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Characterization of Ffh of *Mycobacterium tuberculosis* and its interaction with 4.5S RNA

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ABSTRACT

Signal recognition particle (SRP) mediates targeting of proteins to appropriate cellular compartments, which is an important process in all living organisms. In prokaryotes, SRP consists of Ffh, a protein, and 4.5S RNA that recognizes signal peptide emerging from ribosomes. The SRP (Ffh) of one the most successful intracellular pathogen, *Mycobacterium tuberculosis*, has been investigated with respect to biochemical properties. In the present study, Ffh of *M. tuberculosis* was overexpressed and was confirmed to be a GTPase using thin layer chromatography and malachite green assay. The GTP binding ability was confirmed by GTP overlay assay. The 4.5S RNA sequence of *M. tuberculosis* was synthesized by *in vitro* transcription assay. The interaction between Ffh and 4.5S RNA was confirmed by overlay assay and RNA gel shift assay. The results show that the biochemical properties of *M. tuberculosis* Ffh have been conserved, and this is the first report that shows the interaction of components of SRP in *M. tuberculosis*, namely Ffh protein and 4.5S RNA.

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Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, is one of the most successful intracellular pathogen, killing millions of people annually (WHO 2009). In *M. tuberculosis*, protein export mechanism is important for the vital physiologic processes and virulence (Feltcher et al. 2010). In bacteria, most of the proteins are exported to cytoplasmic membrane *via* two pathways, general secretory pathway or signal recognition particle (SRP) pathway (Fekkes and Driessen 1999; Herskovits et al. 2000; Luirink et al. 2005). The general secretory pathway is a post translational targeting machinery used by a variety of exported proteins, whereas the SRP functions cotranslationally to target subsets of proteins whose final destination is the cytoplasmic membrane (Macfarlane and Muller 1995; Valent et al. 1995; Ulbrandt et al. 1997). The components of prokaryotic SRP were initially identified by sequence comparison with well characterized eukaryotic SRP (Romisch et al. 1989; Bernstein et al. 1989), a ribonucleo protein complex consisting of one 7S RNA molecule as the central core to which six proteins of different sizes (9–72 kDa) were attached (Walter and Blobel 1982). While the prokaryotic SRP pathway is much simpler than eukaryotic counterpart consisting of two proteins, Ffh and FtsY and a 4.5S RNA molecule, all are essential for *Escherichia coli* viability (Brown and Fournier 1984; Gill and Salmond 1990; Phillips and Silhavy 1992).

Ffh is the bacterial homologue of SRP54, the eukaryotic 54-kDa protein that binds to the signal sequence of preprotein, hence the name Ffh (fifty-four homologue). One of the key functions of SRP, is the recognition of signal sequence of a nascent polypeptide as it emerges from the ribosome (Zopf et al. 1990). Based on the primary sequence, Ffh can be divided into three domains: the methionine-rich M domain interacts with signal peptide and 4.5S RNA (Romisch et al. 1990). The G domain has the GTPase activity required for its interaction with the docking protein and the subsequent release of the nascent peptide at the translocon (Samuelsson et al. 1995). Finally, the highly conserved N domain at amino terminus plays a role in the control of the GTP occupancy of the G domain (Freymann et al. 1999). The SRP receptor consists of a conserved docking-protein, FtsY in bacteria, is a homologue of Ffh and has G and N domains. Both require GTPase activity to form a heterodimer at the membrane and target the preproteins to the membrane (Kusters et al. 1995; Samuelsson et al. 1995).

SRP dependent protein targeting can take place in ribosomes containing short nascent peptides that emerge from ribosomes. The preprotein having hydrophobic amino terminal signal sequence is recognized by the Ffh – 4.5S RNA to form RNC (ribosome-nascent chain) complex (Miller et al. 1994; Luirink et al. 2005). Once the RNC binds to the signal sequence, the complex is targeted to the membrane associated SRP docking protein or receptor, FtsY. At the membrane, SRP is released from the preprotein in a GTP-dependent manner.

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The components of SRP have not been reported in *M. tuberculosis* which is having complex cell wall structure and a slow grower. In this study, we are reporting the characterization of SRP components Ffh and 4.5S RNA of *M. tuberculosis* for the first time.

Materials and methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 2. Bacterial strains DH5α (Invitrogen) and XL1-Blue (Stratagene) were used for cloning and TOP 10 (Invitrogen) for the cloning and expression of recombinant proteins. *E. coli* cells were grown and maintained in Luria–Bertani (LB) medium supplemented with ampicillin 50 μg/ml with constant shaking at 37 °C. *M. tuberculosis* H37Rv strain was grown in Middlebrook 7H9 broth supplemented with 10% albumin–dextrose–catalase (ADC) and 0.5% glycerol at 37 °C with constant shaking at 150 rpm for 4–6 weeks. LB-agar and 7H10 agar containing 10% oleic acid–albumin–dextrose–catalase (OADC) and 0.5% glycerol were used for *E. coli* and *M. tuberculosis* H37Rv, respectively.

Plasmids and DNA manipulations

Standard genetic and molecular biology techniques were used for construction of strains and plasmids (Sambrook et al. 1989). Polymerase chain reaction (PCR) oligonucleotide primers were designed (Table 1) to amplify full-length *ffh* gene from whole-genomic DNA of *M. tuberculosis* H37Rv. Each PCR primer set having unique restriction enzyme sites (*Bgl*II in 5′- and *Eco*RI in 3′) were designed to clone into expression vector pBAD b (Invitrogen). PCR-amplified full-length *ffh* gene digested with *Bgl* II and *Eco* RI and inserted inframe with N terminal His tag into similarly digested pBAD b to yield pBAF6.

Hypothetical *M. tuberculosis* 4.5S RNA gene (*ffs* gene) sequence was identified from Signal Recognition Particle DataBase (SRPDB) and primers were designed to amplify *ffs* gene. The amplified 143 bp product was cloned into TOPO TA (Invitrogen) cloning vector and the resulting construct was named pT4.5sc. Restriction site overhangs were created in 4.5S RNA gene by digestion with *Bam*HI and *Xho*I and the released product was cloned into pBluescript SK+ vector to yield pSBS4 construct. This was used as a template for *in vitro* transcription (IVT) to produce 4.5S RNA. All constructs were sequenced to confirm the fidelity of the sequence.

Expression and purification of Ffh

E. coli TOP10 cells harboring pBAF6 construct were grown in LB medium supplemented with ampicillin 50 μg/ml at 30 °C with shaking (200 rpm) until A_{600} reached 0.6. L-Arabinose was added to a concentration of 0.2% (w/v) and growth was continued for additional 3 h at 30 °C. Growth was arrested by keeping the cells in ice for 15 min and pelleted down by centrifugation at 6000 × *g* for 10 min and stored at −80 °C. Pellets were thawed on ice and resuspended in cell lysis buffer A (50 mM Tris–Cl pH 8.0, 300 mM NaCl, 10 mM imidazole, 20% glycerol, and 0.5 mM phenyl methyl sulfonyl fluoride) and lyzed by sonication. The cell lysates were centrifuged 15,000 × *g* for 15 min at 4 °C. The supernatant containing recombinant protein was collected and incubated with ProBond Nickel affinity resin (Invitrogen)-packed column pre-equilibrated with buffer A. After extensive washings with buffer A containing 40 mM imidazole the recombinant Ffh was eluted with 200 mM imidazole. Fractions were run on 10% SDS-PAGE and analyzed by Coomassie brilliant blue staining and western blotting using anti HisG antibodies. Pure fractions were dialyzed (25 mM Tris–Cl pH 8.0, 150 mM NaCl, 5 mM imidazole, 10% glycerol, 0.5 mM PMSF), aliquoted and stored at −80 °C.

GTP blot overlay assay

[α-^{32}P] GTP-binding assay on polyvinylidene difluoride (PVDF) membrane was performed as described by Lapetina and Reep (1987) and Rao et al. (1997). Purified recombinant Ffh protein along with control protein DacB2 (a recombinant penicillin binding protein of *M. tuberculosis*) was resolved on SDS-PAGE and electrophoretically transferred to PVDF membrane (Millipore, USA). Transferred blots from SDS-PAGE were rinsed two times for 15 min with GTP-binding buffer consisting of 50 mM Tris–Cl, pH 7.5, 0.3% Tween 20, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 5 μM ATP. The blots were then incubated with [α-^{32}P] GTP (BRIT, Hyderabad, India) at a concentration of 1 μCi/ml in binding buffer for 90 min. The blots were then washed extensively for several times with binding buffer. All these incubations were carried out at room temperature and finally the blots were air dried and subjected to autoradiography (24 h at − 80 °C).

GTPase assay by thin layer chromatography (TLC)

In this method (Chopra et al. 2003), GTP hydrolysis was measured after purified Ffh (1 μg) was incubated with 1 μCi of [γ-^{32}P] GTP (BRIT, Hyderabad, India) in 20-μl reaction volume in TMD buffer (25 mM Tris–Cl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.4) for different time points at 25 °C. The reaction was terminated after addition of 2 μl of 4% SDS and the aliquots were resolved by polyethylenemine cellulose thin layer chromatography (TLC) using 0.75 M KH_2PO_4 (pH 4.2). The decrease in the amount of [γ-^{32}P] GTP was determined by increase in the amount of ^{32}Pi release.

Malachite green GTPase assay

Malachite green GTPase assay was performed as per the method prescribed by Leonard et al. (2005) and Sharma et al. (2006). The reaction buffer contained 10 μl of 10× TMD buffer, 2 μl of 100 mM GTP. Five microgram of purified Ffh was added to the reaction buffer and incubated at 37 °C. GTP control and protein control (DacB2) reactions were also performed to identify the specificity of the experiment. At various time points (0, 5, 10, 20, 40 and 80 min), 15 μl of aliquots were removed and transferred to microtiter plate wells containing 5 μl of 0.5 M EDTA. When the time course was completed, 150 μl of the malachite green (1 mM malachite green, 10 mM ammonium molybdate in 1 N HCl) was added to each well and the absorbance at 650 nm was measured. The amounts of enzymatically released inorganic phosphate in triplicate samples were measured photometrically by referring a standard curve from 10 to 100 μM Pi was generated for each experiment and read in parallel.

In vitro transcription

To prepare wild-type 4.5S RNA, pSBS4 plasmid was linearized by *Xba*I. *In vitro* transcription was performed using MAXIscript kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. In brief, the *in vitro* transcription reaction contained template DNA 10 μg, 10 mM ATP, 10 mM CTP, 10 mM GTP and 10 μCi [α-^{32}P] UTP (BRIT). The reaction was initiated by addition of *T7* RNA polymerase. After incubation at 37 °C for 1 h, the reaction was stopped and labeled transcripts were purified by NucAway spin Column (Ambion Inc). The presence of RNA transcripts was confirmed by 8 M urea denaturing gel and β-actin used as a positive control.

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