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The heterodimeric subunit SRP9/14 of the signal recognition particle functions as permuted single polypeptide chain

Fabrice Bovia, Nazarena Bui and Katharina Strub*
Département de Biologie Cellulaire, Université de Genève, Sciences III, CH-1211 Geneva 4, Switzerland

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ABSTRACT

The targeting of nascent polypeptide chains to the endoplasmic reticulum is mediated by a cytoplasmic ribonucleoprotein, the signal recognition particle (SRP). The 9 kD (SRP9) and the 14 kD (SRP14) subunits of SRP are required to confer elongation arrest activity to the particle. SRP9 and SRP14 form a heterodimer which specifically binds to SRP RNA. We have constructed cDNAs that encode single polypeptide chains comprising SRP9 and SRP14 sequences in the two possible permutations linked by a 17 amino acid peptide. We found that both fusion proteins specifically bound to SRP RNA as monomeric molecules folded into a heterodimer-like structure. Our results corroborate the previous hypothesis that the authentic heterodimer binds to SRP RNA in equimolar ratio. In addition, both fusion proteins conferred elongation arrest activity to SRP(-9/14), which lacks this function, and one fusion protein could functionally replace the heterodimer in the translocation assay. Thus, the normal N-and C-termini of both proteins have no essential role in folding, RNA-binding and in mediating the biological activities. The possibility to express the heterodimeric complex as a single polypeptide chain facilitates the analysis of its functions and its structure *in vivo* and *in vitro*.

INTRODUCTION

The signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein that plays an essential role in sorting proteins to the endoplasmic reticulum (for review see ref. 1, 2 and 3). According to a model, SRP recognizes and binds to the signal sequence of the nascent chain as it emerges from the ribosome. This interaction effects a pause or an arrest in the synthesis of the polypeptide chain and the ribosome–nascent chain–SRP complex is then targeted to the endoplasmic reticulum via the interaction of SRP with its receptor (docking protein). After dissociation of the SRP receptor and SRP from the nascent chain–ribosome complex and then from each other, they may enter a new targeting cycle. Protein synthesis is resumed at its normal speed and the nascent chain engages in the actual translocation process (for review see ref. 4).

The canine SRP is composed of two heterodimeric protein subunits (SRP68/72 and SRP9/14), two monomeric polypeptides (SRP54 and SRP19) and one RNA molecule (SRP or 7S RNA) of 300 nucleotides. Studies on the assembly of SRP *in vitro* have revealed a great diversity in the RNA-binding characteristics of SRP proteins. SRP19 binds to SRP RNA directly (5). The conserved tetranucleotide loop of stem 6 and sequences in stem 8 of SRP RNA (nomenclature according to 6) are essential elements in binding of SRP19 (7). Efficient SRP54 binding to canine SRP RNA is mediated by SRP19 (5). However, SRP 54 can bind directly to the smaller SRP RNA homologue of *E.coli* (4.5 S RNA, (8–10)) indicating that the evolutionary conserved stem loop structure serves as a contact site for SRP54. Indeed, nucleotides conserved between 4.5S RNA, 7SL RNA of *Schizosaccharomyces pombe* and the mammalian SRP RNA are required for binding of the SRP54 homologous proteins to their respective RNA (11, 12). SRP68 binds alone to SRP RNA and the resulting RNA–protein complex is competent for SRP72 binding. The two proteins synthesized *in vitro* in the absence of SRP RNA associate only weakly (13). However, after assembly into SRP, SRP68 and SRP72 disassemble as a stable heterodimer from the particle and, as such, remain competent for SRP RNA binding (5). In contrast, SRP9 and SRP14 form a heterodimer without SRP RNA that binds specifically to SRP RNA (14). SRP9/14 and SRP RNA form a stable complex ($K_d < 0.1$ nM), and their association results in an allosteric change in the conformation of SRP RNA (15). The affinity of SRP68/72 for SRP RNA is substantially lower ($K_d \approx 7$ nM) and the two heterodimers bind non cooperatively to SRP RNA (15). All SRP proteins lack apparent structural similarities to already characterized RNA binding motifs (for review see (16)).

We have been interested in studying the structure–function relationship in the heterodimer SRP9/14. The two polypeptides are highly polar with an overall basic character as derived from their primary sequences (14, 17). They bind to the sequences at the 5′ end of SRP RNA that are homologous to the Alu family

*To whom correspondence should be addressed

of repetitive sequences. Phylogenetic evidence indicates that the Alu sequences of SRP RNA served as the precursor of this class of repetitive sequences in the rodent and primate genomes (18). More specifically, the binding site of SRP9/14 in SRP RNA is composed of four regions. One mostly single-stranded region is highly conserved in its primary sequence in SRP RNA homologues of organisms as divergent as bacteria and man (19). The conservation of structural elements in the SRP9/14 binding site suggests that the proteins also exist in a large variety of organisms. In SRP, the heterodimer SRP9/14 is required to mediate a specific pause or arrest in the synthesis of ER-targeted proteins *in vitro* (20).

To facilitate the analysis of the different functions of SRP9/14 we wanted to know whether the heterodimer could be replaced by a fusion protein comprising SRP9 and SRP14 sequences in a single polypeptide chain. We found that two single polypeptide chain variants folded into heterodimer-like structures and retained all their biological activities. In addition, further analysis of the fusion protein−RNA complex substantiated the previous hypothesis that SRP9/14 binds to SRP RNA in a equimolar ratio.

MATERIALS AND METHODS

Materials. SP6 RNA polymerase, RNase inhibitors, restriction enzymes and DNA ligase were purchased from Biofinex and New England Bio-Labs. Protein A-Agarose and tRNA from *E.coli* were obtained from Boehringer Mannheim Biochemicals, ribonucleotide triphosphates were from Pharmacia LKB Biotechnology and [^{35}S]-methionine (1,500 Ci/mmol) were from Amersham Corp. All other reagents were obtained from Sigma Chemical Co.

In vitro expression of proteins and formation of RNA−protein complexes. Aliquots of 2 μg of the linearized plasmids were used to produce synthetic transcripts with SP6 RNA polymerase in 40 μl transcription reactions (21). The phenol-extracted and precipitated RNA was resuspended in 40 μl of autoclaved water. Proteins were synthesized with wheat germ lysate in the presence of [^{35}S]-labeled methionine as described in (14). The RNA−protein complexes were formed by incubating the RNA and protein moieties in binding buffer (50 mM HEPES−KOH pH7.5, 350 mM potassium acetate, 3.5 mM magnesium acetate, 0.01 % Nikkol) for 10 min at 0°C and 10 min at 37°C.

Construction of SRP9-14 and SRP14-9 fusion protein genes. The cDNA constructs encoding either SRP9-14 or SRP14-9 fusion proteins were obtained in several cloning steps and by introducing restriction enzyme sites using the polymerase chain reaction. The SRP9-14 fusion protein gene contains in its 5′ region the complete coding sequence of the mouse SRP9 cDNA (N. B., F. B., M. D. Morrical, P. Walter and K. S., submitted) with an Nde I site at the initiator codon followed by a linker sequence of 51 base pairs and the complete coding sequence of the mouse SRP14 cDNA (17). The linker sequence encodes the 17 amino acids shown in Figure 1. The amino acids -EQKLISEED- constitute a well characterzed epitope of the human myc protein (22). The SRP14-9 fusion protein gene contains in its 5′ region the complete coding sequence of the SRP14 cDNA including a Nde I site at its initiator ATG followed by the linker sequence and the complete coding sequence of the SRP9 cDNA. The linker sequence is the same as described above. Both genes were cloned into the plasmid pSP64 (Promega) generating the plasmids pS9-14 and pS14-9 which were linearized with EcoRI and HindIII, respectively, for *in vitro* transcription.

Construction of plasmids encoding SRP9 and SRP14 truncated proteins. The mutated cDNAs, encoding SRP9 and SRP14 proteins lacking amino acids 2 to 11 at the N-terminal ends, were obtained by using the polymerase chain reaction to introduce new initiator codons at position 31 in the coding regions of SRP9 and SRP14 cDNAs. Both inserts were cloned in pSP65 resulting in the plasmids pS9-10N and pS14-10N which were linearized with HindIII for *in vitro* transcription. The mutated SRP14 cDNA, encoding a truncated SRP14 protein lacking 10 amino acids at its C-terminal end, was obtained by using the polymerase chain reaction to introduce a terminator codon followed by an EcoRI site at position 301 in the coding region of the SRP14 cDNA (17). The truncated cDNA was cloned into the EcoRI site of pGem4 (Promega) resulting in the plasmid pGm14-10C which was linearized with HindIII for *in vitro* transcription. The plasmid containing the complete cDNA of the mouse SRP9, pSm9-2 (N. B., F. B., M. D. Morrical, P. Walter and K. S., submitted), was linearized with Sty I which cut within the coding region of SRP9. The synthetic transcript derived from the truncated cDNA by *in vitro* transcription, produced a protein lacking 10 amino acids at its C-terminus. The inserts of all mutated clones were sequenced using the chain termination method (23).

RNA-binding experiment of fusion proteins. Alu RNA was synthesized as described (p7Salu, (19)) and purified on a Quiagene column (Kontron). Alu RNA and tRNA from *E.coli* were covalently linked to a hydrazide resin (Affi-Gel Hz, Bio-Rad) via their 3′-OH ends. To this end, the vicinal hydroxyl groups at the 3′ end of the RNA were first oxidized with sodium periodate and then coupled to the hydrazide resin following a protocol of the manufacturer and the advice of Kent Matlack, (UCSF, San Francisco). An estimation of the coupling efficiency indicated that about 1 pmole of Alu and tRNA were coupled to 1 μl of resin, respectively. Before use, the resin was washed once with water and twice with binding buffer. For each RNA-binding experiment 10 pmole of linked RNA was used. Aliquots of 3 μl of each translation reaction were combined with 10 pmole of RNA-coupled resin in a 50 μl binding reaction. The samples were washed four times with binding buffer. The proteins in the supernatants and in the first wash fractions were combined and analyzed by SDS-PAGE together with the RNA-bound proteins.

RNA-binding experiment of SRP9 and SRP14 truncated proteins. Truncated SRP9 and SRP14 proteins were examined for RNA-binding using biotinylated Alu RNA. Biotinylated Alu RNA was synthesized as described except that the concentrations of each nucleotide triphosphate and of biotinylated UTP in the transcription reactions were 1 mM and 100 μM, respectively. Aliquots of 3 μl of each translation reaction were combined with 1 pmole of biotinylated Alu RNA in a final volume of 20 μl of binding buffer containing 0.1 % Triton X-100. After complex formation, streptavidin beads (10 μl) were added to each sample and the RNA-bound and the free proteins were separated by centrifugation in a microfuge (30 sec, 2500 rpm). The beads were washed 3× for 3 min with 500 μl of binding buffer containing 0.1 % Triton X-100. The proteins in the various factions were analyzed by SDS-PAGE followed.

Cross-linking experiment. The translation reactions (30 μl each) were incubated in wash buffer (50 mM HEPES−KOH pH7.5, 250 mM potassium acetate, 2.5 mM magnesium acetate and 0.01 % Nikkol (Nikkol-BL-85Y, Nikko Chemical Co, Japan) with 80 μl of heparin gel (Bio-Rad) for 1 h. The gel was washed twice with the same buffer and the proteins were eluted with 80 μl of wash buffer containing 2 M potassium acetate and with 80 μl of wash buffer containing 1 M potassium acetate. The two elutions were combined and diluted to a final concentration of 0.5 M potassium acetate and 5 mM magnesium acetate. The protein samples were split and incubated with either 20 pmole of SRP RNA or alone for 10 min at 0°C and for 10 min at 37°C. After addition of glutaraldehyde at a final concentration of 0.1 %, aliquots were removed from each reaction at four time points (0, 2, 4, 8 min). The cross-linking reactions were stopped with 0.1 M Tris buffer. The proteins were analyzed by SDS-PAGE.

Purification of SRPϕ14-9 produced in E.coli. The fusion protein SRPϕ14-9 was produced in *E.coli* using the pEt3c expression vector (25). The SRPϕ14-9 gene contains the entire SRP14-9 coding region preceded the amino acid sequence M A S M T G G Q Q M G R I P G N S P R. Bacteria transformed with pEϕ14-9 were grown in a 200 ml culture and protein synthesis induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside during 3 hr. The bacterial pellet was lyzed with 20 ml 50 mM Tris−HCl pH 7.5, 1 mM EDTA, 10% glycerol, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM phenylmethyl-sulphonyl fluoride, 0.13 mg/ml lysozyme, 20 μg/ml DNAse I and a solution of protease inhibitors (200×stock solution: 20 μg/ml of each pepstatin A, leupeptin, antipain, chymostatin and 100 μg/ml aprotinin), after lysis, sodium deoxycholate was added at a final concentration of 0.05% and the concentration of potassium acetate was adjusted to 200 mM. The lysate was clarified by centrifugation at 4°C for 1 h at 27,500 rpm. The supernatant was mixed with 0.2 % polymin P and centrifuged. SRPϕ14-9 was purified on a heparin column (Bio-Rad, Econo-pac) and on a hydroxylapatite column (Bio-Rad). The purified protein SRPϕ14-9 was subsequently concentrated using 100 μl heparin resin in a batch absorption procedure together. The protein was quantified by comparison with a Coomassie-stained lysozyme standard.

Sedimentation analysis. The protein−Alu RNA complexes (500 mM potassium acetate) were sedimented into a 10−30 % (w/w) sucrose gradient (5 ml) at 4°C for 18 h at 40,000 rpm. Fractions (460 μl) were collected and the proteins analyzed by SDS-PAGE.

To synthesize [^{35}S]-labeled SRP14-9 and SRPϕ14-9 proteins, cultures of the transformed *E.coli* strain BL21(DE3)/LysS were grown to an optical density of 0.6 at 600 nm in L-broth containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. Rifampicin (0.2 mg/ml) was added for 20 min and then 1 ml of each cell culture was incubated with 1 μCi of [^{35}S]-methionine for 30 min The cells were lyzed and the proteins partially purified on a heparin resin. Equal amounts of partially purified radio-labeled SRP14-9 and SRPϕ14-9 alone or bound to SRP RNA were sedimented on sucrose gradients as described. On one hand, the proteins were analyzed by SDS-PAGE. On the other hand, they were incubated with protein A beads loaded with immunopurified anti-ϕ antibodies for 1 h at 4°C in the presence of RNAse inhibitors. The beads were washed three times with low salt buffer (150 mM potassium acetate, Tris−HCl pH 8, 0.01% Nikkol, 0.1% Triton X-100).

Elongation arrest and translocation assays. Elongation arrest assays were performed as described in (14), except that completely reconstituted SRP, SRP(-9/14) or SRP(-14) were added at a final concentration of 35 nM to 10 μl translation reactions programmed with the different synthetic transcripts. After incubation for 1 h at 26°C, 10 μl of a fresh translation reaction programmed with synthetic preprolactin and sea urchin cyclin transcripts was added to each sample. After an additional 25 min, the proteins were precipitated with trichloroacetic acid. Translocation assays were performed as described (20). The results were quantified (20) and the mean values and the standard deviations were calculated for all samples based on three to five independent measurements. The experimental variation is generally bigger in the elongation arrest assay than in the translocation assay. This difference may be explained by the fact that the SRP particles used in the elongation arrest assays are reconstituted by *in vitro* synthesized proteins *in situ* whereas purified proteins are used for reconstitution of SRP particles used in the translocation assays.

RESULTS

Two fusion proteins comprising SRP9 and SRP14 sequences in opposite order bind as monomers to SRP RNA

We have previously isolated cDNA clones encoding the 14 kD (SRP14) (17) and the 9 kD (SRP9) (N. B., F. B., M. D. Morrical, P. Walter and K. S., submitted) subunits of murine SRP. Starting from the murine SRP14 and SRP9 cDNAs, we constructed two fusion protein genes. In one fusion protein gene the SRP9 coding sequences were 5′ of the SRP14 coding sequences (SRP9-14). In the other fusion protein gene the order is reversed (SRP14-9) (Fig. 1A). In both fusion proteins, a peptide of 17 amino acids was inserted between the two polypeptides SRP9 and SRP14. The central portion of the peptide constitutes the epitope for a monoclonal antibody against the human myc protein (22). The glycine residues at its N-terminus were introduced to increase flexibility of the linker peptide and to disrupt the extension of the predicted a-helical structures in the N- and C-terminal regions of SRP9 and of SRP14.

Synthetic transcripts were produced from the two fusion protein genes with SP6 RNA polymerase and used to program wheat germ translation extracts. The [^{35}S]-labeled translation products,

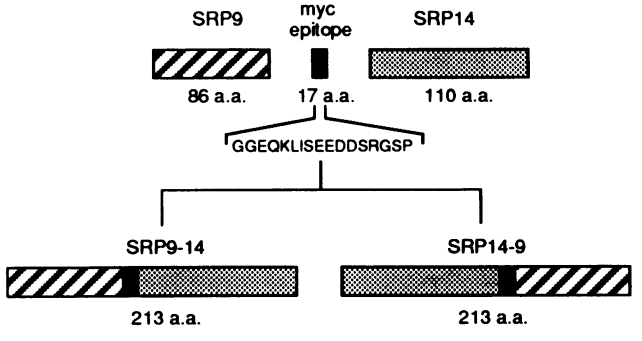


Figure 1. *In vitro* expression of the SRP9-14 and the SRP14-9 fusion proteins. Schematic outline of the construction of the fusion proteins SRP9-14 and SRP14-9. The linker peptide constitutes an epitope for an antibody against the human mycT protein ((EQKLISEED). a.a.: amino acids.

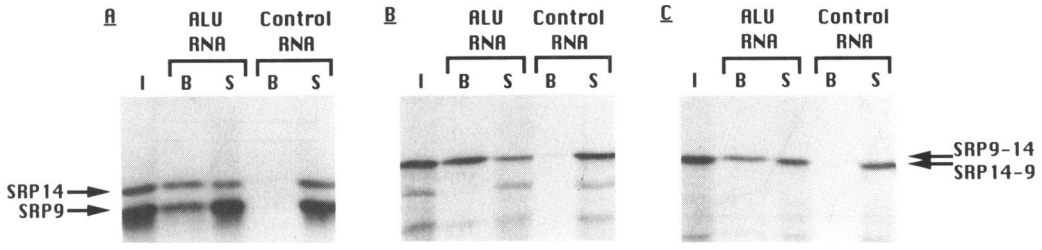


Figure 2. RNA-binding characteristics of SRP9-14 and SRP14-9. The heterodimer SRP9/14 (**A**), the fusion proteins SRP9-14 (**B**) and SRP14-9 (**C**) were bound to Alu RNA which was covalently coupled to a hydrazide resin via its 3′ end. I: Amount of *in vitro* synthesized [^{35}S]labeled protein used in the assay; B: Protein bound to the Alu RNA; S: Free protein. Control RNA: tRNA covalently coupled to a hydrazide resin.

when analyzed by SDS-PAGE, migrated with a slightly higher apparent molecular weight (27 kD) than expected from the calculated molecular weight (24.5 kD)(results not shown). The slower migration is most likely due to the very basic C-terminal domain of SRP14 (9 out of 16 residues are basic).

We then examined whether both fusion proteins could bind specifically to SRP RNA. SRP9-14, SRP14-9 and the two subunits of the heterodimer, SRP14 and SRP9, were synthesized in wheat germ lysate and incubated with an *in vitro* synthesized transcript comprising the Alu portion of SRP RNA. Such a transcript binds the heterodimer SRP9/14 as well as the entire SRP RNA (19). The Alu-transcript was covalently coupled to agarose beads via its 3′ end (see Material and Methods) and the RNA-bound protein could therefore be separated from the free protein by a simple centrifugation. As expected, the murine proteins SRP9 and SRP14 synthesized together in wheat germ lysate specifically bound to the Alu RNA (Fig. 2, panel A) and not to the control RNA. Approximately 30 % of the *in vitro* synthesized SRP14 and SRP9 and the excess of SRP9 over SRP14 was not associated with the RNA (Fig. 2, panel A). Taking into consideration the low dissociation constant of the SRP9/14-SRP RNA complex (Kd< 0.1 nM, (15)), we assumed that these fractions of the *in vitro* synthesized proteins were biologically inactive. In contrast, murine SRP9 and SRP14 alone did not bind to the Alu-portion of SRP RNA (results not shown), as reported before (14). Interestingly, we found that both fusion proteins, SRP9-14 and SRP14-9, also specifically bound to Alu RNA (Fig. 2., panels B and C) and not to the control RNA. Two smaller translation products of 20 kD and 18 kD in the SRP9-14 and SRP14-9 translation reactions, respectively, also bound specifically to Alu RNA suggesting that certain sequences in the fusion proteins were dispensable for the formation of a RNA−protein complex.

Since SRP9 and SRP14 proteins alone do not bind specifically to SRP RNA, these results implied that SRP9 and SRP14 sequences in the fusion proteins had to form a heterodimer to recognize the RNA. Conceivably, both fusion proteins could fold into a genuine heterodimer-like structure, despite the small size of SRP9 and SRP14, and presumably bind to Alu RNA as a monomer. Alternatively, the results could be explained by assuming that SRP9 and SRP14 sequences of two fusion protein molecules would interact to form a heterodimer and the RNA-bound form would therefore consist of a fusion protein dimer (Fig. 3A).

We had previously observed that SRP9 and SRP14 in the heterodimeric complex could be specifically cross-linked with glutaraldehyde (14). We therefore used this reagent to distinguish

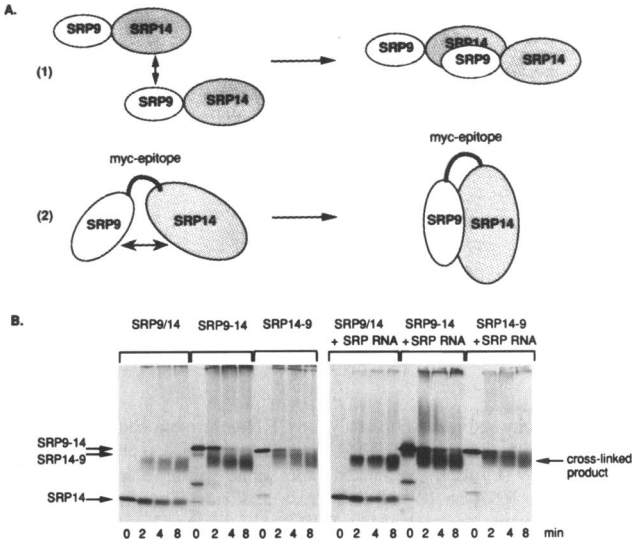


Figure 3. Two possible modes of interaction between SRP9 and SRP14 sequences in the fusion proteins. **A.** Formation of a heterodimer between two fusion protein molecules (1) or of a heterodimer-like structure within one fusion protein molecule (2). **B.** Glutaraldehyde-mediated cross-linking between SRP9 and SRP14 subunits in the heterodimer and in the fusion proteins with and without SRP RNA, respectively.

between the two possibilities. If SRP9 sequences of one fusion molecule formed a heterodimer with the SRP14 sequences of another fusion molecule, we would expect to find a cross-linked product of an apparent molecular size of 50−55 kD. If the SRP9 and SRP14 sequences within one fusion molecule folded into a heterodimer-like structure, we should observe a cross-linked product that migrates slightly faster than the fusion protein due to the fact that the polypeptide chain cannot be extended completely during electrophoresis.

The [^{35}S]labeled fusion proteins and SRP14 were synthesized *in vitro* and the SRP14 translation reaction was complemented with murine SRP9 for dimer formation. Murine SRP9 synthesized in bacteria and purified to homogeneity can functionally replace canine SRP9 in the SRP particle (26). The *in vitro* synthesized proteins were partially purified on a heparin resin (see Material and Methods). We found that this procedure removes a large fraction of the wheat germ components that give rise to non-specific cross-linked products. The cross-linking reactions were carried out at room temperature at a final glutaraldehyde

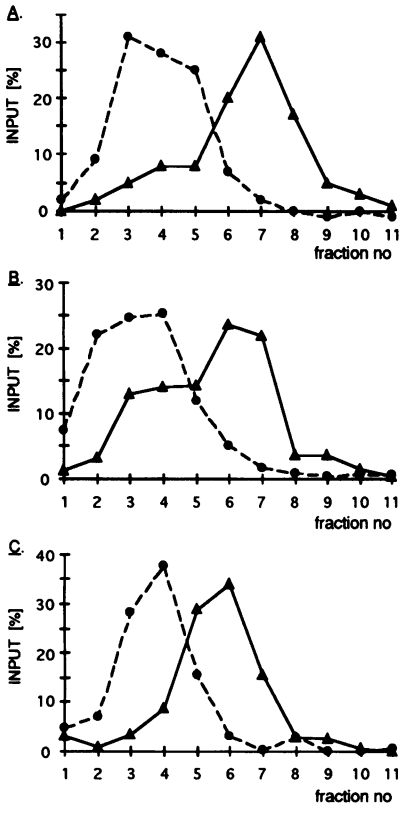


Figure 4. Velocity sedimentation analysis of RNA−protein complexes. The *in vitro* synthesized heterodimer SRP9/14 (**A**) and the two fusion proteins SRP9-14 (**B**) and SRP14-9 (**C**) were sedimented either alone or after binding to 5 pmole of synthetic Alu RNA on 10−30 % sucrose gradients. The protein content of the different fractions was analyzed by SDS-PAGE followed by autoradiography. The amount of protein contained in each fractions was quantified by densitometry. Solid lines: Proteins in the presence of Alu RNA; dashed lines: Proteins alone. [% Input]: Amount of protein in this fraction as percentage of the total amount of protein used in the experiment.

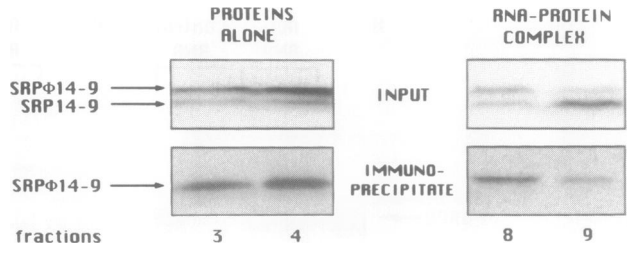


Figure 5. Immunoprecipitation analysis of RNA−protein complexes. Fractions containing radiolabeled SRPϕ14-9 and SRP14-9 proteins (fractions 3 and 4) and radiolabeled SRPϕ14-9 and SRP14-9 proteins bound to SRP RNA (fractions 8 and 9), respectively, were taken from a sucrose gradient, and the proteins were immunoprecipitated with anti-ϕ antibodies. Input: Total amount of proteins used in the immunoprecipitation experiments. Immunoprecipitate: Proteins precipitated by the anti-ϕ antibodies.

concentration of 0.1 %. Samples were removed at the time points indicated in Fig. 3B, and the reactions quenched by adding Tris/HCl buffer. The products were analyzed by SDS-PAGE.

The heterodimer SRP9/14 and both fusion proteins generated cross-linked products of identical size. They accumulated over time and migrated with an apparent molecular weight of approximately 25 kD (Fig. 3B). No cross-linked product of the size expected for a dimer between two molecules of the fusion proteins was observed. The same cross-linked product was observed in the presence of SRP RNA. Based on our previous results, we expected at least half of the protein molecules to be contained within a RNA−protein complex in these samples. These results therefore demonstrated that the fusion protein molecules folded into a heterodimer-like structure. Moreover, they suggested that the fusion proteins specifically bound to SRP RNA as monomeric molecules.

This interpretation was further substantiated by fractionating the RNA-protein complexes by velocity sedimentation in sucrose gradients followed by immunoprecipitation experiments. SRP9-14, SRP14-9 and SRP14 were synthesized *in vitro* and the SRP14 translation reaction was again complemented with purified murine SRP9. We chose to use *in vitro* synthesized Alu RNA, as opposed to SRP RNA, for complex formation to keep the molecular mass of the RNA moiety (50 kD) in the complex as small as possible. The protein−RNA complexes and the proteins alone were analyzed by sedimentation through a 10−30 % w/w sucrose gradient (see Material and Methods). The radiolabeled proteins in the different fractions of the gradients were analyzed by SDS-PAGE and quantified by densitometric scanning of the autoradiography. In the absence of Alu RNA, the two fusion proteins and the heterodimer SRP9/14 were found predominantly in fractions 3 and 4 (Fig. 4, dashed lines). Upon addition of Alu RNA, a large portion of all three proteins, SRP9/14, SRP9-14 and SRP14-9, were shifted into fractions 6 and 7 demonstrating their association with the Alu RNA (Fig. 4, solid lines) and also indicating that the number of molecules contained in the RNA−protein complexes was the same.

To investigate the molecular composition of a the RNA−protein complex, we engineered the fusion protein gene (SRPϕ14-9). It comprises, in addition to SRP9 and SRP14 sequences, a bacteriophage T7 derived, short peptide (ϕ-epitope) at its N-terminus (see Material and Methods), which is recognized by specific antibodies. We argued that if a RNA−protein complex formed in the presence of equimolar amounts of SRPϕ14-9 and SRP14-9 proteins, and if it was comprised of two protein molecules, an antibody against the ϕ-epitope of SRPϕ14-9 should co-immunoprecipitate SRP14-9.

Both proteins, SRP14-9 and SRPϕ14-9, were synthesized individually in bacteria in the presence of [^{35}S]methionine (see Materials and Methods). The two proteins were combined in equimolar amounts, assuming that both proteins had the same specific radioactivity, and bound to SRP RNA. We chose to use SRP RNA in these experiments for complex formation to obtain a better separation of free and complexed proteins in sedimentation on sucrose gradients. Indeed, we found most of the RNA−protein complex migrating in fractions 8 and 9, whereas the migration of the proteins alone remained the same (data not shown). The proteins in the fractions containing the RNA−protein complex (8 and 9), and as a negative control the free proteins (fractions 3 and 4), were immunoprecipitated with antibodies against the ϕ-epitope. We found that SRP14-9 was not co-precipitated with SRPϕ14-9 by the ϕ-antibodies in any fraction assayed containing either the RNA−protein complex or the proteins alone (Fig. 5). These results demonstrated the equimolar composition of the SRPϕ14-9−RNA complex.

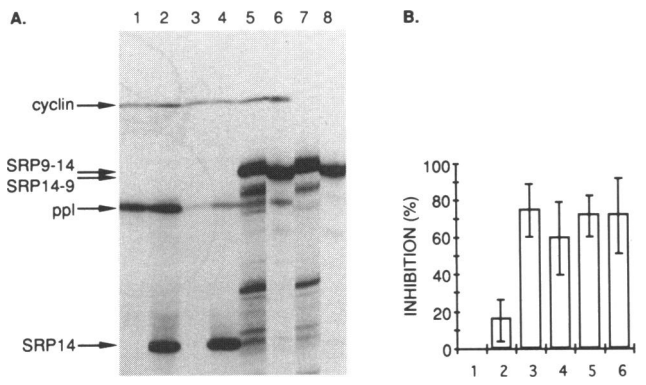


Figure 6. Elongation arrest activity of SRP particles comprising the fusion proteins SRP9-14 and SRP14-9. **A.** Completely reconstituted SRP (lane 3), SRP(-14) (lanes 1 and 4) and SRP(-9/14) (lanes 2, 5 and 6) were added at a final concentration of 35 nM to 10 μl translation reactions primed with synthetic SRP14 RNA (lanes 2 and 4), with SRP9-14 RNA (lane 5), with SRP14-9 RNA (lane 6). No exogenous RNA was added to the reactions shown in lanes 1 and 3. After incubation for 1 hour at 26°C, 10 μl of a fresh translation reaction programmed with synthetic preprolactin and cyclin transcripts was added to each sample. After an additional 25 min, the reactions were stopped and the proteins visualized by SDS-PAGE and autoradiography. Lanes 7 and 8 show SRP9-14 and SRP14-9 synthesized in the absence of all other components. **B.** Quantification of the elongation arrest activity. The relative amount of preprolactin as compared to cyclin in the presence of SRP(-14) was taken as a standard for non-inhibition.

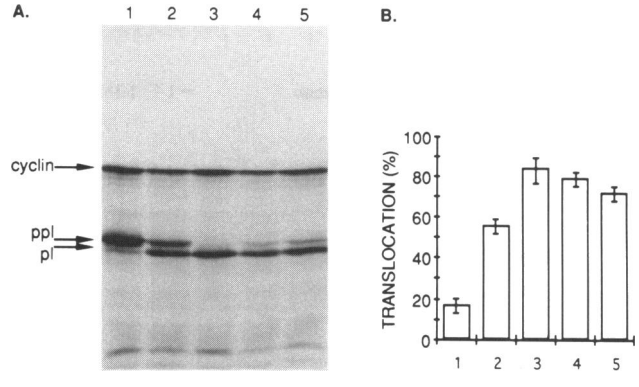


Figure 7. Translocation efficiency of a SRP particle comprising the fusion protein SRPϕ14-9. **A.** Canine SRP (lane 3), SRP completely reconstituted with canine proteins and the purified murine heterodimer SRP9/14 (lane 4), SRP completely reconstituted with canine proteins and the fusion protein SRPϕ14-9 (lane 5) and SRP(-9/14) (lane 2) were each added at a final concentration of 50 nM to translation reactions programmed with synthetic cyclin and preprolactin mRNAs and complemented with one equivalent of salt extracted microsomes (EKRMs, see 32). Synthesis of preprolactin and cyclin without SRP is shown in lane 1. **B.** Quantification of the translocation efficiency.

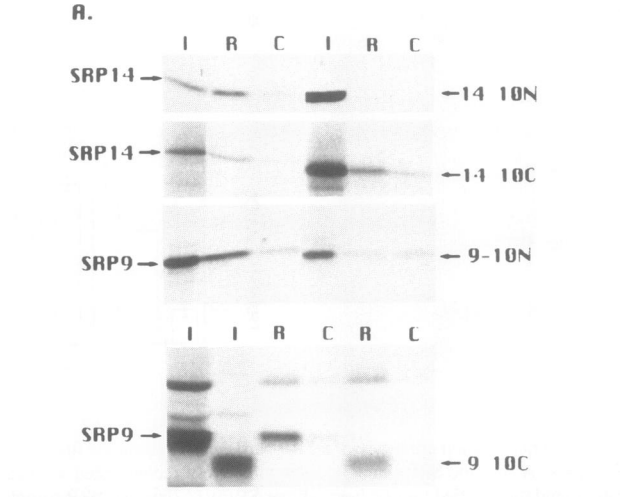
The two fusion proteins retain biological activity

Since the two fusion proteins bound to SRP RNA, we wanted to determine whether they could functionally replace the heterodimer SRP9/14 in canine SRP. Canine SRP can be separated under non-denaturing conditions into five fractions, SRP68/72, SRP54, SRP19, SRP9/14 and SRP RNA. The five fractions together reconstitute a functional SRP. In addition, a partially reconstituted SRP particle, lacking SRP9/14 (SRP(-9/14)), is deficient in elongation arrest activity (20). Using the same procedure, we have reconstituted complete and partial SRP particles which lacked either SRP9/14 (SRP-9/14) or only SRP14 (SRP-14). In our experiments, the canine SRP9/14 was replaced by highly purified murine SRP9 and SRP14 (26) and by *in vitro* synthesized SRP9-14 and SRP14-9, respectively. The elongation arrest activities of the particles were assayed by determining the specific inhibition of the synthesis of a secretory protein, preprolactin, as compared to the synthesis of a cytoplasmic protein, cyclin (Material and Methods, (14). After display of the [^{35}S]labeled proteins by SDS-PAGE and autoradiography (Fig. 6A), the relative amounts of preprolactin and cyclin were determined by densitometry and the specific decrease in preprolactin synthesis as compared to cyclin synthesis was calculated for each sample (see material and methods). The inhibitory effects of the different particles were compared to an SRP particle lacking SRP14, SRP(-14), the particle we chose as our standard for non-inhibition (Fig. 6, lane 1). As expected from their RNA-binding characteristics, we had previously observed that SRP9 and SRP14 alone cannot complement SRP(-9/14) for its lacking elongation arrest activity (results not shown). As compared to SRP(-14), we observed a small inhibitory effect of SRP(-9/14) complemented with SRP14 (Fig. 6, lane 2).

The positive control, completely reconstituted SRP (Fig. 6A, lane 3), specifically reduced the relative amount of preprolactin synthesized as compared to the non-inhibition controls. Furthermore, SRP particles reconstituted with either of the two *in vitro* synthesized fusion proteins, SRP9-14 (lane 5) or SRP14-9 (lane 6), also specifically inhibited the synthesis of preprolactin. As a control, the *in vitro* synthesized SRP9-14 and SRP14-9 proteins that were used for reconstitution, are shown in Fig. 6, lanes 7 and 8. These results demonstrated that both fusion proteins can functionally replace the heterodimer SRP9/14 in elongation arrest activity of the particle.

The arrest in the elongation of the nascent preprolactin chains is released upon interaction of SRP with the SRP receptor (docking protein) and translocation into microsomes occurs co-translationally. The molecular basis of the interactions that trigger release, is as yet unknown. We wanted to ascertain that SRP9-14 and SRP14-9 fusion proteins were also functional in the release of the arrest. However, translocation of preprolactin into microsomes was very inefficient, after reconstitution of SRP particles with *in vitro* synthesized SRP proteins. We therefore decided to use purified fusion proteins overexpressed in bacteria for the reconstitution of SRP particles *in vitro* . The fusion protein SRPϕ14-9 described before was produced in bacteria and purified to homogeneity (see Material and Methods). In contrast, due to rapid degradation, we did not succeed in producing SRP9-14 in sufficient amounts in *E.coli*, and it could therefore not be examined.

Canine SRP proteins and SRP RNA were used together with murine SRP9/14 and SRPϕ14-9 to reconstitute SRP particles *in vitro* . Their translocation activity was determined in wheat germ lysate programmed with preprolactin and cyclin transcripts and complemented with canine microsomes. As already described by (20), SRP-9/14 has a reduced translocation efficiency as compared to authentic canine SRP or to completely reconstituted SRP (Fig. 7, lane 2, 3 and 4). It could be explained by the fact that in absence of elongation arrest, the nascent chain–ribosome–SRP complex only has a limited time window to establish a functional interaction with the microsomes for translocation to occur. Importantly, the defect of SRP(-9/14) was



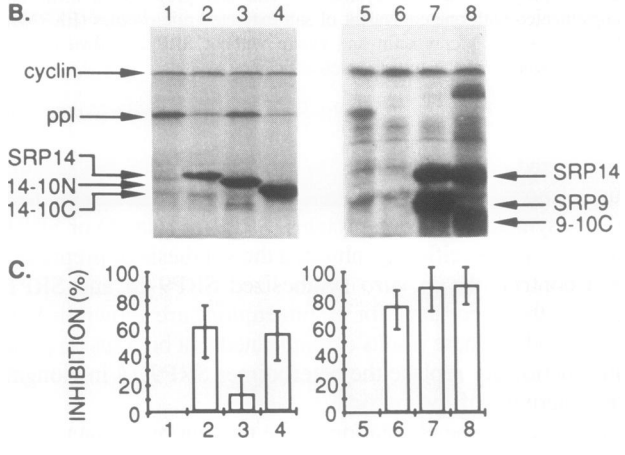


Figure 8. RNA-binding and elongation arrest activities of truncated SRP9 and SRP14 proteins. **A.** I: Amount of proteins used in the RNA-binding assay. R: Proteins bound to biotinylated Alu RNA. C: Proteins bound to a control RNA. Control RNA: A biotinylated transcript of 368 nucleotides representing the antisense strand of the SRP14 coding region (17). **B.** Preprolactin and cyclin synthesized in the presence of SRP(-14) (lane 1), of SRP(-9/14) (lane 5), of SRP(-14) complemented either with *in vitro* synthesized SRP14 (lane 2), or with SRP14-10N (lane 3) or with SRP14-10C (lane 4) and SRP(−9/14) complemented with either *in vitro* synthesized SRP9 and SRP14 (lane 7), or with SRP9-10C (lane 8). Preprolactin and cyclin synthesis in the presence of SRP (lane 6). **C.** Quantification of the elongation arrest activity.

rescued upon the addition of SRPϕ14-9 (Fig. 7, lane 5) indicating that the fusion protein SRP14-9 is functional in the release of the elongation arrest.

Additional sequences at the C-termini of SRP9 and SRP14 are dispensable for RNA-binding and elongation arrest activity

The fact, that N- and C-termini of SRP9 and SRP14 proteins could be located within the fusion protein without loss of function indicated that the normal termini of both proteins are not essential structural elements for SRP9/14 functions. These findings prompted us to examine whether additional amino acids at both ends of SRP9 and SRP14 proteins were also dispensable for function.

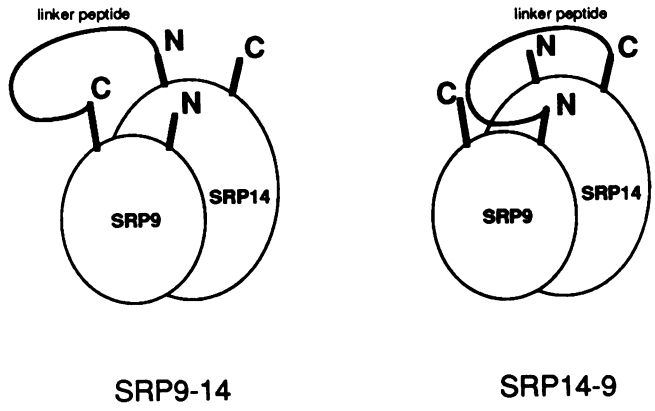


Figure 9. Model for a hypothetical fusion protein structure.

We engineered SRP9 and SRP14 cDNAs which encode truncated proteins lacking amino acids 2 to 11 at the N-termini and 10 amino acids at their C-termini. All proteins were synthesized in wheat germ lysate and the translation reactions of the SRP14 and SRP9 variants were complemented with purified SRP9 produced in bacteria and with unlabeled SRP14 synthesized *in vitro* , respectively. To assay their RNA-binding activity, the protein samples were incubated with biotinylated Alu-RNA, and streptavidin beads were used to remove the RNA-bound proteins from the free proteins. The proteins in the different fractions were displayed by SDS-PAGE followed by autoradiography. The truncated proteins SRP9-10C and SRP14-10C bound together with murine SRP14 and SRP9, respectively, specifically to Alu RNA as well as the positive control, the heterodimer SRP9/14 (Fig. 8A). Only weak non-specific binding to the biotinylated control RNA was observed for all protein samples. Thus, ten amino acids at the C-termini of SRP9 and of SRP14 are dispensable for RNA-binding. In contrast, truncated proteins lacking ten amino acids at the N-termini of SRP9 and SRP14 did no longer bind to Alu RNA and were therefore found in the fractions containing the free proteins (Fig. 8A).

We then examined whether the truncated SRP9 and SRP14 proteins that bound to Alu RNA could also confer elongation arrest activity to SRP-9/14. The same experimental procedure was used as described before. Complementation of SRP(-9/14) with SRP14 and SRP9-10C and, as a positive control, with SRP14 and SRP9, respectively, restored elongation arrest activity of the particle (Fig. 8B, lanes 7 and 8). In addition, *in vitro* synthesized SRP14-10C rescued elongation arrest activity of SRP(-14) as well as SRP14 (Fig. 8B, lanes 2 and 4). As expected, SRP14-10N which together with murine SRP9 did not bind to Alu RNA, also failed to restore elongation arrest activity of SRP(-14) (FIG.8, lane 3).

DISCUSSION

We have shown that SRP9 and SRP14 proteins, which function as a heterodimeric subunit in SRP, can be replaced by two fusion proteins comprising SRP9 and SRP14 primary sequences in two possible permutations. These findings imply that the two

polypeptide chains need not be separate entities in order to generate their biological activities.

Circularly permuted molecules (for review see ref. 27) have been used to study folding and biological functions of DNA, RNA and proteins. They are defined as isomeric molecules in which the normal termini have been connected and new termini were created. If the normal termini are critical for function and/or the new termini disrupt critical structures, these molecules are expected to have lost their activities. In generating the fusion proteins SRP9-14 and in SRP14-9, we did not introduce new termini. Rather, we connected all normal termini in one or the other fusion protein molecule, without loss of activity. These findings demonstrate that the C-termini and the N-termini in both proteins have no essential role in folding, in RNA-binding and in conferring biological activity to SRP. This interpretation was further substantiated by the finding that ten additional amino acids at the C-termini of both proteins were dispensable for all functions. In contrast, residues within the first ten amino acids of both proteins appear to be important for folding and/or binding to SRP RNA.

The sedimentation rate of SRP indicated a molecular weight of about 250 000, consistent with an equimolar composition of all subunits (28). While this assumption was save for the large subunits of SRP, it remained rather uncertain for the two smallest polypeptides SRP9 and SRP14 which contribute only little to the total molecular weight of the particle. However, our results further corroborate this assumption. They demonstrate a monomolecular composition of RNA and protein moieties for the fusion protein SRPϕ14-9 and, taking into consideration the similar sedimentation rates of all complexes, strongly suggest equimolar binding of the heterodimer SRP9/14 to SRP RNA.

Considering the small size of both polypeptides, SRP9 and SRP14, it came as a surprise that both fusion proteins could fold into a biologically active heterodimer-like structure. Our findings could certainly be explained in several ways since, theoretically, the linking peptide could span the extreme poles of a hypothetical globular structure of the heterodimer. However, we find one model particularly attractive. This model assumes a hypothetical structure for SRP9/14 in which the N- and C-termini of each protein are located in proximity (Fig. 9). In such a hypothetical structure, the linking peptide constitutes a separate domain and would therefore not be expected to interfere with SRP9/14 functions by steric hindrance. In addition, its overall position relative to the rest of the fusion protein molecules is similar in both single polypeptide chain variants.

The construction of genes that encode oligomeric proteins in a single polypeptide has recently found several applications. The possibility to express the variable regions of the heavy and light chains of immunoglobulins, which constitute the antigen binding site, as a single fusion molecule has allowed to produce minimal synthetic antibody molecules in bacteria which have similar affinities for the antigen as the parent immunoglobulins (29–31). The gene V protein of bacteriophage f1 binds to single-stranded DNA and to RNA as a homodimer. A fusion protein consisting of two gene V protein molecules has an enhanced stability and rate of folding. This suggests that oligomeric complexes can be thermodynamically stabilized when expressed as a single polypeptide chain.

The possibility to express the heterodimer SRP9/14 as a single polypeptide chain will certainly greatly facilitate the analysis of its structure and functions.

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