GTPase activity of a bacterial SRP-like complex

Tore Samuelsson and Monica Olsson

Department of Medical Biochemistry, University of Göteborg, Medicinareg. 9, S-413 90 Göteborg, Sweden

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ABSTRACT

We have recently identified a protein (SRPM54) in *Mycoplasma mycoides* homologous to SRP54, a subunit of the mammalian signal recognition particle (SRP). This protein forms a complex with a mycoplasma RNA related to the RNA component of SRP. We have now demonstrated that the protein has an intrinsic GTPase activity *in vitro* and kinetic parameters for the enzymatic reaction have been determined. The GTPase activity was not significantly affected by the presence of the mycoplasma SRP RNA. Different regions of the SRPM54 protein were expressed as recombinant proteins in *E.coli* and were purified to near homogeneity. On the basis of the properties of these SRPM54 fragments two different functional domains of the protein could be distinguished. An N-terminal part was found to contain the GTPase activity and this domain had approximately the same kinetic properties as the full-length protein. Another domain corresponding to a C-terminal fragment contained the RNA binding activity as shown using an assay based on the retention of RNA-protein complexes to nitrocellulose filters.

INTRODUCTION

The signal recognition particle (SRP) in mammalian cells is important for the targeting of secretory proteins to the membrane of the endoplasmic reticulum (ER) (1, 2). The SRP is composed of one RNA molecule (~300 nucleotides) and six different polypeptides (9, 14, 19, 54, 68 and 72 kDa, respectively). As the signal sequence of the secretory protein emerges from the ribosome it is bound by the SRP. The resulting complex of SRP with the ribosome is recognized by the SRP receptor (SR, docking protein) associated with the ER membrane (3, 4). SRP is finally released from the signal peptide and the receptor to join a new transport cycle (5).

The SRP54 subunit of SRP is known to play an important role in the identification of the signal sequence and this protein is composed of two different structural domains (6, 7). The aminoterminal G-domain carries a GTP-binding site and the C-terminal M-domain has binding sites for both signal sequence and SRP RNA (8–11). Proteins homologous to mammalian SRP54 have been identified in yeast (12, 13), *E.coli* (6, 7, 14) and *M.mycoides* (15). It has been demonstrated that the *E.coli* protein (denoted ffh or P48) is part of an SRP-like particle where the

protein is complexed with 4.5S RNA (16, 17). This RNA has a structural domain in common with mammalian SRP RNA and related RNAs have been found in a number of bacteria (18, 19).

We have recently obtained evidence of a ribonucleoprotein complex similar to the *E.coli* SRP-like complex in the very simple prokaryote *Mycoplasma mycoides* (15). The fact that an SRP-like complex is found also in mycoplasma suggests that it has a fundamental biological role as these organisms have a highly limited coding capacity and appear to have abandoned many non-essential functions (20). The mycoplasma RNP is composed of a small RNA (77 nucleotides) (21) and a 50 kDa polypeptide (denoted SRPM54). A region of the RNA was defined which interacts with the protein and this region contains structural elements that have been highly conserved during evolution of SRP RNAs (15).

GTP hydrolysis is known to be important for transport of secretory proteins to the ER membrane in the mammalian cell. Not only the SRP54 subunit of SRP has a GTP binding site (6, 7) but also the SRP receptor subunits α and β (68 and 30 kDa, respectively) (17, 22). GTP hydrolysis is required for dissociation of SRP from the signal sequence of a nascent polypeptide (23). It has recently been shown that the GTP binding site of the SR α subunit is important in this process but the role of the corresponding site in the SRP54 protein has not yet been examined (2, 24).

The bacterial counterparts of the mammalian SRP54 protein also have a GTP binding site as judged by the amino acid sequence information (6, 7, 14, 15, 25). We have attempted to study the functional role of this site of the mycoplasma SRPM54 protein and we have now shown that the protein has an intrinsic GTPase activity. Furthermore, we present evidence that the enzymatic activity resides in the G domain and that this domain is sufficient for GTPase activity. We also show that the SRP RNA binding site of the SRPM54 protein is located in the C-terminal M domain of the protein and that this domain by itself effectively binds to RNA.

MATERIALS AND METHODS

Construction of expression plasmids

Recombinant DNA techniques were as described (26). Oligonucleotides were either purchased from Symbicom, Umeå, Sweden or synthesized on a Beckman DNA synthesizer (DNA SM). The programs of the University of Wisconsin Genetics Computer Group were used for analysis of nucleotide and amino

acid sequences (27). The nucleotide sequence encoding SRPM54 is in the GenBank database with the accession number M91593. For nucleotide and amino acid position numbers the reader is referred to this sequence and ref (15).

The construction of plasmids pET3a/M54 (used for expression of protein SRPM54) as well pUC9/M54/BamHI is described in ref (15). A construct for the production of the C-terminal M domain was produced in the following way. A *NdeI* site in nucleotide position 2190 was created by PCR amplification of plasmid pUC9/M54 using one oligonucleotide (5'-GAATG GCCGA TAGAC ATATG GGAAT GGG-3') that matched positions 2169–2196 but also contained a novel *NdeI* site and one oligonucleotide (5'-CTTTA TTAAT AGGTA ACATT TTC-3') that matched the *MaeIII* site at position 2360. The resulting PCR fragment was digested with *NdeI* and *MaeIII* and was then ligated to a fragment of plasmid pUC9/M54/BamHI reaching from the *MaeIII* site at position 2360 up to the *BamHI* site. Finally, a resulting *NdeI/BamHI* fragment was isolated and introduced into the vector PET3a to obtain the plasmid designated pET3a/M54c.

The constructs containing an oligo-histidine tag were obtained in the following way. A DNA fragment was produced by annealing the synthetic oligonucleotides 5'-TATGA GCCAC CATCA CCACC ATCAC GGTAC CGG-3' and 5'-TACCG GTACC GTGAT GGTGG TGATG GTGGC TCA-3'. This fragment was introduced at the *NdeI* site of pET3aM54 and pET3aM54/c, respectively. The oligonucleotide construct encodes the amino acid sequence MTHHHHHHVTV. The resulting plasmid constructs were designated pET3aM54His and pET3aM54cHis and were used for the production of full-length protein and the C-terminal M-domain (SRPM54-C), respectively (Fig. 1). To obtain the construct for the production of the G-domain (SRPM54-N, Fig. 1) the plasmid pET3aM54His was digested with *EaeI* and *XbaI*. A fragment (940 bp) corresponding to the G-domain of the SRPM54 protein was isolated and introduced between the *XbaI* and *BamHI* sites of pET3a with the aid of an adaptor produced by annealing the oligonucleotides 5'-GGCCG ATAGA TTGAT GTAAT AG-3' and 5'-GATCC TATTA CATCA ATCTA TC-3'. This synthetic adaptor also contains two consecutive stop codons. The resulting plasmid was designated pET3aM54HisN. For the production of protein SRPM54-ΔC a similar approach was used. The plasmid pET3aM54His was digested with *XbaI*, *TaqI* and *AccI*. A 1260 bp fragment was isolated and introduced between the *XbaI* and *BamHI* sites of pET3a. A synthetic adaptor that contains two consecutive stop codons was constructed by annealing the oligonucleotides 5'-CGATA ATAGG CAGCA G-3' and 5'-GATCC TGCTG CCTAT TAT-3'. The resulting plasmid construct was designated pET3aM54HisΔC.

Expression and purification of recombinant proteins

In order to express proteins in *E. coli* we made use of the system described in ref (28) with pET vectors (Novagene) in combination with *E. coli* BL21(DE3) pLysS as the host strain. The growth of cells has been described previously (15). During protein purification all operations were carried out at +4°C. SDS-polyacrylamide gel electrophoresis was performed out as described (29). Scanning of gels was performed with Molecular Dynamics Computing Densitometer model 300A. Purification of the individual proteins shown in Fig. 2 and that contained a histidine tag were as follows.

SRPM54. The initial steps of the purification procedure were essentially as described previously for the corresponding protein without histidine linker (15). Cells corresponding to a volume of 4 L of growth medium were thawed and suspended in 50 ml of buffer E (50 mM Na⁺/Hepes pH 7.6, 2 mM EDTA, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). After the addition of 5 ml 10% NP-40 the mixture was kept on ice for 10 min and 55 ml of 0.4M NaCl, 20% glycerol was then added. The resulting mixture was homogenized with Ultraturrax and centrifugation was for 20 min at 10,000 rpm using a Beckman JA-10 rotor. The supernatant was diluted with 220 ml of buffer A (20 mM Hepes pH 7.6, 50 mM NaCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, 0.1 mM PMSF). It was then loaded to a 200 ml column of S-Sepharose Fast Flow (Pharmacia) and the column was washed with 200 ml of buffer A. Elution was carried out with the same buffer using a linear gradient of a total volume of 1 L from 50 mM NaCl to 1 M NaCl. Fractions of 10 ml were collected and assayed for the SRPM54 protein using the RNA binding assay described below. Fractions containing the major portion of RNA binding activity were pooled and dialyzed against 2 L of buffer N (10 mM Na⁺/Hepes pH 7.6, 150 mM NaCl, 10% glycerol, 0.1 mM PMSF, 10 mM imidazol). The protein was then applied to a 20 ml column of Ni²⁺-NTA-Agarose (QIAGEN) (30) equilibrated with buffer N. The column was washed with 50 ml of the same buffer and the SRPM54 protein was finally eluted with buffer N containing 0.2 M imidazol. Fractions of 1 ml were collected. Fractions containing the protein were pooled and dialyzed against a buffer containing 20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 50% glycerol, 2 mM DTT, 1 mM EDTA and the protein was finally stored at -20°C.

SRPM54-C. An extract of cells was prepared as described above. After dilution with 220 ml of buffer A it was loaded on a 100 ml column of Heparin-Sepharose. The column was eluted with a linear gradient of a total volume of 200 ml from 0.2 M NaCl to 1 M NaCl in buffer A. Fractions of 2 ml were collected and RNA binding was monitored. Fractions containing RNA binding activity were pooled and dialyzed against buffer N. The protein was then chromatographed on Ni²⁺-NTA-Agarose as described above except that a 10 ml column was used instead.

SRPM54-ΔC and SRPM54-N. Cells corresponding to a volume of 1.3 L of growth medium were suspended in 25 ml of buffer E. After the addition of 2.5 ml 10% NP-40 the mixture was kept on ice for 10 min and 30 ml of 0.4M NaCl, 20% glycerol was then added. The resulting mixture was homogenized and centrifuged as described above. The supernatant was diluted with 120 ml of buffer N and was applied to a 4 ml column of Ni²⁺-NTA-Agarose. Washing and elution of this column was as described above for the other protein constructs.

The yields of protein were in the range 0.3 mg–2 mg /L of growth medium. In general the recovery of SRPM54-C was relatively poor as compared to the other constructs, possibly due to a physical instability of this protein. The best yields were obtained with the SRPM54-N construct. The purity of the preparations shown in Fig. 2 as estimated by laser densitometer scanning of the Coomassie stained gel was 84% (SRPM54), 62% (SRPM54-C), 31% (SRPM54ΔC) and 86% (SRPM54-N), respectively. The full-length protein and the M domain were also produced as proteins without a histidine tag. In the case of the full-length protein it was purified as described previously (15).

and SRPM54-C was purified by a combination of DEAE-Sepharose and Heparin-Sepharose chromatography.

Assay of GTPase activity

Unless otherwise indicated the incubation mixture for assay of GTPase activity contained 20 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 10% glycerol, 2 μ M GTP, 50 nCi [γ -³²P]GTP (Amersham) and BSA (0.1 mg/ml) in a final volume of 20 μ l. Incubation was for 20 min at 37°C. GTPase activity was measured by the release of ³²P-labeled phosphate from [γ -³²P]GTP. For analysis by TLC 1 μ l of the reaction mixture was applied to a sheet of PEI-cellulose and development was carried out with 0.5 M potassium phosphate pH 3.5. For the assay based on the absorption of GTP to charcoal the reaction was terminated by the addition of 0.4 ml of a suspension of Norit A (12% in 0.1 M HCl, 10 mM KH₂PO₄). The mixture was briefly vortexed and then centrifuged for 15 seconds. The radioactivity of 200 μ l of the supernatant was finally determined. The enzyme-dependent release of phosphate was calculated by subtracting the release of phosphate in samples without enzyme.

Binding of SRP RNA to SRPM54 constructs

The production of radioactively labeled mycoplasma SRP RNA by T7 RNA polymerase transcription *in vitro* of a synthetic gene has been described previously (15). After transcription the RNA was extracted with phenol and purified using a QIAGEN column. For binding studies the RNA was incubated with protein as described in the legend to Fig. 8. The binding of RNA to the full-length protein was found to be optimal between 0.1 M and 0.3 M NaCl.

RESULTS

Expression and purification of recombinant mycoplasma SRPM54 proteins

We have previously described the expression of mycoplasma SRPM54 in *E. coli* (15). This protein was purified to near homogeneity using S-Sepharose chromatography. We have now improved the purification procedure of this protein and describe also the expression and purification to near homogeneity of three different recombinant fragments of the protein.

It has previously been shown that the mammalian SRP54 protein is composed of two different structural domains as shown by the observation that the protein may be proteolytically cleaved to obtain two fragments with different properties (8, 9). The N-terminal G domain has a GTP binding site (6, 7) and the C-terminal M domain contains an RNA binding site (9, 10) and may be crosslinked to a signal sequence (8, 9, 11). The mycoplasma protein is structurally similar to the mammalian protein (15) and we therefore expected it to have a similar two-domain structure. In order to test this experimentally different regions of the SRPM54 protein were expressed as recombinant proteins. These constructs are outlined in Fig. 1. The full-length protein contains 447 amino acids (for the amino acid sequence of the protein as well as numbering of amino acid residues the reader is referred to ref (15)). SRPM54-C, (amino acids 296–447) contains the 'M domain', SRPM54- Δ C (amino acids 1–399) is identical to the full-length protein except that 48 amino acids at the C-terminal end have been deleted and SRPM54-N (amino acids 1–295) corresponds to the 'G domain'.

For the expression of proteins a system was used based on T7 RNA polymerase expression with the vector pET3a in combination with *E. coli* BL21(DE3) as the host organism (28). Two different kinds of constructs were used, one in which the protein coding sequence is linked directly to the *E. coli* translation initiation signal and another in which the N-terminus of the authentic protein is linked to an oligo-histidine tag to facilitate the purification procedure as such histidine tags are specifically retained on a column of Ni²⁺-agarose (30).

The results from expression and purification of the constructs with the histidine tag is shown in Fig. 2. For each construct is shown the unfractionated *E. coli* extract as well as the purified protein. All of the protein constructs were purified by chromatography on Ni²⁺-agarose but in the case of SRPM54 and SRPM54-C it was necessary to include a chromatography on S-Sepharose and Heparin-Sepharose, respectively, in order to achieve a high degree of purification. The full-length protein and the M domain were also produced as proteins without a histidine tag and these proteins were also purified to near homogeneity. However, for the majority of experiments shown below and unless otherwise indicated the constructs with the histidine tag were used. The purification procedures are described in more detail under 'Materials and Methods'.

Figure 1. Domain structure of SRPM54 and recombinant fragments of the protein. Numbers refer to the amino acid positions as indicated in ref (15). The boxes I, II and III refer to the sequence elements of a GTP binding site (25).

SRPM54 exhibits a GTPase activity which resides in an N-terminal part of the protein

Examination of the amino acid sequence of SRPM54 reveals a putative GTP binding site (15, 25). In order to see whether the SRPM54 protein (full-length protein as well as truncated constructs) exhibits a GTPase activity *in vitro* we made use of assays dependent on the release of radioactive phosphate from [γ - ^{32} P]GTP. Such an assay is shown in Fig. 3 where the phosphate released was monitored by TLC chromatography of products obtained after incubation of SRPM54 proteins with [γ - ^{32} P]GTP. Evidently, the SRPM54 protein exhibited a GTPase activity both with and without the histidine tag (Fig. 3, lanes 2 and 3, respectively). Also the constructs with the G domain, SRPM54-N (lane 6) and SRPM54- Δ C (lane 7), were able to catalyze GTP hydrolysis whereas the M domain was inactive in this respect (both with and without histidine tag, SRPM54-C, lanes 4 and 5, respectively). Thus, we are able to conclude from these experiments that the GTPase activity resides in the N-terminal G-domain of the protein and that this domain is sufficient for activity. It is highly likely that the GTPase activity is associated with the SRPM54 protein and is not a contaminating activity of the *E. coli* extract from which it was purified. Thus, the full-length protein SRPM54 and SRPM54-N were extensively purified as shown in Fig. 2 and we consistently found GTPase activity in preparations of these proteins whereas an activity was never observed with highly purified SRPM54-C. Furthermore, during chromatography of the SRPM54 protein and its G domain the peak of GTPase activity always coincided with that of respective SRPM54 protein. This is illustrated by the chromatograms in Fig. 4.

It was also possible to monitor the GTPase reaction with an assay in which unreacted [γ - ^{32} P]GTP was absorbed to charcoal. Using this assay we were able to optimize the conditions for the enzymatic reaction. Enzyme-dependent GTP hydrolysis was linear for at least 20 minutes. The G domain (SRPM54-N)

appeared more stable than the full-length protein under the experimental conditions (Fig. 5). The GTPase activity was dependent on the presence of Mg^{2+} and there was a broad pH optimum in the range 6.5–8.0 (data not shown). The reaction was optimal at low ionic strength; increasing concentrations of both NaCl and Tris inhibited the reaction. Glycerol was found to stimulate the reaction both for the full-length protein and the G domain alone (Fig. 6). Consistent with the data shown in Fig. 3 no reaction was detected with the SRPM54-C fragment (the M domain, data not shown).

By measuring the enzymatic rate as a function of the substrate concentration Lineweaver-Burk plots (Fig. 7) were obtained for the estimation of the K_m and V_{max} parameters. For the full-length protein K_m was 3 μ M indicating a rather high affinity for the GTP substrate as previously noted with other GTPases (31–33). The V_{max} was estimated as 0.26 mol [mol enzyme] $^{-1}$ min $^{-1}$. The corresponding K_m and V_{max} values for the G-domain (SRPM54-N) were 5 μ M and 0.32 mol [mol enzyme] $^{-1}$ min $^{-1}$, respectively. This experiment indicated that there are no large kinetic differences between the full-length protein and the G-domain.

SRP RNA binds to a C-terminal domain of SRPM54

We have previously shown that the mycoplasma SRPM54 protein specifically binds *in vitro* to its homologous SRP RNA. With the aid of RNase footprinting we were also able to define a highly conserved region of the RNA that interacts with the protein (15). We have now carried out similar foot-printing studies with the purified SRPM54-C construct and showed that this protein leaves the same foot-print on the RNA as does the full-length protein (data not shown). This result suggests that the RNA binding domain is located in the C-terminal M domain of the protein as previously shown for the mammalian counterpart of the protein (9, 10).

We have previously noted that the binding of SRP RNA to SRPM54 protein may be monitored with an assay based on the retention of RNA-protein complexes to nitrocellulose filters (15). We have now made use of this assay to study the interaction of SRP RNA and the different protein constructs (Fig. 8). The fraction RNA bound to the filter was studied as a function of total protein incubated with the RNA. From the protein

Figure 2. Purification of recombinant SRPM54 proteins. Proteins purified as described under 'Materials and Methods' were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue R-250. For each protein construct the extract of BL21 cells overproducing the protein is shown to the left and to the right the protein after purification.

Figure 3. SRPM54 and its G domain catalyzes GTP hydrolysis. Proteins (1 μ g each) were incubated with 20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 10% glycerol, 2 μ M GTP, 50 nCi [γ - ^{32} P]GTP (Amersham) and BSA (0.1 mg/ml) in a final volume of 20 μ l. Incubation was for 20 min at 37°C. 1 μ l of the reaction mixture was applied to a sheet of PEI-cellulose and development was carried out with 0.5 M potassium phosphate pH 3.5. The proteins added to the incubation mixture were: none (lane 1), SRPM54 with or without histidine-linker (lanes 2 and 3, respectively), SRPM54-C with or without his-linker (lanes 4 and 5, respectively), SRPM54-N (lane 6) and SRPM54- Δ C (lane 7).

concentration at 50% saturation (Fig. 8) the apparent dissociation constant was estimated as $0.2 \mu\text{M}$ for the complex between full-length protein and RNA. The C-terminal domain (SRPM54-C) also bound to the RNA but with a reduced affinity as compared to the full-length protein; the dissociation constant was approximately $1 \mu\text{M}$. The relative binding of SRPM54 and SRPM54-C varied to some extent with the protein preparations tested. However, the SRPM54-C constructs consistently bound to the RNA with less affinity than the full-length protein. The binding of the other constructs, SRPM54- ΔC and SRPM54-N was very poor (Fig. 8). The conclusion from our experiments of the type shown in Fig. 8 is therefore that the C-terminal M domain is important for binding to the RNA; this domain is sufficient for a fairly effective binding *in vitro* whereas the G domain by itself has a very low affinity for the RNA.

We also tested the binding of SRP RNA to protein constructs that did not contain a histidine tag. The data (not shown) demonstrate that the binding efficiency was essentially the same as with the constructs with histidine tag. Therefore, the presence

of the histidines in the N-terminal end of the respective protein does not seem to influence its RNA binding properties.

SRPM54 is an effective GTPase also in the presence of SRP RNA

In order to see whether the GTPase reaction is affected by the presence of mycoplasma SRP RNA we carried out assays with varying concentrations of SRP RNA or, as controls, phenylalanine tRNA and polyU. The proteins tested were SRPM54 full-length protein as well as SRPM54-N. The results showed that when the protein ($0.4 \mu\text{M}$) was challenged with a large excess of mycoplasma SRP RNA ($50 \mu\text{M}$) under conditions where the protein should be saturated with RNA (compare Fig. 8)

Figure 4. Copurification of SRPM54 protein and GTPase activity. Upper panel: A crude extract of *E. coli* BL21 expressing SRPM54 protein was subjected to chromatography on S-Sepharose (for details see 'Materials and Methods'). Fractions were monitored for GTPase activity (open circles) and analyzed by SDS-PAGE. The resulting Coomassie stained gel was scanned with a laser densitometer and the amount of SRPM54 protein in each fraction was determined (filled circles). Lower panel: A sample of the G domain (SRPM54-N) that had been purified by Ni^{2+} -agarose (see 'Materials and Methods' and Fig. 2) was subjected to gel filtration on a column of Sephacryl S-200 ($1.6 \times 86 \text{ cm}$). The elution buffer was $10 \text{ mM Na}^+/\text{Hepes pH } 7.6$, 0.1 M NaCl , 5% glycerol, 1 mM EDTA . Fractions of 1.0 ml were collected and GTPase activity was monitored (open circles). In both panels A_{280} is shown by a continuous line.

Figure 5. Time course of GTP hydrolysis catalyzed by SRPM54 protein and the G domain. Proteins were incubated with $20 \text{ mM Tris-HCl pH } 7.5$, 2 mM MgCl_2 , 10% glycerol, $2 \mu\text{M GTP}$, $50 \text{ nCi } [\gamma\text{-}^{32}\text{P}]\text{GTP}$ (Amersham) and BSA (0.1 mg/ml) in a final volume of $500 \mu\text{l}$. Duplicate aliquots of $20 \mu\text{l}$ were removed after the times indicated and assayed for release of radioactive phosphate using the charcoal method (Materials and Methods). Full-length SRPM54 is represented by filled circles and SRPM54-N by open circles.

Figure 6. Effect of glycerol on GTPase activity. GTPase activity was monitored as described under 'Materials and Methods' with varying concentrations of glycerol. Full-length SRPM54 is represented by filled circles and SRPM54-N by open circles.

there was only a 2-fold decrease in the rate of GTP hydrolysis (data not shown). This decrease in activity was not observed when tRNA or polyU was used instead of SRP RNA. However, a decrease in activity was noted also with the G domain in the presence of SRP RNA. Therefore, it would seem that the inhibition of GTPase activity was not dependent on the specific binding of SRP RNA to the M domain of the SRPM54 protein. At any rate, we are able to conclude that the SRPM54 protein had a significant GTPase activity even under conditions when it was fully saturated with the mycoplasma SRP RNA.

DISCUSSION

We have recently identified a protein from *M. mycoides* which is structurally similar to the SRP54 protein from mammalian cells and the *E. coli* ffh (P48) protein. We have presented evidence that this protein (SRPM54) forms an SRP-like complex with the mycoplasma SRP RNA (15). In the present investigation we have demonstrated that there is a GTPase activity associated with the SRPM54 protein. This finding is an important step forward in the analysis of its function. Furthermore, we have been able to define two separate functional domains of the protein. Thus, the GTPase activity is associated with the N-terminal G domain whereas RNA binding is confined to the C-terminal M domain. These data are in good agreement with those obtained for the mammalian protein. Thus, the mammalian SRP54 contains a GTP binding site (17) and it has been shown that both the RNA binding and signal sequence recognition sites are in the M domain and overlap to a large extent (8–11). Preliminary observations suggest that also the mycoplasma protein is able to recognize a signal peptide and that the M domain (SRPM54-C) is sufficient for this recognition (Lütcke, H., Dobberstein, B. and Samuelsson, T., in preparation). Therefore, all available data support the notion that the bacterial protein has the same type of domain structure as the mammalian protein.

Proteins that catalyze the hydrolysis of GTP are ubiquitous and are involved in a variety of cellular processes such as protein synthesis, transmembrane signalling, and vesicular transport (31,

34). A common theme is that two alternate conformations (the GTP and GDP-bound forms, respectively) differ in their affinity for specific target macromolecules. The rate of exchange between the two forms of the protein is influenced by molecules that affect k_{cat} (GTPase activating proteins) or $k_{diss-GDP}$ (guanine nucleotide release proteins). In general, the role of the GTPase is to make a specific biochemical event thermodynamically favourable, but the GTPase activity sometimes also seems to serve as a timing device, for instance in the case of EF-Tu, where the GTPase activity is believed to regulate the rate and fidelity of protein synthesis (32).

GTP hydrolysis is known to play an important role also in the targeting of a secretory protein to the ER membrane in mammalian cells. Thus, three different polypeptides with sequence elements of a GTP binding site have been identified that are related to the function of the mammalian SRP; the SRP54 subunit of SRP (6, 7), and the SRP receptor subunits α and β (17, 22). All three polypeptides have been shown to bind GTP as demonstrated by UV-crosslinking of ^{32}P -labeled GTP to the respective proteins. Interestingly, the GTP-binding domain of the SRP receptor subunit α is structurally similar to the corresponding domain of the SRP54 protein and, therefore, these molecules seem to form a separate class of GTP-binding proteins (31). GTP hydrolysis is required for dissociation of SRP from the signal sequence of a nascent polypeptide. A nonhydrolyzable analog can replace GTP in the signal sequence displacement reaction, but the SRP then fails to dissociate from the membrane (23). Furthermore, a functional GTP binding site in SR α has been shown to be important for protein translocation across ER (24). However, the GTP binding site of SRP54 may also be important in this context (2).

When SRP and the SRP receptor are combined GTP hydrolysis is observed but neither SRP nor its SRP54 component alone has a significant GTPase activity (17). For the moment it is not clear whether the bacterial protein that we have studied differs

Figure 7. Lineweaver-Burk plots of the GTPase activity of SRPM54 and its G-domain. GTPase activity was monitored as described under 'Materials and Methods'. Full-length SRPM54 is represented by filled circles and SRPM54-N by open circles.

Figure 8. Binding of SRPM54 constructs to mycoplasma SRP RNA. The RNA (1.9 nM [^{32}P]adenosine-labeled RNA, 5×10^{18} cpm/mol) was incubated with protein in a mixture of 7 μ l containing 10 mM Tris-HCl, 10 mM Mg $_2$ Cl $_2$, 100 mM NaCl, 0.03 mM EDTA, 3% glycerol, 0.3 μ g polyU/ μ l. Incubation was for 1 min at room temperature and the mixture was then diluted with 0.5 ml of 20 mM Tris-HCl, 10 mM Mg $_2$ Cl $_2$, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol. The resulting solution was immediately filtered through nitrocellulose filters and radioactivity was determined. Full-length SRPM54 is represented by filled circles, SRPM54-C by open triangles, SRPM54- Δ C by filled triangles and SRPM54-N by open circles.

kinetically from the mammalian protein or whether the GTPase activity of the mammalian protein escaped detection due to unfavourable conditions. Future studies directly comparing the GTPase activity of the different proteins will reveal to what extent these proteins differ with respect to their kinetic properties.

The function of the GTP binding site of SRP54 has not yet been studied. A GTPase of the mammalian SRP54 could play a role not only during the interaction with the receptor as discussed above (2). It may also be important at an earlier event when it is specifically bound to the nascent peptide at the ribosome. In this case the GTPase may be activated by the interaction with the signal sequence and the ribosome. Possibly, it could serve as a timing device in analogy with EF-Tu to improve the fidelity in the selection of the signal peptide (6).

The bacterial SRP54 proteins may have a function related to that of the mammalian SRP. This idea is supported by the recent observations that the *E. coli* homologue of SRP54, ffh, is important in protein translocation (35) and that it may be crosslinked to a signal sequence (36). In addition, there may be bacterial macromolecules that are homologous to the SRP receptor. A candidate for a bacterial homologue of the SRP receptor subunit α is the structurally related protein encoded by the ftsY gene (37). This bacterial protein, like its mammalian equivalent, has the structural elements of a GTP binding site. The ftsY gene is part of an operon that encode proteins involved in cell division (37). So far, we do not know whether there is a similar protein in *Mycoplasma* but future work will have to involve the search for additional mycoplasma macromolecules related to the function of the SRPM54 protein.

In conclusion, we have in the present study shown that the mycoplasma SRPM54 protein has a significant intrinsic GTPase activity. The maximal rate measured is comparable to other GTPases such as *E. coli* EF-Tu and IF-2 (32, 33) and the GTPase activity resides in a domain distinct from the RNA binding domain of the protein. With our demonstration of this activity a detailed analysis of the function of this enzyme is now close at hand and it should be possible to further characterize the physical parameters related to enzyme activity ($k_{\text{cat}} \cdot \text{GTP}$, $k_{\text{diss}} \cdot \text{GDP}$, and $k_{\text{diss}} \cdot \text{GTP}$) and determine the effect of different macromolecules on these parameters.

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