RelA and SpoT are products of duplication and divergence of the ancestral bifunctional, ribosome-dependent Rel protein (Atkinson et al, 2011; Mittenhuber, 2001) (**Fig. 7**).

Rel proteins from *Mycobacterium tuberculosis* and its non-pathogenic relatives have been quite extensively investigated *in vitro* (Avarbock et al, 2005; Avarbock et al, 2000; Jain et al, 2007; Sajish et al, 2009), and a crystallographic structure is available for truncated Rel from *Streptococcus dysgalactiae subsp. equisimilis* (Hogg et al, 2004). Just like RelA, Rel's synthetic activity is strongly activated by the ribosome, with deacylated A-site tRNA acting as an ultimate inducer (Avarbock et al, 2000). An inter-domain auto-inhibitory cross-talk was suggested to regulate Rel activity (Jain et al, 2007), similarly to RelA (Mechold et al, 2002). This inbuilt auto-inhibition was suggested to work as an internal timer for switching the activated RelA after dissociation from the ribosome during prolonged 'hops' (English et al, 2011).

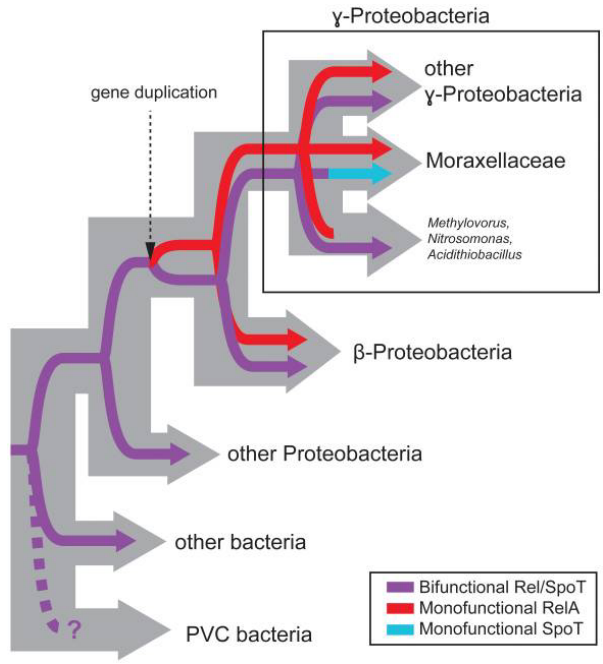

Figure 7. Schematic diagram for the evolution of long RSHs in bacteria. Thick gray branches indicate the divergence of bacterial groups, while the inner line shows the divergence of long RSH proteins and their functionality, as per the inset box. Reproduced with permission from (Atkinson, Tenson et al. 2011).

In addition to 'long' RSHs (RelA, Rel and SpoT) numerous 'short' RSHs have been discovered recently (Lemos et al, 2007; Murdeshwar & Chatterji, 2012). These proteins are monofunctional, i.e. have only synthetic or hydrolytic activity, and are considerably smaller, containing no ribosome-binding domains. Rarely, they can contain additional domains, such as RNAse (Murdeshwar & Chatterji, 2012).

4.2. Targets of ppGpp

The main molecular target of ppGpp is RNA polymerase (Reddy et al, 1995). However, despite almost forty years of research, the exact molecular mechanism of ppGpp-mediated regulation of RNA polymerase and the location of the ppGpp-binding site are still a matter of debate (Vrentas et al, 2008). In *E. coli*, binding of ppGpp and auxiliary factor DksA to the polymerase changes its specificity, down-regulating transcription of rRNA (Murray et al, 2003) as well as of genes coding for ribosomal proteins (Lemke et al, 2011), while activating transcription of genes involved in amino acid biosynthesis (Paul et al, 2005). Conversion of GTP to ppGpp during the stringent response results in a decrease in the GTP concentration, affecting transcription of mRNAs using G as an initiator nucleotide – an effect which works together with ppGpp-mediated regulation in *E. coli* (Murray et al, 2003), and is the sole regulatory mechanism in *Bacillus subtilis*, since *B. subtilis* RNA polymerase is insensitive to ppGpp (Krasny & Gourse, 2004). Numerous global transcriptome analyses of ppGpp-mediated regulation of the transcriptional program both in *E. coli* and other organisms, reveal global regulatory effects on cellular metabolism (Traxler et al, 2006; Traxler et al, 2011; Vercruysse et al, 2011), as well as specific regulatory pathways, such as regulation of antibiotics production in *Streptomyces coelicolor* (Hesketh et al, 2007).

Regulation of transcription is not the only regulatory pathway exploited by ppGpp (Dalebroux & Swanson, 2012). Several other targets of ppGpp have been identified over the years: translational GTPases (Legault et al, 1972), DNA primase (Wang et al, 2007), polynucleotide phosphorylase (Gatewood & Jones, 2010) and lysine decarboxylase (Kanjee et al, 2011) to name a few. Therefore it is no surprise that the stringent response is involved in regulation of bacterial virulence (Dalebroux et al, 2010), antibiotic tolerance (Nguyen et al, 2011), the bacterial cell cycle (Ferullo & Lovett, 2008) and biofilm formation (He et al, 2012). Therefore control of the stringent response is potentially a very powerful tool, with both biotechnological (Hoffmann & Rinas, 2004) and medical (Wexselblatt et al, 2010) applications.

RESULTS AND DISCUSSION

Aims of the study

In this thesis I have investigated by means of *in vitro* experimentation several cases of molecular regulation by nucleotides. The specific questions addressed in this work are:

- What are the affinities of *E. coli* translational GTPases IF2 and EF-G to GTP, GDP and ppGpp? (Papers I, II)
- Is there an interplay among G nucleotide and initiator tRNA binding to IF2? (Paper II)
- What is the interplay among G nucleotide and SRL of rRNA binding to IF2 and EF-G? (Paper V)
- How does complex formation between Hbs1 and Dom34 affect the kinetics of GTP dissociation from Hbs1? (Paper III)
- Is there a regulatory effect of ppGpp on *E. coli* stringent response enzyme RelA? (Paper IV)

Regulation of translational GTPases by G nucleotides and other ligands

1.1 Binding of G nucleotides and initiator tRNA to IF2 are independent

A powerful method for investigation of interactions *in vitro* is Isothermal Titration Calorimetry (ITC) (Ghai et al, 2012). There are several benefits of this method. First, one can use natural, non-labeled substrates. Second, it provides full thermodynamic characterization of the system, determining directly the heat that is absorbed or generated during any binding reaction. This parameter, which is proportional to the enthalpy of binding (ΔH) is used to calculate the rest of thermodynamic values such as entropy (ΔS) and Gibbs free energy (ΔG) from the following equation

$$\Delta G = \Delta H - T\Delta S = -RT\ln Ka \quad (1)$$

(where R is the gas constant and T is the absolute temperature). The association constant (Ka) and reaction stoichometry (n) can be calculated as well providing a full set of data for thermodynamic profiling of binding in a single experiment.

Partitioning the entropic and enthalpic members into the Gibbs free energy of the interaction is useful for understanding the nature of binding reaction (Ladbury et al, 2010). And third, plotting the enthalpy of interaction vs the temperature of measurement one can calculate the change in heat capacity of the interaction (Δc_p),

$$\Delta c_p = d(\Delta H)/d\Delta T \quad (2)$$

The heat capacity parameter is a powerful tool for assessment of the change in the solvent-accessible area of the molecule upon ligand binding. In turn this value reflects protein structural rearrangement accompanying complex formation. Large negative value of Δc_p corresponds to a reduction in the solvent-accessible value of the protein typical, for instance, for protein-protein interaction or simple folding of peptide chain. Th relation between the Δc_p parameter and change in solvent-accessible area is described by expression

$$\Delta c_p = 0.27\ \Delta A_{aromatic} + 0.4\ \Delta A_{nonaromatic} \quad (3)$$

where $\Delta A_{aromatic}$ and $\Delta A_{nonaromatic}$ are protected areas for aromatic and non-aromatic aminiacids, respectively, in $Å^2$ (Samanta et al, 2002). The main drawback of using ITC is that this method requires very high amounts (≈5000 pmoles per experiment) of the starting material.

In papers I (Hauryliuk et al, 2009) and II (Mitkevich et al, 2010) we have investigated EF-G and IF2 interactions with G nucleotides and initiator tRNA by means of ITC. By analyzing the interactions at different temperatures and calculating the Δc_p values, we have demonstrated that binding of GDP to IF2 promotes structural rearrangements in the protein (Hauryliuk et al, 2009). This result was later supported by other groups using different methods – Nucleic Magnetic Resonance, NMR (Wienk et al, 2012) and SAXS (Vohlander Rasmussen et al, 2011). Available cryoEM reconstructions of apo-, GDP- and GDPNP-bound IF2 on the ribosome also support the existence of a GDP-induced rearrangement in IF2 (Myasnikov et al, 2005). Comparing affinities of EF-G and IF2 to GTP, GDP and ppGpp determined by ITC (**Table 1**) with *in vivo* concentrations of these nucleotides (Buckstein et al, 2008), we came to the conclusion that of the two trGTPases, IF2 is the main *in vivo* target of ppGpp-mediated inhibition (Mitkevich et al, 2010). This result provides quantitative support for an earlier work suggesting that IF2 acts as a ppGpp sensor *in vivo* (Milon et al, 2006). Lastly, we demonstrated that the interaction between IF2 and initiator tRNA is insensitive to binding of G nucleotides, including binding of ppGpp, which is known to be a strong inhibitor of translation (Mitkevich et al, 2010). This somewhat surprising result, however, corroborates well with earlier observations that complex formation between IF2 and initiator tRNA is insensitive to GDP and GTP (Petersen et al, 1979; Wu & RajBhandary, 1997). Despite slight differences in affinities, binding of G nucleotides to the IF2 significantly changes entropy-enthalpy partititioning of initiator tRNA binding to the different IF2 forms.

Table 1. Thermodynamic parameters of IF2 and EFG binding to GTP, GDP and ppGpp nucleotides determined by ITC. All measurements were performed two to four times in phosphate buffer with following composition: 5 mM K_2HPO_4, 10% glycerol, 1mM DTT, 95 mM KCl, 5 mM $MgCl_2$, pH 7.5. K_a and K_d stand for association and dissociation equilibrium constants, respectively. Standard deviation for K_a did not exceed ± 20%, for ΔH did not exceed ± 10%. K_d was calculated as $1/K_a$.

GTPase	Ligand	K_a, M^{-1}	K_d, μM	ΔG^o, kcal/mol	ΔH^o, kcal/mol	$T\Delta S^o$, kcal/mol
IF2	GTP	1.5×10^5	6.7	–7.05	–19.3	–12.25
	GDP	6.1×10^5	1.6	–7.88	–4.62	–12.20
	ppGpp	3.6×10^5	2.8	–7.57	–12.81	–5.24
EF-G	GTP	1.2×10^5	8.3	–6.95	–1.70	5.25
	GDP	1.1×10^5	9.1	–6.86	–5.90	0.96
	ppGpp	7.2×10^5	13.9	–6.62	–5.09	1.53

1.2. Binding of G nucleotides and SRL rRNA to IF2 and EF-G are mutually exclusive

The original report by Munishkin and Wool (Munishkin & Wool, 1997) demonstrated that complex formation between EF-G and the SRL RNA oligonucleotide is inhibited in the presence of GDP, and is insensitive to the non-hydrolysable GTP analogue, GDPNP. Several questions, however, remained unanswered. First, provided that formation of the SRL:EF-G complex is inhibited by GDP, is the reverse true as well, i.e. is binding of GDP to EF-G inhibited in the presence of the SRL RNA oligonucleotide? Second, does GTP – not GDPNP - affect SRL binding to EF-G? Given that the SRL RNA oligonucleotide does not induce EF-G GTPase (Clementi et al, 2010), this experiment should be technically feasible. And third, is this a general mechanism, i.e. is the interaction between other trGTPases and SRL governed by the same rules?

We have answered all these questions by means of ITC measurements (paper V). First, we show that, indeed, binding of GDP to EF-G is inhibited by SRL. Second, we show that GTP, just as GDPNP, has no effect on EF-G:SRL complex formation. Lastly, we show that interaction of IF2 with SRL follows the same rules as interaction of EF-G with SRL does, indicating the general nature of the phenomenon (**Fig. 8**).

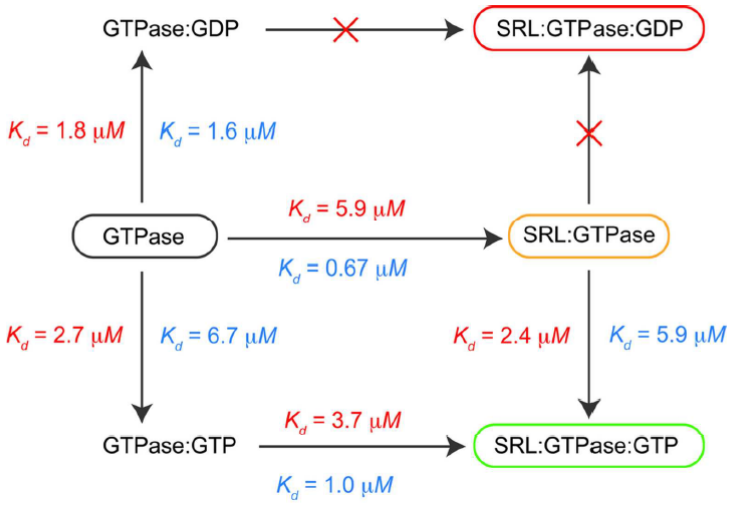

Figure 8. A scheme of the interplay among G nucleotides and SRL binding to the bacterial translational GTPases IF2 and EF-G. The affinity constants for IF2 complexes are shown in blue and for EF-G in red, as measured at 25°C.

We have also examined by ITC EF-G interactions with another ribosomal element, ribosomal protein L7/12. An earlier report demonstrated that interaction between apo-EF-G and isolated L7/12 is weak (K_d in mM range) (Mulder et al, 2004), but it was hypothesized that it could be promoted in the presence of GDP or GTP. We have performed measurements both with apo-EF-G and in the presence of GTP and GDP and detected no stable binding (**Fig. 9**).

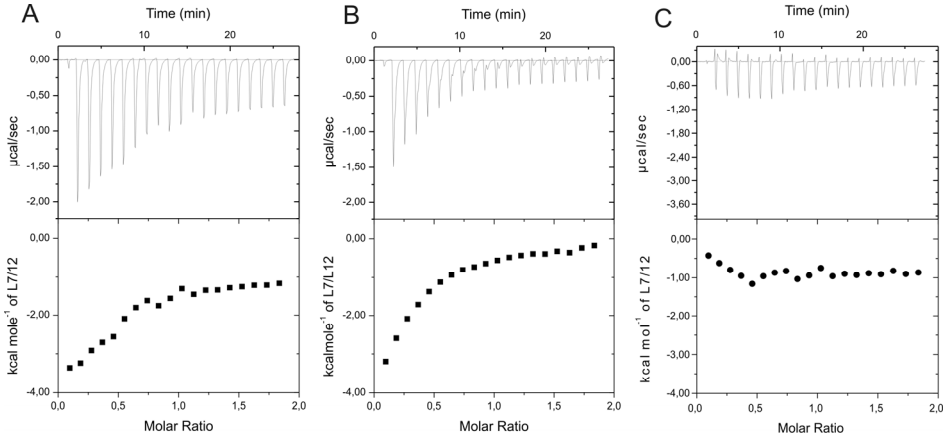

Figure 9. EF-G and L7/12 interact weakly. ITC titration curves (upper panel) and binding isotherms (lower panel) for L7/12 titration into EF-G (A), L7/12 dilution in to the buffer (B) and L7/12 titration into to EF-G after subtraction of L7/12 dilution (C).

1.3 Cross-talk between binding of G nucleotides and Dom34 to Hbs1

The eukaryotic translational GTPase Hbs1 forms a tight complex with Dom34, and formation of this complex promotes GTP binding by Hbs1 (Graille et al, 2008). Given the high degree of homology between eRF1/eRF3 and Dom34/Hbs1 pairs (Atkinson et al, 2008; Inagaki et al, 2003) and high degree of structural similarity between the two complexes (Chen et al, 2010; Cheng et al, 2009), it was highly likely that just like eRF1 for eRF3 (Pisareva et al, 2006), Dom34 acts as a GDI for Hbs1.

We have validated this hypothesis by measuring the GTP dissociation rate from the Dom34:Hbs1:GTP ternary complex (Chen et al, 2010) (**Fig. 10**). The dissociation rate constant (k_{-1}) was similar to that determined for the eRF1:eRF3:GTP complex (Pisareva et al, 2006), underscoring the functional similarities of these two systems. Guided by the X-ray structure of the Dom34:Hbs1 complex, we have investigated the effects of the two point mutations in Dom34 (R162A, R169A) and validated the functional importance of these two residues in Dom34 functionality as a GDI.

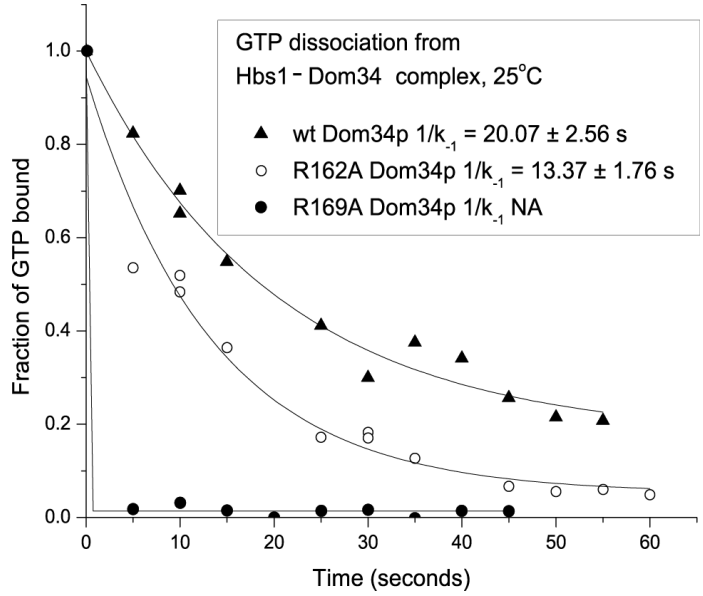

Figure 10. GTP and Dom34 bind to Hbs1 cooperatively and Dom34 acts as a GDI. The effect of Dom34 Kinetics of GTP dissociation from the Dom34-Hbs-GTP complex. Dissociation kinetics were followed in the presence of wild type and mutant (R169A and R192A) Dom34.

2. Regulation of *E. coli* stringent response protein RelA by ppGpp

Examination of the time courses of ppGpp production in *in vitro* stringent response systems (Payoe & Fahlman, 2011) revealed deviations from linearity in earlier time points due to a lag effect. A priori there can be several possible reasons for this behavior. Frist, it can be the effect of incubation at 37°C on the active RelA concentration. It has been suggested that RelA forms dimers (Gropp et al, 2001), and one could envision that over the course of the reaction, the ratio of dimers vs monomers changes, thus affecting RelA activity. Indeed, this kind of behavior was suggested for RelA homologue from *M. tuberculosis*, Rel (Avarbock et al, 2005). Second, it could be that accumulation of one of the products of RelA activity – ppGpp or AMP – results in activation of the RelA ppGpp-synthetic activity. To investigate the nature of the lag effect, we used a poly(U)-dependent *in vitro* stringent response system similar to that used in (Jenvert & Schiavone, 2007).

We have shown that production of ppGpp is responsible for the effect. The stimulatory effect is specific for ppGpp, and other nucleotides neither induce RelA activation nor interfere with the activating effect of ppGpp (**Fig. 11**).

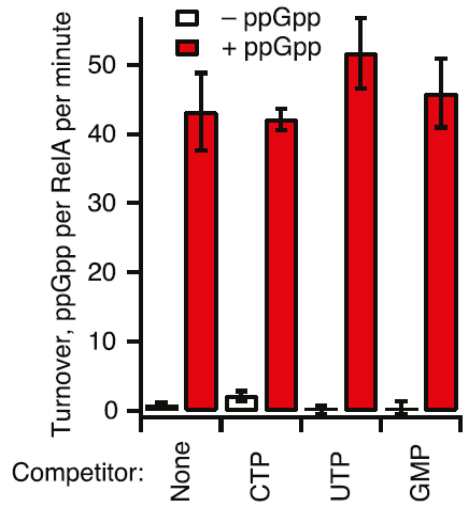

Figure 11. Effect of CTP, UTP and GMP on RelA phosphotransferase activity in the presence (solid red bars) and absence (hollow bars) of 100 µM ppGpp. The reaction mixture contains RelA, 70S, ppGpp, 3H-GDP, ATP and competing nucleotides.

Unlike the case of Rel (Avarbock et al, 2005), the preincubation time did not affect RelA activity. The ppGpp-mediated activation is strongly L11-dependent, and our titration experiments suggest that ppGpp acts by increasing RelA's

catalytic constant (k_{cat}) rather than altering its sensitivity to ribosome-mediated activation, i.e. changing the Michaelis constant, K_M (**Fig. 12**).

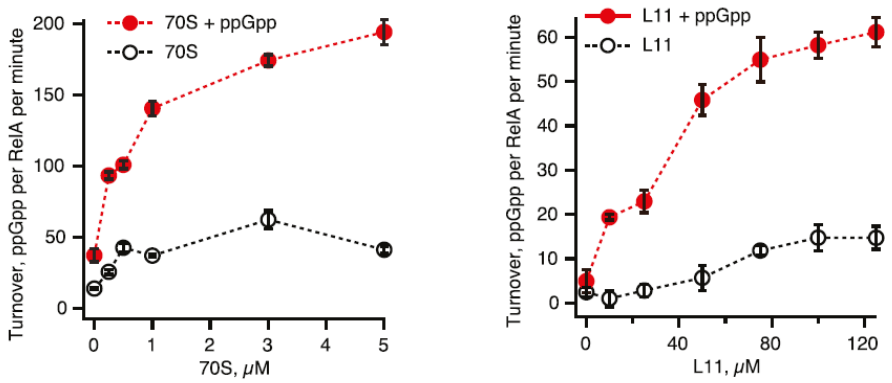

Figure 12. RelA synthetic activity as a function of the 70S ribosome concentration (left panel) and L11 concentration (right panel) in the presence (solid red cycles) or absence (hollow black cycles) of 100 μM ppGpp. Error bars represent standard deviation of the turnover estimated by linear regression. Each experiment was performed at least three times.

The activating effect of ppGpp is not masked in the presence of other RelA activators, such as A-site tRNA, suggesting that these two regulatory mechanisms act via different routes (**Fig. 13**).

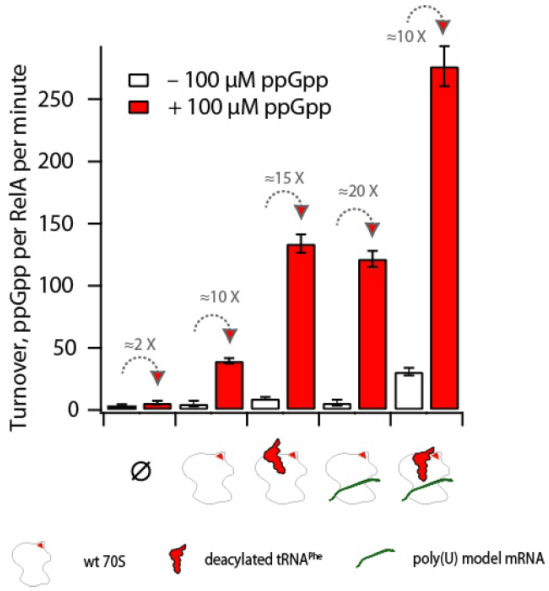

Figure 13. Effect of 70S ribosomes, poly(U) and deacylated $tRNA^{Phe}$ on on RelA synthetic activity in the presence (solid red bars) or absence (hollow bars) of ppGpp.

Our surprising result raises numerous questions, and opens new avenues in research on the stringent response.

First is the mechanism of ppGpp-mediated activation. Product-mediated enzyme activation is an exceedingly rare phenomenon. One previously documented mechanism is activation via change in the oxidative environment (Coleman et al, 1978), which is unlikely to be at play in the case of RelA – ppGpp is hardly an oxidative agent. Another possible – and more likely – mechanism is direct allosteric regulation of RelA by ppGpp. Several proteins are regulated by ppGpp this way, including RNA polymerase (Reddy et al, 1995). This hypothesis can be proven using the same techniques used for studying the RNA polymerase:ppGpp interaction: demonstrating the interaction using fluorescently-labelled ppGpp (Reddy et al, 1995), co-crystallization of ppGpp and its target (Artsimovitch et al, 2004) or crosslinking with thio-6-ppGpp (Toulokhonov et al, 2001). However, given how challenging it is to work with RelA *in vitro* (Pedersen & Kjeldgaard, 1977), implementation of none of these would be easy. Yet another possibility is that ppGpp activates RelA by means of some exchange reaction, and more detailed investigation of the molecular mechanism of ppGpp synthesis by RelA is due.

The second question is highly complementary to the first: which RSH molecules are activated by ppGpp, and which are not? What is the architecture of ppGpp-mediated cross-talk between RSH proteins? In order to answer this question, we plan to test several 'small' and 'long' RSH proteins from several organisms *in vitro*. Unfortunately, some of the RSH proteins are notoriously hard to purify, e.g. *E. coli* SpoT. Our *in vitro* experimentation is to be complemented by following RSH activity in the living cells on the single molecule level (English et al, 2011) using engineered *E. coli* strains coding several RSH genes.

CONCLUSIONS

- Of the trGTPases initiation factor IF2 is the main target of ppGpp-mediated inhibition of translation in bacteria
- Binding of G nucleotides and initiator tRNA to IF2 are independent of each other
- Binding of GDP and the rRNA element SRL to translational GTPases IF2 and EF-G are mutually exclusive
- Dom34 acts as a GDI for eukaryotic translational GTPase Hbs1
- ppGpp stimulates the bacterial stringent response enzyme RelA, creating a direct positive feedback loop

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SUMMARY IN ESTONIAN

Translatsiooniliste GTPaaside ja poomisvastuse faktori RelA reguleerimine G nucleotiidide poolt

G-nukleotiidide GTP, GDP ja alarmooni ppGpp kontsentratsioonid mõjutavad oluliselt translatsiooni bakterirakus. Käesoleva töö raames uurisin mehhanisme ppGpp sünteesiks, selle alarmooni märklaudu ning translatsioonis osalevate GTPaaside regulatsioonimehhanisme.

Stressitingimustes, eeskätt toitainete nappuse korral, tõuseb alarmooni ppGpp tase bakterirakus. Käesolevas töös näitasime, et ppGpp stimuleerib iseenda RelA valgu vahendatud sünteesi. Selline positiivse tagasisidestusega mehhanism tagab kiire stressivastuse.

Eelnevalt oli teada, et ppGpp inhibeerib mitmeid ribosoom-seoselisi translatsioonifaktoreid. Samas ei olnud teada, milline neist võiks olla selle alarmooni peamine märklaud. Käesoleva too käigus tegime kindlaks, et initsiatsioonifaktor kaks (IF2) seondab seda alarmooni oluliselt tugevamini kui teised translatsioonifaktorid. Seega võiks olla tegemist ppGpp põhilise märklauaga.

IF2 on üks translatsiooni masinavärgi põhikomponente. Eelpool märkisin ära, et stressitingimustes võib selle valguga võib seonduda ppGpp. Tavaolekus toimuva translatsiooni käigus seondub IF2 initsiaator tRNA, GTP/GDP ja ribosoomiga. Käesoleva töö käigus tegime kindlaks, et G-nukleotiidide ja initsiaator-tRNA seondumine on üksteisest sõltumatud sündmused. Samas näitasime, et G-nukleotiidid mõjutavad IF2e ja elongatsioonifaktor G (EF-G) seondumist ribosoomile. Täheldasime, et oma märklauaks oleva ribosomaalse RNA fragmendiga seonduvad need valgud eelistatud GTP vormis.

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