

The 54-kD Protein of Signal Recognition Particle Contains a Methionine-rich RNA Binding Domain

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Abstract. Signal recognition particle (SRP) plays the key role in targeting secretory proteins to the membrane of the endoplasmic reticulum (Walter, P., and V. R. Lingappa. 1986. *Annu. Rev. Cell Biol.* 2:499–516). It consists of SRP7S RNA and six proteins. The 54-kD protein of SRP (SRP54) recognizes the signal sequence of nascent polypeptides. The 19-kD protein of SRP (SRP19) binds to SRP7S RNA directly and is required for the binding of SRP54 to the particle. We used deletion mutants of SRP19 and SRP54 and an in vitro assembly assay in the presence of SRP7S RNA to define the regions in both proteins which are required to form a ribonucleoprotein particle. Deletion of the 21 COOH-terminal amino acids of

SRP19 does not interfere with its binding to SRP7S RNA. Further deletions abolish SRP19 binding to SRP7S RNA. The COOH-terminal 207 amino acids of SRP54 (M domain) were found to be necessary and sufficient for binding to the SRP19/7S RNA complex in vitro. Limited protease digestion of purified SRP confirmed our results for SRP54 from the in vitro binding assay. The SRP54M domain could also bind to *Escherichia coli* 4.5S RNA that is homologous to part of SRP7S RNA. We suggest that the methionine-rich COOH terminus of SRP54 is a RNA binding domain and that SRP19 serves to establish a binding site for SRP54 on the SRP7S RNA.

SECRETORY and membrane proteins contain signal sequences required for their targeting to the ER (Walter and Lingappa, 1986). Signal recognition particle (SRP)¹ recognizes the signal sequence of nascent polypeptide chains, binds to them and retards elongation (Walter and Lingappa, 1986; Wolin and Walter, 1989). Upon interaction with docking protein (DP), the SRP receptor in the ER membrane, elongation resumes and the nascent chain is translocated (Gilmore et al., 1982; Meyer et al., 1982).

SRP consists of SRP7S RNA (Walter and Blobel, 1982) and six different protein subunits of 9, 14, 19, 54, 68 and 72 kD molecular mass (Siegel and Walter, 1988c). 7S RNAs and smaller RNAs that are similar to parts of SRP7S RNA have been found in a wide range of organisms (Brennwald et al., 1988; Gundelfinger et al., 1984; Ribes et al., 1988; Haas et al., 1988; Struck et al., 1988). Whereas the primary sequence may vary considerably the secondary structure of these molecules is highly conserved (Poritz et al., 1988). We have recently shown that secretion of β -lactamase in *Escherichia coli* involves a ribonucleoprotein complex composed

of 4.5S RNA that is homologous to stem-loop 2 of SRP7S RNA (Poritz et al., 1988) and a 48-kD protein (*fth*) homologous to SRP54 (Byström et al., 1983; Römisch et al., 1989; Bernstein et al., 1989; Ribes et al., 1990).

Of the protein components SRP9 and SRP14 bind to the SRP7S RNA as heterodimer and are required for elongation retardation (Siegel and Walter, 1988b,c). SRP68 and SRP72 also form a heterodimer and are essential for interaction with DP in the ER membrane (Siegel and Walter, 1988b,c). SRP19 binds to SRP7S RNA alone (Siegel and Walter, 1988b; Lingelbach et al., 1988). Footprinting experiments indicate that SRP19 contacts the two stem-loops of SRP7S RNA (Siegel and Walter, 1988a). The binding of SRP54 to the particle requires the presence of SRP19 (Siegel and Walter, 1988b; Römisch et al., 1989). We have shown recently that SRP54 alone binds to *E. coli* 4.5S RNA (Ribes et al., manuscript submitted for publication) and suggested therefore that SRP19 binding to SRP7S RNA exposes a binding site for SRP54 on the RNA. SRP54 has also been shown to bind to the signal sequence of nascent polypeptide chains by cross-linking experiments (Krieg et al., 1986; Wiedmann et al., 1987).

SRP54 has been found to be similar to three other proteins (Römisch et al., 1989; Bernstein et al., 1989): DP, which is the receptor for SRP at the ER membrane (Lauffer et al., 1985; Hortsch et al., 1988); FTS Y, which is an *E. coli* protein of unknown function (Gill et al., 1986) and an *E. coli* 48-kD protein (Byström et al., 1983). The COOH-terminal 300-amino acid residues of DP and FTS Y are similar to the

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1. **Abbreviations used in this paper:** DP, docking protein; SRP, signal recognition particle; SRP54M, methionine-rich COOH terminus of SRP54 (amino acids 297–504); SRP54N+G domains, amino acids 1–295 of SRP54, containing a putative GTP-binding site.

first 300 amino acids of SRP54, whereas the *E. coli* 48-kD protein can be aligned with SRP54 over its entire length (Römisch et al., 1989; Bernstein et al., 1989). The alignments of both DP and FTS Y with SRP54 end at almost exactly the same position within SRP54, namely at amino acid 297 and 299, respectively. We assume that this indicates the end of a functional domain that SRP54 has in common with the other proteins and possibly also the end of a structural domain.

The protein components of SRP can be released from the RNA under non-denaturing conditions (Siegel and Walter, 1988b; Scoulica et al., 1987) and reassembly of a complete SRP (Siegel and Walter, 1988b) or a subparticle (Siegel and Walter, 1988b; Lingelbach et al., 1988; Römisch et al., 1989) is possible in vitro. We used this approach to locate the regions in SRP19 and SRP54 which are required for assembly into a SRP subparticle. Proteolytic cleavage of SRP purified from dog pancreas and subsequent analysis of the resulting SRP54 fragments support our in vitro data.

Materials and Methods

Constructs

SRP19 and Derivatives. Plasmid pSRP19 has been described previously (Lingelbach et al., 1988). Truncated SRP19 polypeptides were created by three methods: (a) linearization of pSRP19 within the coding region by Xba I causes synthesis of only the N₂-terminal 68 amino acids of SRP19 in the subsequent in vitro transcription and translation. (b) The other carboxy-terminal truncations were generated by adding synthetic oligonucleotides complementary to part of the coding region to the in vitro translation reaction (Haeuptle et al., 1986). The wheat germ endogenous RNase H cleaves the message at the hybridization site and the truncated mRNA gives rise to a shortened SRP19. The oligonucleotides used are SRP19/3 (complementary to coding bases 460–476), SRP19/8 (404–420) and SRP19/7 (344–360). (c) Plasmid pSRP19X/H was constructed as follows: pSRP19 was digested with Xba I and the vector pDS12/RBSII (Bujard et al., 1987) with Sal I. Both linearized DNAs were made blunt end with Klenow (Maniatis et al., 1982) and then digested with Hind III. The resulting Hind III/blunt fragment from pSRP19 containing the coding region for the COOH-terminal 75 amino acids was ligated to the pDS12/RBSII vector.

SRP54 and Derivatives. pSRP54TE_x and pSRP54-I were described previously (Römisch et al., 1989). In vitro transcription and translation of pSRP54-I gives rise to the full length 54-kD protein. pSRP54TE_x contains the coding region for only the COOH-terminal 35-kD segment of SRP54 (SRP54Ex). COOH-terminal deletion mutants were created by cutting pSRP54-I within the coding region with Bgl II, Bam HI, Eco RI, Hind III, or Sph I before transcription.

pSRP54B* was generated as follows: the nucleotides around the starting methionine of SRP54 were changed to a Nco I site by site-directed mutagenesis in M13. The Nco I/Bam HI* fragment was cut out and introduced into pDS56/RBSII-6xHis (Hochuli et al., 1988). This construct contains the coding region for the first 466 amino acids of SRP54 plus an additional 6 histidine residues at the COOH-terminus, which can be used for affinity purification on a metal chelating column (Hochuli et al., 1988).

The Nco I/Bam HI fragment encoding the first 166 amino acids of SRP54 and the Bam HI/Bam HI* fragment encoding amino acids 167–466 of SRP54 were introduced into pDS56/RBSII-6xHis (pSRP54^{1–166}, pSRP54^{167–466}). These plasmids were used for overproduction of the encoded SRP54-derived peptides.

The SRP54N+G polypeptide was generated by translation of the SRP54 mRNA in presence of an oligonucleotide complementary to coding bases 886–897. pSRP54M, site-directed mutagenesis in M13 was to create a Nco I site around the methionine at position 297 of SRP54. The mutated fragment was cut out with Nco I/Pst I, made blunt end, and cloned into the Sma I site of pGEM2 (Promega Biotec, Madison, WI).

General molecular biology techniques were performed according to Maniatis et al. (1982).

Binding Experiments

Formation of a RNP was tested essentially as described previously (Römisch et al., 1989). Linearized DNA was transcribed in vitro and translated in the wheat germ cell-free system in the presence or absence of truncating oligonucleotides for 30 min at 25°C. EDTA was added to 5 mM final concentration to release nascent chains from ribosomes and incubation was continued for 15 min. Then the translation reactions were adjusted to 5 mM magnesium acetate and 500 mM potassium acetate. 10 µl translation of SRP54, SRP19, or truncation thereof were mixed with 1 µg SRP7S RNA or tRNA and incubated at 25°C for 30 min. The volume was adjusted to 200 µl with 500 mM potassium acetate, 5 mM magnesium acetate, 50 mM Tris-acetate, pH 7.5 (wash buffer). 40 µl were removed and TCA precipitated to monitor for total protein synthesis (T). 30 µl DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in wash buffer was added to the remainder and incubated at 4°C for 15 min. After centrifugation the unbound supernatant fraction (U) was TCA precipitated. The DEAE-Sepharose was washed three times with 1 ml wash buffer and DEAE-bound material was released by a 15 min incubation at 4°C with 200 µl of the same buffer adjusted to 2 M potassium chloride. Eluted protein was TCA precipitated and all samples were analyzed on 10–15% Laemmli-type SDS polyacrylamide gels (Laemmli, 1970) and subjected to fluorography.

Antibodies

Rabbit antisera 908 and 907 were raised against SRP54 derived peptides overexpressed in *E. coli* from pSRP54^{1–166} and pSRP54^{167–466}, respectively, and purified on a nickel chelating column as described (Hochuli et al., 1988). All other antisera were raised in rabbits against synthetic peptides derived from SRP54. Numbers in brackets indicate the position of the peptide antigen used within the amino acid sequence of SRP54: 901 (88–100), 730 (103–117), 832 (131–149), 903 (149–161), 765 (180–196), 906 (197–208), 981 (267–280), 982 (321–334), 831 (496–504).

V8 Protease Digestion and Immunoblotting

SRP was purified from dog pancreas as described (Scoulica et al., 1987), but the sucrose gradient centrifugation was omitted. 100 µl of 125 nM SRP in 50 mM TEA-HCl pH 7.5, 325 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT was incubated with varying concentrations of V8 protease (endoproteinase Glu-C, Boehringer, Mannheim, FRG) as indicated in the figure legends for 1 h at 25°C. Protein was either TCA precipitated or V8 protease activity was inhibited by addition of PMSF to a final concentration of 20 µg/ml and the proteolytic fragments tested for DEAE binding as described for a binding assay. DEAE-unbound and eluted material were TCA precipitated. After PAGE on 10–15% Laemmli-type gels, protein was blotted onto nitrocellulose filters (Schleicher & Schüll, Dassel, FRG). Rabbit antisera were used at a dilution of 1:200 and blots were developed with alkaline phosphatase-coupled goat anti-rabbit antibodies (Dianova).

Results

Deletion Mapping of the SRP7S RNA Binding Site of SRP19

To map regions of SRP19 that are important for its binding to SRP7S RNA we generated various truncated derivatives of SRP19 (Fig. 1). Full-length SRP19 consists of 144 amino acids. COOH-terminally shortened derivatives were obtained by translating SRP19 mRNA, which was truncated by RNase H at sites specified by complementary oligonucleotides. The resulting polypeptides made up the NH₂-terminal 123, 104, and 84 amino acids respectively. Linearization of pSRP19 within the coding region by Xba I results in expression of the N-terminal 68 amino acids. The Xba I/Hind III fragment of pSRP19 subcloned into the bacterial expression vector pDS12/RBSII codes for the COOH-terminal 75 amino acids. SRP19 and the truncated polypeptides derived from it were transcribed in vitro and translated in a wheat

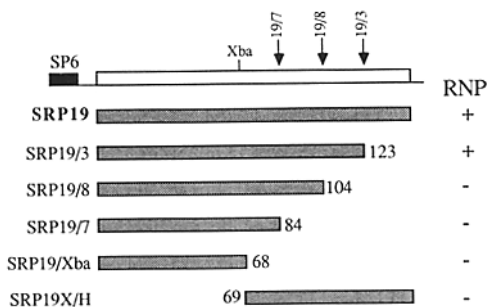


Figure 1. Schematic representation of SRP19 truncations. Plasmid pSRP19 is outlined on top of the figure (Lingelbach et al., 1988). The coding region is indicated as an open bar. Full-length SRP19 and truncated proteins are shown underneath. They were generated by oligonucleotide truncation (SRP19/3, SRP19/8, SRP19/7), cutting within the coding region of pSRP19 (SRP19/Xba) or subcloning (SRP19X/H as described in Materials and Methods. For COOH-terminal truncations the last amino acid is indicated, for the SRP19X/H the starting amino acid is numbered. The capability of the polypeptides to form a ribonucleoprotein particle (RNP) with 7S RNA is indicated.

germ cell-free system. 10 μ l of translation reaction were incubated with 1 μ g SRP7S RNA or 1 μ g tRNA as a negative control. 20% of the incubation mixture was TCA precipitated (T) to monitor for protein synthesis. Protein associated with RNA was recovered by batch adsorption to DEAE-Sepharose. The unbound supernatant fraction (U) was TCA precipitated and protein which had bound to the DEAE (B)

via the RNA was eluted and TCA precipitated. All samples were subsequently analyzed by SDS-PAGE and fluorography. The resulting fluorographs were scanned to quantitate bound and unbound fractions (B+U=100%). The fact that the sum of bound plus unbound fractions is on average only 2.5 times rather than four times T reflects loss of protein during the washing steps. As was shown previously by Lingelbach et al. (1988) SRP19 binds specifically to SRP7S RNA (86% B, 14% U) and not to tRNA (100% U) (Fig. 2 A, compare 19/7S RNA and 19/7tRNA). Deletion of the 21 COOH-terminal amino acids by truncating the SRP19 mRNA with oligonucleotide 19/3 has no influence on the binding of the polypeptide to SRP7S RNA (90% B, 10% U) (Fig. 2 A, 19/3/7S RNA and 19/3/tRNA). It remains unclear why this truncation gives rise to two polypeptide chains of slightly different molecular weight, but both can still bind specifically to SRP7S RNA.

Further deletions from the COOH terminus, i.e., truncation with oligonucleotides 19/8 and 19/7 or linearization of the plasmid within the coding region with Xba I, completely abolished binding to SRP7S RNA. As an example the effect of the truncation with oligonucleotide 19/7 is shown in Fig. 2 A (compare 19/7/7S RNA and 19/7/tRNA). The COOH-terminal 75 amino acids when expressed from the Xba I/Hind III fragment subcloned in pDS12/RBSII were also incapable of binding to SRP7S RNA on their own as shown in Fig. 2 B (compare 19X/H/tRNA and 19X/H/7S RNA).

Our results suggest that at least the COOH-terminal 21 amino acid residues of SRP19 are dispensable for binding to SRP7S RNA in vitro.

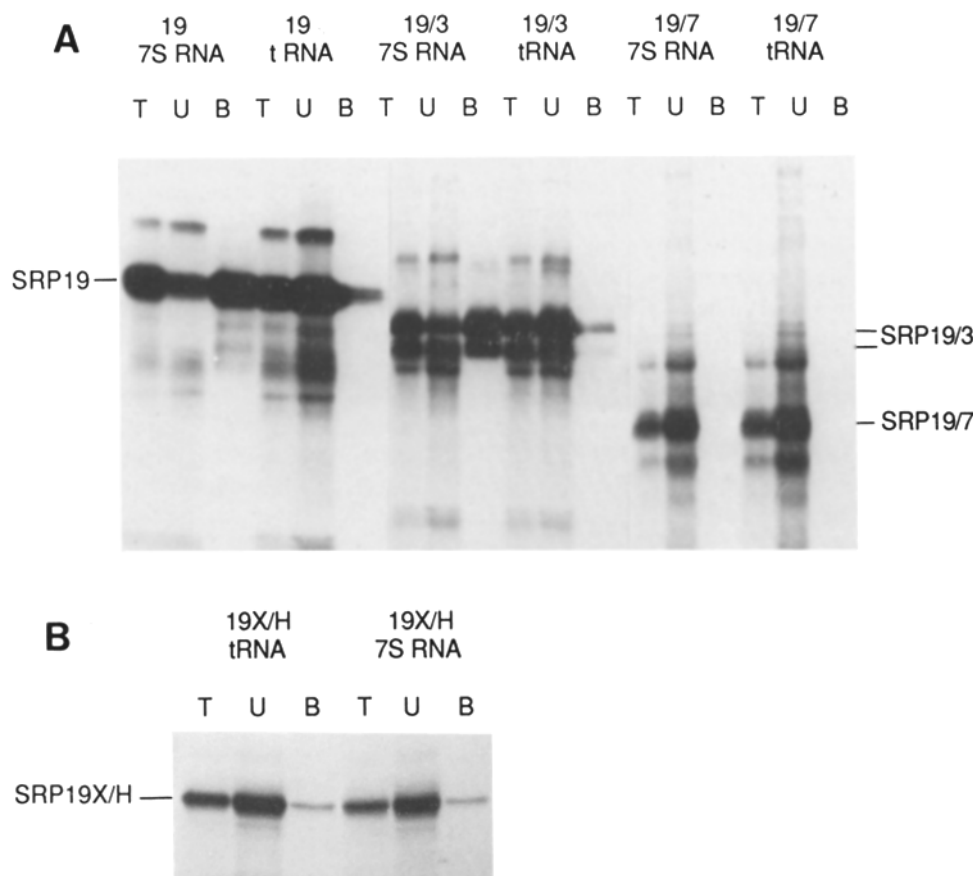


Figure 2. In vitro binding of truncated SRP19 to SRP7S RNA. After in vitro transcription and translation COOH-terminally deleted versions of SRP19 (A) and one NH₂-terminal truncation of SRP19 (B) were bound to SRP7S RNA in vitro and the assembled SRP subparticle recovered by binding to DEAE-Sepharose. tRNA was used instead of SRP7S RNA as negative control. 20% of the total incubation mixture (T), material which bound to DEAE (B) and material not bound to DEAE (U) were analyzed by SDS-PAGE and fluorography. SRP19/3 and SRP19/7 are translation products of mRNAs truncated by the presence of the respective oligonucleotides during translation. SRP19X/H is the translation product of the Xba I/Hind III fragment of pSRP19 subcloned into pDS12/RBSII.

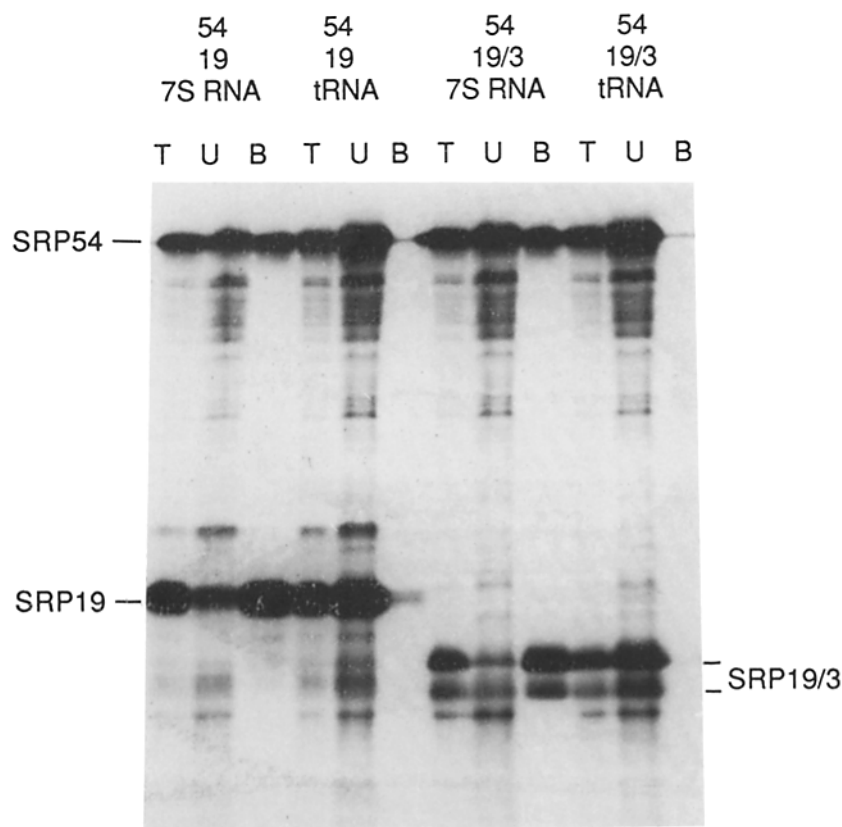


Figure 3. In vitro assembly of SRP7S RNA, SRP54 and a COOH-terminally truncated SRP19 protein. Full-length SRP54 and either full-length SRP19 or SRP19/3, which lacks the COOH-terminal 21 amino acids were incubated with SRP7S RNA or tRNA and total (T), DEAE-bound (B), and unbound (U) material were analyzed as described in Fig. 2.

Effect of SRP19 Truncations on Binding of SRP54 to the SRP Subparticle In Vitro

As SRP19 binds to 7S RNA and mediates the binding of SRP54 to the ribonucleoprotein particle, we wanted to know whether two distinct regions of SRP19 are responsible for SRP54 binding to the SRP19/7S RNA subparticle and SRP19 binding to SRP7S RNA. Since only the COOH-terminal 21 amino acids of SRP19 are dispensable for 7S RNA binding, we tested whether this part of SRP19 is necessary for SRP54 binding to the subparticle in vitro. The binding assay was performed as described above but this time in vitro synthesized SRP54 in addition to SRP19 or SRP19/3 was incubated with SRP7S RNA or tRNA. As shown in Fig. 3, truncation of the COOH-terminal 21 amino acids of SRP19 does not impair binding of SRP54 to the particle (compare lanes B in the presence of 7S RNA). As expected the SRP19 mutants which were unable to bind to SRP7S RNA on their own as described above did not promote SRP54 binding to the particle (data not shown).

While 80% of SRP19 and 93% of SRP19/3 bind to 7S RNA only 43 and 36% of SRP54 are in the bound fraction. This considerable difference in binding efficiency between SRP19 and SRP54 is consistent in all our experiments. One possible explanation for this observation is that SRP19 forms a binary complex with SRP7S RNA and the amount of SRP7S RNA in the binding assay is not limiting, thus the chance for SRP19 to bind to SRP7S RNA is high compared with that of SRP54 to bind to the small fraction of SRP7S RNA that is complexed with SRP19.

Deletion Mapping of the SRP Binding Region of SRP54

Our initial approach to identify the region of SRP54 responsible for its binding to the SRP was similar to our experiments with SRP19: as shown in Fig. 4, a set of COOH-terminal truncations of SRP54 were generated by linearizing the pSRP54-1 plasmid with restriction enzymes cutting within the coding region. In the SRP54B* truncation the COOH-terminal 38 amino acids of SRP54 were replaced by six histidine residues. In addition, a NH₂-terminally truncated SRP54 cDNA was used which codes for the COOH-terminal 35-kD segment of SRP54 (SRP54Ex).

All SRP54 deletions were tested for binding to the SRP7S RNA in the presence and also in the absence of SRP19. As shown previously (Römisch et al., 1989; Siegel and Walter, 1988b) both SRP54 and SRP19 bind specifically to SRP7S RNA (36 and 62% B, respectively) and not to tRNA (0% B for both) (Fig. 5 A). The translation product from the SRP54B* truncation was still capable of binding to the SRP subparticle (Fig. 5 A) with an efficiency similar to SRP54: in this experiment 86% of SRP19 was bound to 7S RNA and 49% of SRP54B* could bind to the subparticle. SRP54B* could not bind to 7S RNA in the absence of SRP19 (Fig. 5 A). Deletion of a 14kD fragment from the COOH terminus by cutting pSRP54-1 with BglII prior to in vitro transcription and translation completely impaired binding of the truncated polypeptide to the SRP19/7S RNA complex (lanes B for 54Bgl in Fig. 5 B). Further truncations from the COOH-terminus also led to polypeptides incapable of binding to the subparti-

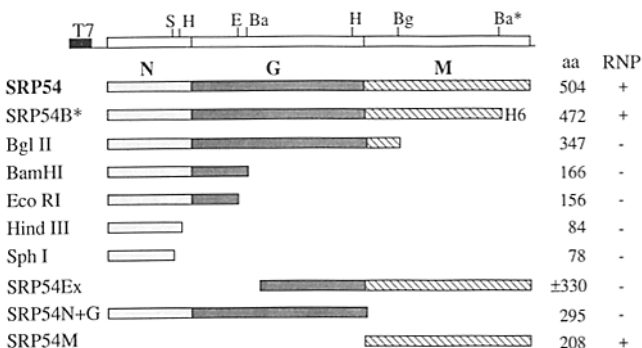


Figure 4. Schematic outline of SRP54 and derivatives. Fig. 4 shows an outline of pSRP54-1 (Römisch et al., 1989). The coding region is represented by an open bar and the following restriction sites are indicated: S, Sph I; H, Hind III; E, Eco RI; Ba, Bam HI; Bg, Bgl II. Full-length SRP54 is shown underneath. N, G, and M designate the proposed domain structure of the protein based on the presence of a GTP-binding consensus motif (G) and accumulation of methionine residues (M) (Römisch et al., 1989). Coding regions and size of truncated derivatives of SRP54 are outlined underneath. The names of the resulting polypeptides are given on the lefthand side. H6 indicates the addition of six histidine residues to the COOH terminus of the SRP54B* coding region. Size in number of amino acids and capability of the polypeptides to form a RNP with SRP19/7S RNA are indicated on the right hand side.

cle (data not shown). SRP54Ex lacking the NH₂-terminal 19 kD of the full-length protein also did not bind to SRP19/7S RNA (Fig. 5 C).

Identification of the SRP54M Domain as the Region Binding to SRP19/7S RNA In Vitro

The finding that all our random large truncations of SRP54 rendered it incapable of binding to SRP19/7S RNA led us to believe that they might cause a severe perturbation of the tertiary structure of the protein. Therefore they might give negative results in the binding assay even though the primary sequence responsible for binding is still contained in the polypeptide. We therefore tried to maintain the tertiary structure by generating a mutant protein that ends at the predicted domain boundary between the G and the M domain of SRP54 (SRP54N+G; Fig. 4 and Römisch et al., 1989). We also created a mutant which expresses only the COOH-terminal M-domain of SRP54 (SRP54M; Fig. 4 and Römisch et al., 1989).

Binding experiments to SRP7S RNA were performed as described above in presence or absence of SRP19. As shown in Fig. 6 A the SRP54N+G domain does not bind to SRP19/7S RNA (lanes B). However the SRP54M domain on its own is capable of specifically binding to the subparticle (37%B) but not to SRP7S RNA alone (Fig. 6 B). We therefore conclude that the SRP54M domain is the part of the protein that is necessary and sufficient for binding of SRP54 to the SRP.

SRP54 Binds to SRP via its M Domain In Vivo

We wanted to test whether binding of SRP54 via its M domain to SRP19/7S RNA in vitro reflects the situation in vivo.

Therefore we looked for a protease that would cleave SRP54 into large fragments that could easily be identified. It has been shown that elastase cleaves a 35-kD fragment off SRP54 and that this fragment does not bind to SRP anymore (Scoulica et al., 1987). The 35-kD fragment is comparatively protease resistant but the rest of SRP54 is completely digested by elastase into small peptides (data not shown). We used V8 protease (endoproteinase-GluC) in increasing concentrations to digest SRP purified from dog pancreas. The digested SRP was TCA precipitated, proteolytic fragments separated on 10–15% polyacrylamide gels and blotted onto nitrocellulose. Blots were probed with an antiserum raised against a protein fragment corresponding to amino acids 167–466 of SRP54 (907), an antiserum against amino acids 1–166 of SRP54 (908) and an antiserum against the COOH-terminal 9 amino acids of SRP54 (831). At 10 µg/ml V8 protease cleaves SRP54 into two fragments (Fig. 7 A, 907): immunoblotting revealed that the 35-kD fragment contains the NH₂-terminal part of the protein (Fig. 7 A, 908) and the 24-kD fragment contains the COOH terminus (Fig. 7 A, 831). One or both fragments migrate slightly aberrantly in the polyacrylamide gel and therefore add up to >54 kD. Increasing concentrations of V8 protease lead to a further digestion of the NH₂-terminal 35-kD fragment to a 32- and finally an 18-kD fragment, which are still recognized by antiserum 908 (Fig. 7 A, open arrowheads). The COOH-terminal 24-kD fragment is processed to a 21-kD polypeptide (Fig. 7 A, black arrowheads). Undigested SRP54 is shown as a control.

To test which of the proteolytic SRP54 fragments are still associated with SRP we digested SRP with 10 µg/ml V8 and incubated the fragments with DEAE-Sepharose as described above for the binding experiments. DEAE-bound (B) and unbound fractions (U) were analyzed by SDS-PAGE and immunoblotting. Undigested SRP was used as a positive control for DEAE-binding. The 35-kD NH₂-terminal fragment does not bind to SRP as it is recovered in the DEAE-unbound fraction (Fig. 7 B). The 24-kD COOH-terminal fragment is found in the DEAE-bound fraction and therefore still associated with the particle (Fig. 7 B). At the V8 protease concentration used the SRP19 in the particle is completely protease resistant (data not shown).

We characterized the proteolytic fragments from the V8 digestion more precisely by immunoblotting with a number of antisera raised against SRP54-derived peptides. In Fig. 7 D the position of the peptides used as antigens for the respective sera is indicated on a schematic outline of SRP54. Sera reacting with the 35-kD fragment are indicated by an open arrowhead, sera reacting with the 24-kD fragment by a black arrowhead. We conclude that the 35-kD fragment comprises most of the SRP54N+G domain and the 24-kD fragment most of the SRP54M domain.

We used SDS-PAGE and autoradiography in combination with immunoblotting to compare the relative molecular sizes of the in vitro expressed SRP54N+G and M domains to the SRP54 derived V8 fragments. In vitro expressed [³⁵S]methionine-labeled SRP54, SRP54N+G and SRP54M were separated on a 10–15% polyacrylamide gel. Dog pancreas SRP undigested or digested with 10 µg/ml V8 protease was applied to the same gel. Protein was blotted onto nitrocellulose and visualized by autoradiography and immunoblotting.

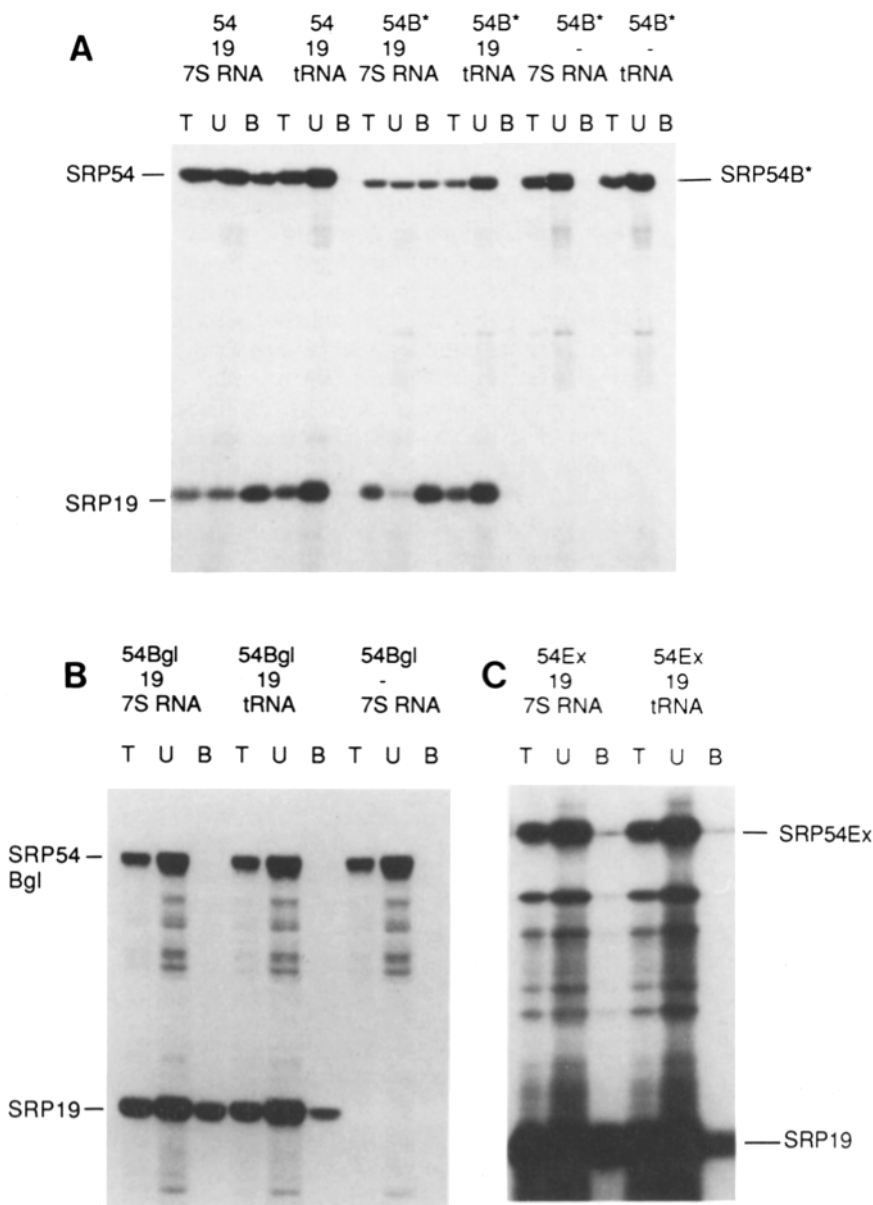


Figure 5. In vitro binding of SRP54 deletion mutants to the SRP19/7S RNA subparticle. **A** shows a binding experiment performed with the SRP54B* truncation. Binding of SRP54 was done as a positive control, incubation with tRNA as negative control. Total (T), unbound (U), and bound (B) fractions were analyzed as described in Fig. 2. **B** shows binding experiments using a SRP54 COOH-terminal deletion created by cutting within the coding region with Bgl II (SRP54Bgl). **C** shows the same type of experiment done with the NH₂-terminal SRP54 deletion mutant SRP54Ex.

The first three lanes of Fig. 7 C show the in vitro translation products, lanes 4 and 5 show the immunoblot with antiserum 907 on the undigested and digested SRP, respectively. The in vitro synthesized and dog pancreas SRP54 comigrate. As judged from the migration in the SDS-gel, the in vitro synthesized SRP54N+G is ~1 kD smaller than the 35-kD V8 fragment and the SRP54M is ~1 kD larger than the 24-kD V8 fragment. We therefore conclude that the V8 cleavage site in SRP54 is located 8–10 amino acids COOH-terminal to our assigned G-M domain boundary at amino acid 297 (Fig. 7 D).

The SRP54M Domain Binds to *E. coli* 4.5S RNA

E. coli 4.5S RNA is homologous to stem-loop 2 of SRP 7S RNA (Poritz et al., 1988). As we could show previously that SRP54 can bind to *E. coli* 4.5S RNA in the absence of SRP19 (Ribes et al., 1990 and Fig. 8), we wanted to test

whether the SRP54M domain is also responsible for this interaction. Binding to 4.5S RNA was performed as described above for SRP7S RNA. Fig. 8 shows that the SRP54N+G domain does not bind to 4.5S RNA (compare 54N+G/4.5S and 54N+G/tRNA, lanes B). However, the SRP54M domain specifically binds to *E. coli* 4.5S RNA (Fig. 8, compare 54M/4.5S and 54M/tRNA). In this experiment 57% of the SRP54M domain are in the bound fraction that is comparable to 45% B for full-length SRP54. These bound ratios are higher than those found for SRP54M and SRP54 binding to SRP19/7S RNA (37% B and on average 38% B, respectively). The bound fractions are consistently larger in repeated experiments (data not shown) if binding of SRP54 and derivatives to 4.5S RNA rather than to the SRP19/7S RNA complex is tested. This might reflect that the chance for SRP54 to form a binary complex with nonlimiting amounts of 4.5S RNA is higher than to interact with the small fraction of SRP7S RNA that has complexed SRP19.

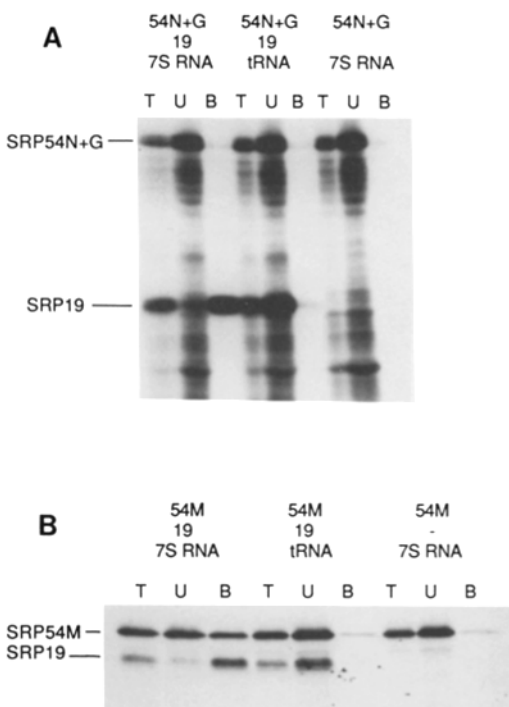


Figure 6. Binding of SRP54N+G and SRP54M to the SRP19/7S RNA subparticle in vitro. (A) SRP54N+G was synthesized in vitro by translating SRP54 mRNA in the presence of an oligonucleotide complementary to the nucleotides coding for the end of the G domain. Assembly with SRP7S RNA in the presence or absence of SRP19 was assayed as described above and total (T), unbound (U), and bound (B) fractions were analyzed. (B) The SRP54M domain was expressed in vitro from pSRP54M and binding to SRP7S RNA was tested in the presence or absence of SRP19 as described above.

Discussion

Using a deletion-mapping and limited proteolysis approach we have studied the interactions between SRP7S RNA, SRP19, and SRP54. Our deletion experiments with SRP19 revealed that the integrity of a major part of the protein is essential for binding to SRP7S RNA. We could only delete the COOH-terminal 21 amino acids without interfering with binding and thereby rule out the possibility that the seven lysine residues clustered in this region are responsible for an electrostatic interaction with the negatively charged RNA. Truncation of 400 amino acids from the COOH terminus led to a complete abolishment of binding. Deletion of the NH₂-terminal 68 amino acid residues also resulted in a loss of the SRP7S RNA binding function. This may indicate that the binding of SRP19 to 7S RNA is not mediated by a single small stretch of amino acids but rather by multiple contacts between protein and RNA which require an intact tertiary structure of the protein. This interpretation is also supported by footprinting data from Siegel and Walter (1988a), who could show that SRP19 interacts with the two large stem-loops of SRP7S RNA. Alternatively, the larger deletions might create an incomplete domain which could interfere with correct folding of the RNA-binding domain and thus abolish binding to SRP7S RNA even though the deleted portion of SRP19 is not itself physically interacting with the RNA.

The in vitro translation products derived from truncated mRNAs we use in our binding assays are released from the ribosomes with EDTA. It is therefore conceivable that initially they still have tRNA bound to their COOH terminus which may interfere with binding to SRP7S RNA. Peptidyl-tRNA would be expected to bind to DEAE-Sephacel in the absence of SRP7S RNA. No such background binding was observed (Fig. 2 A, compare 19/tRNA to 19/3/tRNA and 19/7/tRNA) and one of the translation products derived from a truncated mRNA (19/3) was still capable of binding to 7S RNA (Fig. 2 A). Therefore we assume that the 15-min incubation time in presence of EDTA at the end of the translation is sufficient for removal of the tRNA from the polypeptide chains.

The COOH-terminal 21 amino acids of SRP19 are also not essential for the promotion of SRP54 binding to the particle in vitro. SRP19 mutants that did not bind to SRP7S RNA also did not mediate SRP54 binding to SRP7S RNA, suggesting that these two functions are closely related and are not contained in two different domains of SRP19 as we thought initially. It is not clear whether the COOH terminus of SRP19 is also dispensable for SRP function in vivo.

By two different approaches, deletion mapping and limited proteolysis, we could show that the methionine-rich COOH-terminal M domain of SRP54 (Fig. 4) is responsible for its binding to the SRP. The exact binding site within this domain has not yet been determined. Removal of the COOH-terminal 38 amino acids from SRP54 (SRP54B*, Fig. 4) does not interfere with binding to SRP19/7S RNA. Bernstein et al. (1989) proposed that the SRP54M domain forms four amphipathic α -helices (1, 2, 3A and 3B). Due to amino acid sequence homology the authors further suggest that helix 3B is a repeat of helix 3A. In SRP54B*, helix 3B is truncated approximately in the center. Only one of the 3A-, 3B-type helices is present in the *E. coli* 48-kD protein that is homologous to SRP54 and can bind to SRP7S RNA in vivo if 7S RNA is expressed in *E. coli* (Ribes et al., manuscript submitted for publication). Together, this information suggests that helix 3B is dispensable for binding of SRP54 to SRP19/7S RNA. Truncation of SRP54 at the Bgl II site located between helix 1 and 2 shows that the NH₂-terminal 50 amino acids of the M domain are not sufficient for particle binding. The COOH-terminal proteolytic V8 fragment of SRP54 has potentially lost some of α -helix 1, which would further reduce the minimal required unit for binding to SRP19/7S RNA. We will answer this question by protein sequencing to locate the V8 cleavage site exactly.

That a truncated SRP54 that contains part of the G domain in addition to the M domain (SRP54Ex, Fig. 4) cannot bind to SRP (Fig. 5 C) suggests that correct folding of the G domain is required for particle binding of the M domain.

We have recently shown that SRP54 and its 48-kD *E. coli* homologue can bind to *E. coli* 4.5S RNA (Ribes et al., manuscript submitted for publication) that is homologous to stem-loop 2 of SRP7S RNA (Poritz et al., 1988). We have shown here that it is the SRP54M domain that mediates this RNA binding (Fig. 8). We suggest that in SRP the SRP19 changes the conformation of SRP7S RNA such that the binding site for SRP54 becomes accessible. However, footprinting data from Siegel and Walter speak against this hypothesis (1988a). They showed that the pattern of protected bands

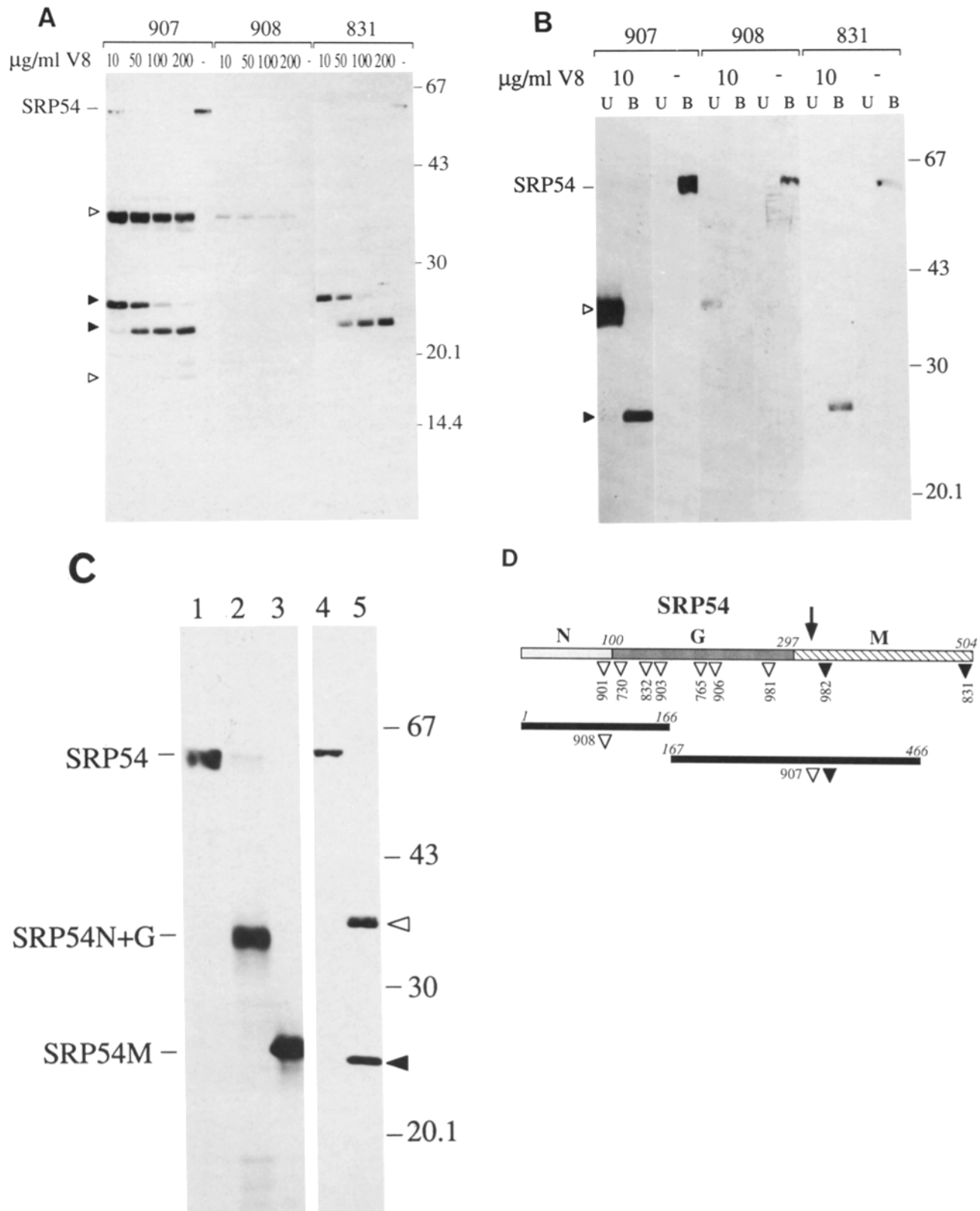


Figure 7. The SRP54M domain binds to SRP in vivo. (A) Per lane 100 μ l of 125 nM dog pancreas SRP was digested with the indicated amounts of V8 protease for 1 h at 25°C, the fragments separated on a 10–15% polyacrylamide gel and blotted onto nitrocellulose. SRP54 fragments were visualized by probing with rabbit antisera 907, 908, and 831 (raised against the SRP54 derived peptides indicated in D). SRP54 fragments containing the NH₂-terminal part of the protein are indicated by open arrowheads, fragments containing the COOH-terminus are indicated by black arrowheads. (B) Dog pancreas SRP was digested with 10 μ g/ml V8 protease as described above and the resulting fragments were incubated with DEAE-Sepharose as described for the in vitro binding assays. DEAE-bound (B) and unbound (U) fractions were analyzed by SDS-PAGE and the rabbit antisera described above. (G) In vitro synthesized [³⁵S]met-labeled SRP54,

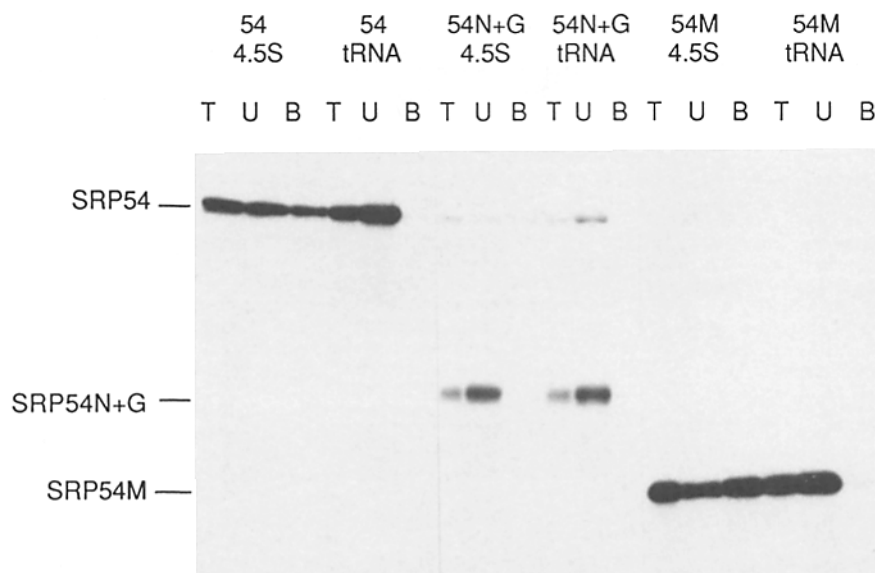


Figure 8. Binding of SRP54N+G and SRP54M to *E. coli* 4.5S RNA in vitro. SRP54N+G and SRP54M were synthesized in vitro and association with 4.5S RNA was tested as described in Fig. 2. Binding of full-length SRP54 was done as positive control, assembly in the presence of tRNA as negative control. Total (T), DEAE-bound (B), and unbound (U) material was analyzed by SDS-PAGE and fluorography.

does not change whether SRP19 alone or SRP19 and SRP54 are bound to SRP7S RNA. One possible explanation compatible with a SRP54/7S RNA interaction is that stem-loops 1 and 2 of SRP7S RNA are stacked on top of each other in presence of SRP19 alone (Zwieb and Ullu, 1986). Both loops could thus be protected from RNase digestion. In the presence of both SRP19 and SRP54 the 7S RNA may have a different conformation in which SRP19 is bound to stem-loop 1 and SRP54 to stem-loop 2. Different conformations for SRP7S RNA have been proposed previously by Zwieb and Ullu (1986). We assume that in the absence of SRP19 stem-loop 1 interferes with SRP54 binding to stem-loop 2 of SRP7S RNA since SRP54 can bind to 4.5S RNA which is homologous to stem-loop 2 and SRP19 does not bind to 4.5S RNA (data not shown). We are currently testing our hypothesis by using deletion mutants of SRP7S RNA to identify the regions which interact with SRP19 and potentially with SRP54.

Many proteins that are associated with RNA-like mammalian A1 hnRNP proteins or yeast poly(A)-binding protein contain a RNP consensus sequence (Adam et al., 1986; Bandziulis et al., 1989). This consensus sequence is a continuous stretch of 10–13 amino acids that can be repeated several times (Robinow et al., 1988) and that is thought to be responsible for the interaction with the RNA. However, there are precedents for RNP proteins, which like SRP54 lack the RNP consensus sequence (Sillekens et al., 1988). We have identified here a novel type of RNA binding domain that is very polar and has an overall basic character and an unusually high methionine content (11%) (Römisch et al., 1989; Bernstein et al., 1989). The RNA binding of this do-

main is very specific and clearly not only due to ionic interactions between the basic polypeptide and the negatively charged RNA since SRP54 binds only to the SRP7S RNA-related 4.5S RNA and not to tRNA (Fig. 8).

SRP54 has two known functions: binding to SRP and binding to the signal sequence of nascent secretory proteins (Krieg et al., 1986; Wiedmann et al., 1987). Here we were able to show that SRP binding of SRP54 is mediated by its M domain. Bernstein et al. (1989) discussed a possible signal sequence binding function for the SRP54M domain because the hydrophobic face of the four predicted amphipathic α -helices could interact with the hydrophobic part of the signal sequence. Our results do not rule out this possibility, but we think it is more likely that the signal binding function of SRP54 is not contained in the same region as SRP binding. As discussed previously (Römisch et al., 1989), we favor the hypothesis that the signal sequence interacts with a region in the G domain, possibly between the first and the second GTP binding consensus motif. The homologous region in Ras proteins has been identified as the effector binding site (Dever et al., 1987) and undergoes a conformational change upon GTP hydrolysis (Pai et al., 1989).

In this work, we characterized the SRP54M domain as a novel type of RNA binding domain. We are now aiming at the identification of the signal sequence binding site of SRP54.

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SRP54N+G, and SRP54M and dog pancreas SRP digested with 10 μ g/ml V8 protease were separated on a 10–15% polyacrylamide gel and blotted onto nitrocellulose. Lanes 1–3 show an autoradiography of the [35 S]met-labeled proteins, lanes 4 and 5 show an immunoblot with antiserum 907 on undigested and digested dog pancreas SRP. D shows a schematic outline of SRP54 and the protein segments against which antibodies were raised. Positions of the antigenic peptide for the respective anti-SRP54 rabbit antisera are indicated by arrowheads. Open arrowheads specify antisera reacting with the 35-kD V8 fragment of SRP54, black arrowheads specify antisera reacting with the 24-kD V8 fragment of SRP54. The black bars indicate SRP54 fragments against which antisera were raised (amino acid numbers are given in italics). The approximate position of the V8 cleavage site is indicated by an arrow.

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