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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.

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.

Table 1
Primers used in the study.

Primer name	Sequence (5′–3′)[a]	Use
Ffh f	CGGA**AGATCT**TCCGTGTTTGAATCGCTGTCT	Cloning Ffh
Ffh r	**GAATTC**CGCCACTACTTCTTGCCT	Cloning Ffh
4.5s temp1	TCGTCTCCGGGCAAGCTCAG	Cloning 4.5S RNA gene
4.5s temp1c	CGAATCCTCCGCAGCATATGG	Cloning 4.5S RNA gene
4.5sc temp 2	GCGTTCCAGCCCAGGTATTAGAC	Cloning 4.5S RNA gene
4.5sc temp2c	ACCGAGGTCCAGTCAGTGCC	Cloning 4.5S RNA gene

[a] Bold letters indicate bases comprising the restriction sites *Bgl*II (AGATCT) and *Eco*RI (GAATTC).

Table 2
Strains and plasmids.

Strain or plasmid	Description	Reference/source
DH5α	*E. coli* strain used for propagation of plasmids	Invitrogen
TOP 10	*E. coli* strain used for cloning and recombinant protein purification	Invitrogen
XL1-Blue	*E. coli* strain used for pBluescript phagemids propagation	Stratagene
TOPO TA	Cloning vector used to clone PCR products	Invitrogen
pBAD b	*E. coli* vector used to generate His-tagged recombinant proteins contains *ara*BAD promoter	Invitrogen
pBluescript SK+	Phagemid used for *in vitro* transcription.	Stratagene
pBAF6	pBAD vector used to purify recombinant Histidine tagged Ffh protein	In this study
pT4.5st	TOPO TA vector contains 4.5S RNA gene used for sub cloning	In this study
pT4.5sc	TOPO TA vector contains 4.5S RNA gene sub cloning	In this study
pSBS4	pBlue script vector contains 4.5S RNA gene	In this study

4.5S RNA blot overlay assay

4.5S RNA blot overlay assay (Boyle and Holmes 1986) was performed to identify interaction between 4.5S RNA and Ffh protein similar to GTP blot overlay assay with little modifications. Transferred blots from SDS-PAGE were washed two times with binding buffer containing 50 mM Tris–Cl, pH 7.5, 0.3% Tween 20, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10 μg yeast t-RNA and 20 units of RNA guard (Amersham Pharmacia). The blots were then incubated with 2 μl of purified i*n vitro* transcription reaction product containing [α-^{32}P] UTP-labeled 4.5S RNA. After 90 min of incubation, the blots were then washed extensively with binding buffer and autoradiography was performed. [α-^{32}P] UTP-labeled β-actin was used as a control to know the specificity of binding of 4.5S RNA.

RNA gel shift assay

RNA gel shift assays to examine binding of 4.5S RNA with Ffh were done as previously described (Bradshaw and Walter 2007). [α-^{32}P] UTP labeled-4.5S RNA was allowed to bind with 2 μg of purified Ffh protein of *M. tuberculosis* in a reaction mixture (20 μl) containing 10 mM Tris–Cl, pH 8.0, 50 mM KCl, 10% glycerol, and 1 μg yeast tRNA. The reaction mixture was incubated at room temperature for 30 min. The mixture was separated in TAE buffer (40 mM Tris–acetate, 20 mM sodium acetate, and 1 mM EDTA) supplemented with 2.5 mM magnesium acetate on a 6% non denaturing polyacrylamide gel.

Results and discussion

Over expression and purification of Ffh

M. tuberculosis Rv2916c encodes for *ffh*, the gene product which has 525 amino acids long protein and a calculated molecular mass of 54.9 kDa and an estimated pI of 8.47. The predicted Ffh sequence was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using sequence-specific primers containing restriction enzyme overhangs. The full-length Ffh was cloned into in pBAD b expression vector in frame with N-terminal His tag and induced with L-arabinose. pBAD expression vector has araBAD promoter that provides tight, dose-dependent regulation of heterologous gene expression (Guzman et al. 1995). Attempts to purify the full-length Ffh protein from *E. coli* TOP10 cells harboring pBAF6 were tried with varying concentration of L-arabinose (0.1–1%) and different induction temperatures (16–37 °C). We found that *E. coli* TOP10 cells with pBAF6 induced with 0.2% L-arabinose at 30 °C for 3 h yielded full-length Ffh at an approximate size of 54 kDa from soluble fractions. SDS-PAGE and western blot analysis of purified fractions of Ffh showed triplet bands (Fig. 1A and B). In order to determine the nature of the protein, individual bands from triplet band were gel extracted separately and subjected to mass spectrometry analysis. The results clearly indicated that all three bands were Ffh protein and ruled out contaminating protein (Supplementary material S.1). Also, the protein has been degraded at the C-terminal region as the peptides of lower molecular weight bands corresponding to this region have not been highlighted (Supplementary material S.2). Moreover, the protein has been tagged with N-terminal His tag and since the western blot also shows triplets, it clearly indicates that some form of proteolysis

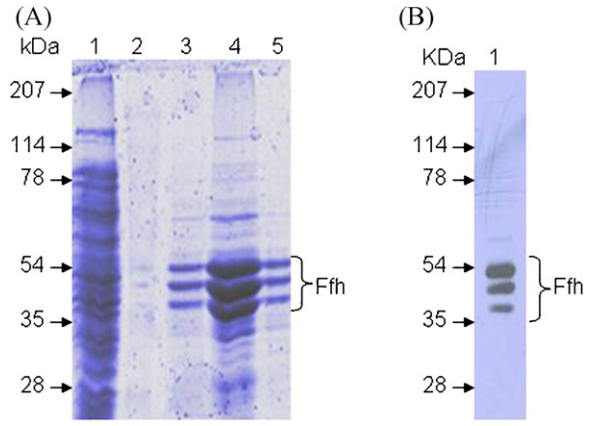

Fig. 1. (A) Expression and purification of *M. tuberculosis* Ffh. SDS-PAGE and Coomassie blue stained gel analysis of L-arabinose-induced *E. coli* TOP10 cells crude lysate harboring Ffh construct (lane 1) and Ni-NTA affinity chromatography-purified fractions of recombinant Ffh (lanes 2–5 indicate different elutions of recombinant Ffh. Maximum yield was observed in elution 3). Molecular weight markers were indicated in kDa. (B) Western blot analysis of crude *E. coli* lysate harboring Ffh construct probed with Anti-His antibodies (lane 1). Molecular weight markers were indicated in kDa.

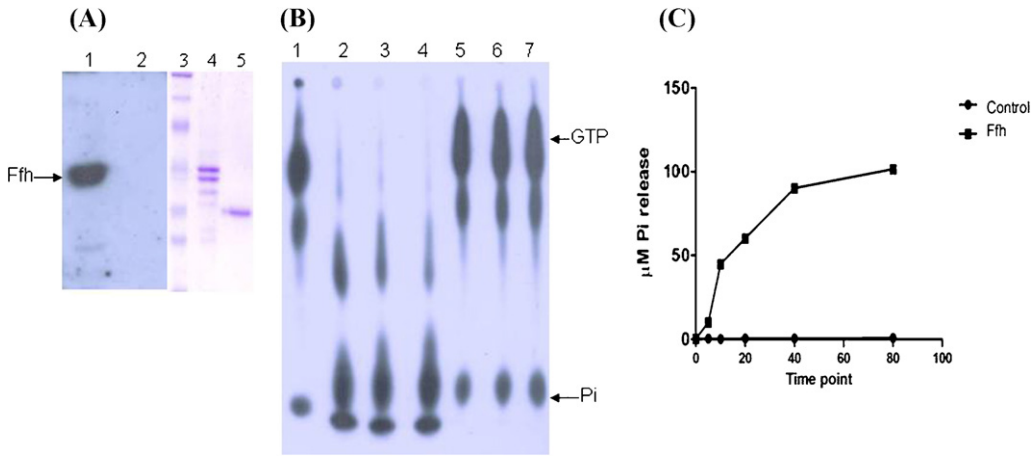

Fig. 2. (A) GTP-binding assay of recombinant Ffh. Recombinant Ffh was separated by SDS-PAGE and transferred to PVDF membrane and GTP overlay assay performed using [α-^{32}P] GTP. Lane 1, Ffh; lane 2, control protein (DacB2); lane 3, molecular weight marker (bands in kDa from top to bottom: 207, 114, 78, 54, 35, 28); lane 4, Ffh; lane 5, DacB2 (lanes 3–5 Coomassie blue stained gel). (B) GTP hydrolysis by Ffh. [γ-32P] GTP hydrolysis and release of Pi by recombinant Ffh was determined using thin layer chromatography. Lane 1, [γ-^{32}P] GTP alone; lanes 2, 3, and 4, [γ-^{32}P] GTP incubated with Ffh aliquoted after 5, 15, and 30 min, respectively. Lanes 5, 6, and 7 were buffer control aliquoted after 5, 15, and 30 min, respectively. (C) Time course of GTP hydrolysis by Ffh. The release of Pi was measured, using malachite green method, at various time points. The Pi release was assayed when GTP was hydrolyzed by Ffh. Recombinant DacB2 used as a protein control. Each time point is the average of the values obtained from three independent experiments.

has occurred specifically at the C-terminal. It is not clear by what process this proteolysis occurs or if it is physiologically significant. Although, it is important to mention that a similar proteolysis was reported in Ffh of *Neisseria gonorrhoea* where the recombinant expressed Ffh protein appeared as doublets (Frasz and Arvidson 2003).

GTP binding and GTPase activity of purified Ffh

GTP binding with Ffh is required for initial steps of cotranslational targeting reaction and GTP hydrolysis to cause release of SRP from its receptor (Connolly et al. 1991). We proved GTP binding to Ffh by GTP blot overlay assay using [α-^{32}P] GTP. This assay has been used previously to analyze GTP-binding proteins from various samples and the technique relies on the ability of the protein to bind GTP ligand following separation by SDS-PAGE and blotting to nitrocellulose membrane (Lapetina and Reep 1987; Huber et al. 1994; Rao et al. 1997). Fig. 2A illustrates the purified Ffh showing its affinity to [α-^{32}P] GTP after separated by SDS-PAGE and transferred to PVDF membrane. The specificity of the GTP binding with Ffh was tested by preincubating the membrane with unlabeled GTP with 0.2–2.0 μM concentration, while incubation with 2.0 μM concentration of unlabeled GTP completely inhibited the binding of labeled GTP (data not shown).

GTP hydrolysis activity of Ffh and release of Pi was determined by TLC and malachite green assay. In TLC, we observed that within 5 min incubation with Ffh showed maximum GTPases activity (Fig. 2B). The buffer control did not show any hydrolysis of GTP. More than 95% GTP was hydrolyzed after 30 min of incubation with purified Ffh. The purified Ffh showed a GTPase activity of 0.25 μM of phosphate min^{-1} μg^{-1} protein as determined by malachite green assay (Fig. 2C). This datum clearly indicates the GTPase activity of recombinant Ffh protein of *M. tuberculosis*.

Cloning and expression of M. tuberculosis 4.5S RNA

The SRP Database website (http://rnp.uthct.edu/rnp/SRPDB/srprna.html) includes 4.5S RNA sequence of *M. tuberculosis* (from CDC 1551 sequence) in alignment of SRP RNAs (Rosenblad et al. 2003). This sequence was BLASTed with *M. tuberculosis* whole-genome sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and we identified the region which codes for 4.5S RNA (*ffs* gene). This sequence was used to design oligonuleotide primers for amplification and cloning of *M. tuberculosis* H37Rv *ffs* gene (Table 1). One

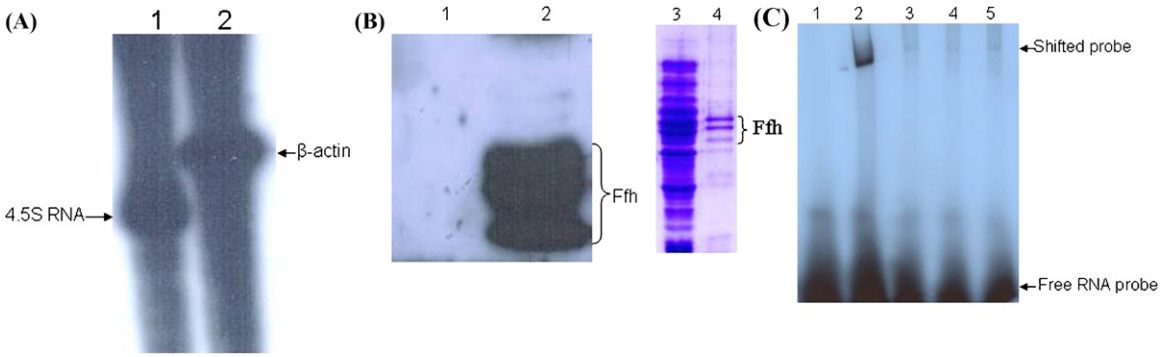

Fig. 3. (A) *In vitro* transcription of 4.5S RNA. *In vitro* transcription and [α-^{32}P] UTP labeling of 4.5S RNA using *T7* RNA polymerase (MAXIscript, Ambion). Lane 1, 4.5S RNA; lane 2, β-actin as control. (B) Ffh and 4.5S RNA interaction. The interaction between Ffh and 4.5S RNA was analyzed by 4.5S RNA Blot over lay assay using [α-^{32}P] UTP labeled 4.5S RNA. Lane 1, *E. coli* (TOP 10) cells whole lysate; lane 2, purified Ffh; lane 3, *E. coli* (TOP 10) cells whole lysate; lane 4, purified Ffh (lanes 3 and 4 Coomassie blue stained gel). (C) RNA gel shift analysis of 4.5S RNA by Ffh. Assays were performed as described in Materials and methods. The experiment was performed with [α-^{32}P] UTP-labeled 4.5S RNA along with control (β-actin). Lane 1, labeled 4.5S RNA; lane 2, addition of Ffh with labeled 4.5S RNA; lane 3, addition of unlabeled 4.5S RNA as a competitor (100-fold excess compared to labeled probe); lane 4, [α-^{32}P] UTP-labeled β-actin; lane 5, addition of Ffh with labeled β-actin.

clone, pSBS4, was selected and used as a template for IVT to produce 4.5S RNA. The [α-^{32}P] UTP-labeled 4.5S RNA was identified by autoradiography and showed 143 nt size and β-actin was used as a positive control (Fig. 3A). The 4.5S RNA prepared in this method was purified and used in the 4.5S RNA blot overlay assay and gel shift assay to study the interaction with Ffh.

Ffh and 4.5S RNA interaction

Direct interaction between Ffh and 4.5S RNA of *M. tuberculosis* has been demonstrated by 4.5S RNA blot overlay assay and RNA gel shift assay. In RNA blot overlay assay, [α-^{32}P] UTP-labeled 4.5S RNA was used to determine the interaction with Ffh. Intriguingly, this assay also showed three bands indicating that it is also binding to the cleavage products (Fig. 3B). Since 4.5S RNA can bind to the NG domains of Ffh, it is possible that even after losing a considerable portion of M domain the RNA may bind to the NG domain giving the triplet bands. To show the specificity of the experiments [α-^{32}P] UTP-labeled β-actin was over laid on the membrane and it clearly showed no bands on the membrane detected by autoradiography. Interaction between Ffh and 4.5S RNA has been demonstrated in other prokaryotes by biochemical methods (Poritz et al. 1990; Zheng and Gierasch 1998; Frasz and Arvidson 2003) as well as in crystal structure studies (Batey et al. 2000). Here we are reporting for the first time *M. tuberculosis* SRP homologue Ffh and its interaction with 4.5S RNA. This proves the evolutionary conservation among prokaryotes (Gribaldo and Cammarano 1998).

To further confirm the interactions, we performed RNA gel shift assay. This experiment is based on the observation that the migration of nucleic acids through polyacrylamide gels can be altered when proteins are bound to it (Carey 1991). The migration is affected by the shape of the nucleic acid, as well as charge to mass ratio of the proteins bound to the nucleic acid. Column purified [α-^{32}P] UTP-labeled 4.5S RNA was incubated with different concentrations of purified Ffh. A representative of autoradiography of such an experiment is shown in Fig. 3C. We found that 25 pmole of Ffh was sufficient to retard the mobility of 1.5 pmole of 4.5S RNA. The specificity of the binding was demonstrated by incubation of Ffh with [α-^{32}P] UTP-labeled β-actin where there was no change in the mobility of β-actin (Fig. 3C). In our experiment, the free probe and shifted band did not appear as single band and it always forms smear in autoradiography. Since it is not possible in our assay to determine stoichiometry of the components of the complex, we have demonstrated the protein–RNA interaction. The specificity of the binding was confirmed by cold chase experiments where 100-fold excess unlabeled 4.5S RNA was added and the shifted labeled band had disappeared.

We present the evidence that SRP system exists in *M. tuberculosis* and *in vitro* experiments demonstrated that Ffh has GTPase activity and interacts with 4.5S RNA. This also confirms that biochemical properties are also evolutionary conserved in *M. tuberculosis*. Further work is needed to identify the target proteins transported *via* SRP pathway will provide further insights into protein secretion and transport mechanism of *M. tuberculosis*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.micres.2012.03.002.

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