

A truncation in the 14 kDa protein of the signal recognition particle leads to tertiary structure changes in the RNA and abolishes the elongation arrest activity of the particle

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ABSTRACT

The signal recognition particle (SRP) provides the molecular link between synthesis of polypeptides and their concomitant translocation into the endoplasmic reticulum. During targeting, SRP arrests or delays elongation of the nascent chain, thereby presumably ensuring a high translocation efficiency. Components of the Alu domain, SRP9/14 and the Alu sequences of SRP RNA, have been suggested to play a role in the elongation arrest function of SRP. We generated a truncated SRP14 protein, SRP14–20C, which forms, together with SRP9, a stable complex with SRP RNA. However, particles reconstituted with SRP9/14–20C, RC(9/14–20C), completely lack elongation arrest activity. RC(9/14–20C) particles have intact signal recognition, targeting and ribosome binding activities. SRP9/14–20C therefore only impairs interactions with the ribosome that are required to effect elongation arrest. This result provides evidence that direct interactions between the Alu domain components and the ribosome are required for this function. Furthermore, SRP9/14–20C binding to SRP RNA results in tertiary structure changes in the RNA. Our results strongly indicate that these changes account for the negative effect of SRP14 truncation on elongation arrest, thus revealing a critical role of the RNA in this function.

INTRODUCTION

In cells, secretory and membrane proteins have to be translocated into the endoplasmic reticulum (ER) for their final dispatching. The signal recognition particle (SRP) is a cytoplasmic ribonucleo-protein complex that is a crucial component in this process. On the basis of biochemical studies, a model of the functional cycle of mammalian SRP has been elaborated (for recent reviews 1–3). SRP recognizes and binds co-translationally to signal sequences present in nascent chains of secretory and membrane proteins. This interaction causes an arrest or a delay in elongation of the nascent chains. The SRP–nascent chain–ribosome complex is

then targeted to the ER via an interaction between SRP and an ER membrane protein complex, the SRP receptor. Once the nascent chain–ribosome complex becomes bound to the translocon, the SRP–SRP receptor complex is released from the ribosome, protein synthesis resumes and the protein is translocated co-translationally across or into the ER membrane. SRP then dissociates from the receptor and is free to engage in another targeting round. SRP-mediated targeting is regulated by GTP binding and hydrolysis of three GTPases, SRP54 and both subunits of the SRP receptor. GTP binding of SRP54 was found to be stimulated by a ribosomal component (4), indicating a regulatory link between translation and translocation.

Mammalian SRP is composed of an RNA molecule of 300 nt and six polypeptides named according to their molecular weight. Reconstitution of subparticles *in vitro* has allowed assignment of the signal recognition and targeting functions to specific components of SRP. SRP(S), a subparticle composed of SRP54, SRP19, SRP68/72 and the central domain of SRP RNA, promotes co-translational translocation of proteins (5), whereas the same subparticle without SRP68/72 allows constitutive translocation of elongation-arrested nascent chains (6).

Elongation arrest activity of SRP requires the presence of all SRP subunits and is detected as a complete arrest or as a pause in elongation of nascent chains bearing a signal sequence in a wheat germ translation system. The arrest or pause is released in the presence of salt-washed mammalian microsomes to allow translocation of the nascent polypeptide. In reticulocyte lysate, SRP effects a kinetic delay in the accumulation of full-length polypeptide (7). Molecular analysis of the translation process has shown that ribosomes pause naturally at specific sites on mRNAs and that SRP enhances pausing at these sites during synthesis of ER-targeted proteins (8). A mathematical model describing the effects of SRP on translocation predicts that an extended pause in elongation would suffice to attach ribosomes with maximal efficiency to the ER membrane *in vivo*, thereby presumably preventing misfolding and aggregation of newly synthesized polypeptides into a translocation-incompetent form (9).

Elongation arrest activity implies a direct interaction between the translational machinery and SRP. *In vitro*, two modes of interaction between SRP and ribosomes can be distinguished

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based on their different sensitivities to salt (6,10). The formation of high salt-resistant complexes is due to interactions between SRP54 and the signal sequence. Formation of complexes that are stable at physiological and lower salt concentrations requires complete SRP and ribosomes with or without a nascent chain. Thus, these complexes reveal direct interactions between the ribosome and SRP. Interestingly, SRP54 can be cross-linked to a non-functional signal sequence and can be loaded with GTP by a ribosomal component in these complexes (4,6), suggesting that positioning of SRP on the ribosome is independent of the presence of a functional signal sequence. Genetic experiments confirmed a direct interaction between SRP and the ribosome and indicated that it occurs at a specific step in the elongation cycle, just before the peptidyl-tRNA undergoes translocation from the A to the P site of the ribosome (11).

How SRP interacts with the ribosome to effect elongation arrest remains to be elucidated. Since elongation arrest activity can only be observed with complete SRP, positive evidence for the specific role of a SRP subunit in this activity cannot be obtained by reconstituting subparticles. The Alu domain of SRP, composed of the Alu sequences at the 5'- and 3'-ends of SRP RNA and the heterodimer SRP9/14, has been suggested to play an essential role in this function based on the observation that its removal or removal of the heterodimer SRP9/14 abolishes the elongation arrest function of the particle (12,13). The same particles were later found to be defective in low salt, signal sequence-independent binding to ribosomes (6,10). This finding may indicate an important role of the Alu domain in ribosome binding or may reveal a more general defect of the particle which may indirectly account for the observed loss of elongation arrest activity.

To define more conclusively the components and the nature of the interactions essential for elongation arrest function, we decided to search for a mutant SRP9/14 protein that specifically interferes with elongation arrest activity of the particle. Mammalian SRP9 and SRP14 proteins form a stable heterodimer and it is the heterodimer which binds specifically to SRP RNA (14). Here we report the characterization of a truncated SRP14 protein which assembles efficiently into SRP and concomitantly abolishes elongation arrest activity of the reconstituted particles. All other functions of the mutant particle comprising the truncated SRP14 protein are the same as for particles comprising wild-type SRP14. Our results provide convincing evidence for a direct role of the Alu domain in effecting elongation arrest. Using hydroxyl radicals as a probe for RNA structure, we found that the heterodimer comprising the truncated SRP14 protein induces changes in the structure of SRP RNA as compared with the wild-type complex. This finding suggests a model in which truncation of the SRP14 protein interferes with elongation arrest activity by changing the tertiary structure of the Alu portion of SRP RNA.

MATERIALS AND METHODS

SP6 RNA polymerase and RNase inhibitor (RNasin) were purchased from Biofinex and DNA ligase from New England Biolabs. Restriction enzymes were obtained from New England Biolabs and Fermentas. The T4 polynucleotide kinase was bought from Fermentas. The plasmids pET-3a, pET-3b and pET-9a were from Invitrogen. Ribonucleotide triphosphates were obtained from Pharmacia LKB Biotechnology and [γ - 32 P]ATP (5000 Ci/mmol) and [35 S]methionine (1500 Ci/mmol) from

Amersham Life Science. Avian myeloblastosis virus reverse transcriptase and *Escherichia coli* tRNA were purchased from Boehringer Mannheim. CM and heparin-Sepharose resins were obtained from BioRad.

Expression, purification and RNA binding assay of the recombinant heterodimers and SRP19

For expression of SRP9/14 and SRP9/14–20C in *E.coli*, we engineered pET plasmids (15) that carry the coding regions for the two polypeptides in two independent T7 transcription units arranged in tandem on the same plasmid. To this end, we introduced an *Nde*I site at the initiator ATG of SRP14–20C cDNA by the polymerase chain reaction (PCR) and ligated the amplified fragment, which was digested with *Nde*I and *Bam*HI, into the linearized plasmid pET-9a (pE14–20C). The SRP14–20C cDNA and the adjacent T7 promoter and terminator sequences were then amplified from the pE14–20C plasmid using an oligonucleotide complementary to vector sequences comprising the *Bgl*III site and another oligonucleotide that introduces a *Bcl*I site at the *Eco*RV site in pET-9a. The amplified DNA was digested with *Bcl*I and *Bgl*III and inserted into the *Bgl*III site of the pE9C plasmid (16), resulting in plasmid pE14–20C-9dim. A similar construct was made for expression of SRP9 and SRP14 and is described elsewhere (17). SRP9 was more highly expressed than SRP14 from both plasmids. For expression of SRP19, we introduced a *Nde*I restriction site at the initiator ATG of the SRP19 cDNA by PCR using plasmid pG19 (18). The amplified fragment was digested with the restriction enzymes *Nde*I and *Bam*HI and inserted into pET-3b linearized with the same enzymes. The coding regions of all expression plasmids were sequenced. The two heterodimers SRP9/14 and SRP9/14–20C and SRP19 were expressed for 3 h in BL21(DE3) cells by the addition of 1 mM IPTG. Bacterial pellets were resuspended in 50 mM Tris, pH 7.5, 50 mM sodium chloride, 10 mM EDTA, 10% glycerol, 10 mM magnesium chloride, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 0.5 μ g/ml aprotinin and 500 mM ammonium chloride was added before lysing the bacteria in a French press. After lysis, the ammonium chloride concentration was adjusted to 250 mM and cell debris was removed by centrifugation at 4°C for 10 min at 30 000 g. Polymyxin P was added at a final concentration of 0.05%, nucleic acids were allowed to precipitate for 30 min at 4°C and then removed by centrifugation at 30 000 g for 20 min. Proteins were purified from the supernatants by heparin and CM chromatography. The excess of SRP9 over SRP14–20C was removed from the heterodimer on the CM column. Fractions containing the purified proteins were pooled and dialyzed against 500 mM potassium acetate, pH 7.5, 20 mM HEPES–KOH, pH 7.5, 5 mM magnesium acetate, 0.01% Nikkol, 10% glycerol and 1 mM DTT (HKMD^N + glycerol). The proteins were quantified by comparison with canine SRP on a Coomassie stained gradient SDS–polyacrylamide gel. For complex formation, the two components (45 pmol SRP RNA and 30 pmol SRP9/14 or SRP9/14–20C) were combined in 50 μ l HKMD^N containing 200 mM potassium acetate under reconstitution conditions. The complexes were fractionated on linear 10–31% glycerol gradients at 40 000 g for 15 h. The RNA was analyzed on 6% urea–polyacrylamide gels and visualized by ethidium bromide staining. The proteins were analyzed by SDS–PAGE and SRP14 visualized by immunoblotting with affinity purified anti-SRP14 antibodies. The recombinant SRP19 protein is shown

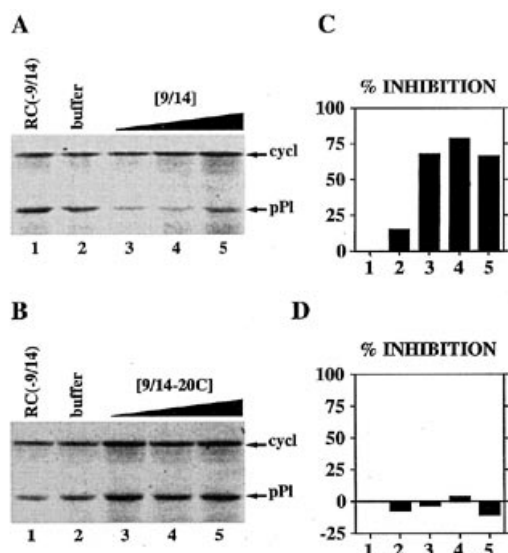


Figure 2. Heterodimer SRP9/14-20C fails to confer elongation arrest activity to the particle. SRP was reconstituted in the presence of increasing amounts of the recombinant heterodimers SRP9/14 (A) and SRP9/14-20C (B). The concentration of the partially reconstituted SRP lacking 9/14 [RC(-9/14)] was 37.5 nM in the translation reactions shown in lanes 1 and 3-5. The concentrations of the recombinant heterodimers were 0 (lane 1), 37.5 (lane 3), 112.5 (lane 4) and 375 (lane 5) nM. The translation reactions shown in lane 2 were complemented with SRP buffer. (C and D) Relative inhibition of accumulation of preprolactin as compared with cyclin. The ratio of preprolactin to cyclin in lane 1 was taken as 0% inhibition.

the sample and P_0 and C_0 are the amounts of preprolactin and cyclin synthesized in the absence of elongation arrest (SRP buffer or SRP9/14); $T = 100 \times P/(pP + P)$, where T is percent translocation, P is the amount of prolactin and pP is the amount of preprolactin (5). All calculated values represent the average of two or more independent experiments. The experimental error is $\pm 10\%$ in the elongation arrest assay.

Ribosome binding assay

Binding assays were performed as described (10). Truncated transcripts encoding the first 143 amino acids of PAI-2 (-hB, I₈/I₁₄ in 21) (PAI-2/143) and encoding the first 130 amino acids of cyclin (cycl/130) were generated by digestion of PAI-2-I₈ I₁₄ (pDB5909) and cyclin (pcyclin) cDNAs with *Ssp*I and *Pst*I respectively, followed by transcription with SP6 RNA polymerase. Wheat germ translations (10 μ l) containing [³⁵S]methionine were performed in the absence and presence of the truncated transcripts PAI-2/143 and cycl/130 for 20 min at 26°C. The stable ribosome-nascent chain complexes were purified by pelleting through a sucrose cushion (150 μ l) containing 50 mM HEPES, pH 7.5, 500 mM KOAc, 2.5 mM Mg(OAc)₂, 1 mM DTT, 0.01% Nikkol, 0.25 mM CH and 0.5 M sucrose. After centrifugation in a Centrikon TFT 80.2 rotor at 70 000 r.p.m. for 60 min at 4°C, the pellets were resuspended in 10 μ l buffer (RSB) containing 50 mM HEPES, pH 7.5, 50 mM KOAc, 2.5 mM Mg(OAc)₂, 1 mM DTT, 0.01% Nikkol, 0.25 mM CH. The supernatants were kept for analysis (S1). Reconstituted SRP samples (1 pmol) were added to the ribosomes and incubated for 10 min at 26°C and for 1 h on ice. The volumes of the samples were adjusted to 50 μ l using RSB (final [KOAc] = 70 mM) prior to centrifugation through a 150 μ l

sucrose cushion as described above. The supernatants (S1 and S2) and the pellets (P) were analyzed by 12 and 15% SDS-PAGE. Nascent chains were visualized by autoradiography and proteins were detected by immunoblotting with anti-SRP68 and anti-SRP14 antibodies. Western blots were revealed using the Enhanced Chemiluminescence system (Amersham).

Hydroxyl radical cutting reactions

The cleavage reactions were carried out as described (19). For milder cleavage reaction the concentrations of ferrous ammonium sulfate and EDTA were reduced to one half, 12.5 and 25 mM respectively. SRP RNA (0.1 pmol) and SRP9/14 were combined at three different RNA:protein ratios (1:5, 1:10 and 1:50) in 3 μ l HKMD^N buffer. In the negative control, SRP9/14 was replaced by the same amount in weight of bovine serum albumin (BSA). RNA fragments were phenol/chloroform extracted and ethanol precipitated. Pellets were resuspended and annealed to 0.3 pmol ³²P-labeled primer in 12 μ l annealing buffer (AB) containing 50 mM Tris, pH 8.5, 30 mM KCl for 3 min at 65°C and 30 min at 42°C. Reverse transcription was carried out by combining 2 μ l primer/template sample, 1 μ l 2 mM dNTPs in AB, 1 μ l ddNTPs or AB (either 0.4 mM ddATP, 0.4 mM ddGTP, 0.2 mM ddCTP or 0.125 mM ddTTP for the sequencing reactions) and 1 μ l reverse transcriptase (1.6 U) in 50 mM Tris, pH 8.5, 40 mM MgCl₂, 150 mM KCl, 5 mM DTT. After an incubation of 1 h at 50°C, reactions were stopped by addition of 4 μ l denaturing sample buffer and the extension products separated on 8% sequencing gels.

RESULTS

SRP9/14-20C competes with SRP9/14 for binding to SRP RNA and lacks the capacity to confer elongation arrest activity to the particle

To examine the role of SRP9/14 in the elongation arrest function of SRP, we decided to search for mutant SRP9 and SRP14 proteins that had specifically lost their capacity to confer elongation arrest activity to the particle. However, the altered proteins would still dimerize with the partner protein and form stable complexes with SRP RNA. A previous analysis had shown that 10 amino acids at the C-termini of murine proteins SRP9 and SRP14 were dispensable for RNA binding and for elongation arrest activity of the particle (22). Analysis of the RNA binding capacities of truncated SRP14 proteins (36), together with experiments in which the truncated SRP14 proteins were assayed for their capacities to confer elongation arrest activity to SRP particles (results not shown), indicated that the heterodimer comprising an SRP14 protein lacking the C-terminal 20 amino acids, SRP9/14-20C, may have the wanted phenotype. Hence, we decided to produce large amounts of the heterodimers SRP9/14-20C and, as a positive control, SRP9/14 in bacteria to study the effect of SRP9/14-20C on SRP functions in more detail.

To express SRP14-20C, we introduced a stop codon into the SRP14 cDNA which replaced Met91 in the SRP14 protein (Fig. 1A). This truncation removes a highly conserved region in SRP14. SRP9/14-20C and SRP9/14 were overproduced in bacteria by simultaneous expression of SRP9 and SRP14 proteins from two independent, tandemly arranged, T7 polymerase-controlled transcription units (Materials and Methods). SRP9 was more highly expressed than SRP14 from both plasmids. The

purified proteins SRP9/14 and SRP9/14–20C are shown in Figure 1. Mouse SRP9 co-migrated with canine SRP9, whereas mouse SRP14 migrated slightly slower than canine SRP14 (Fig. 1B, lanes 2 and 3), possibly, because it still contains the initiator methionine which is removed in canine SRP14 (23). The SRP9 and SRP14–20C proteins migrated in close proximity (Fig. 1C, lane 2). However, the SRP14–20C protein could be visualized individually by immunoblotting with affinity purified anti-SRP14 antibodies (Fig. 1C, lane 4). The heterodimers were assumed to have an equimolar composition based on the following criteria: (i) equal intensities of Coomassie stained SRP9 and SRP14 protein bands; (ii) the excess of SRP9 was removed during ion exchange chromatography (Materials and Methods); (iii) no excess of free SRP14 protein could be detected in the RNA binding experiments discussed below.

To confirm the RNA binding activity of the recombinant heterodimer, SRP9/14–20C was bound at 200 mM potassium acetate to SRP RNA and the free and RNA-bound proteins were separated on 10–31% glycerol gradients. Based on the purification procedure, we could not entirely exclude the possibility that the heterodimeric protein samples contained an excess of SRP14 protein. In addition, SRP9 and SRP14–20C co-migrate on SDS–PAGE. We therefore chose to analyze the proteins by immunoblotting with anti-SRP14 antibodies. The RNA content was revealed by ethidium bromide staining (Fig. 1D and E). SRP9/14–20C and the positive control SRP9/14 co-migrated with SRP RNA in fractions 8–11, demonstrating that they were bound to the RNA. Free proteins migrate in the uppermost fractions in these gradients (24), whereas free RNA migrates at a similar position to the RNA–protein complex because of the small difference in molecular mass between free RNA and the complex (100 and 122 kDa respectively). Thus, the RNA:protein ratio in the different fractions was determined by the presence of free RNA and RNA–protein complex. The apparent absence of SRP RNA in the flanking fractions where the protein could be detected is most likely explained by a lower sensitivity of detection of the RNA. As expected from the previous findings that neither protein alone binds to SRP RNA (14), we detected a single broad protein band co-migrating with SRP RNA in silver stained gels of SRP9/14–20C glycerol gradients; consistent with co-migration of SRP9 and SRP14–20C.

The recombinant proteins were then used in complementation and competition experiments. Mammalian SRP can be reconstituted *in vitro* from the canine proteins SRP68/72, SRP54 and SRP19, murine SRP9/14 and *in vitro* synthesized SRP RNA (16). The elongation arrest activities of the reconstituted particles was subsequently assayed by quantifying their specific effect on synthesis of a secretory protein standardized to synthesis of a cytosolic protein. In the following reconstitution experiments, canine SRP19 was substituted by human SRP19 produced in bacteria (see Materials and Methods and Fig. 1B). Equimolar amounts of SRP components were combined in the presence of increasing concentrations of either SRP9/14 or SRP9/14–20C and the reconstituted particles were added to translation reactions programed with synthetic preprolactin and cyclin transcripts to determine elongation arrest activity (Fig. 2). As a negative control, the translation reactions contained either buffer or a partially reconstituted particle lacking SRP9/14, which we called RC(–9/14) to distinguish reconstituted particles from proteins. RC(–9/14) is deficient in elongation arrest activity (5). The concentrations of the SRP components in the translation reactions

was 37.5 nM, whereas the concentrations of SRP9/14 and SRP9/14–20C were equimolar (lane 3), 3- (lane 4) and 10-fold (lane 5) higher than the concentrations of the other SRP components. The ³⁵S-labeled proteins were displayed by SDS–PAGE (Fig. 2A and B) and quantified using phosphorescence imaging. The relative accumulation of preprolactin as compared with cyclin in the different samples was standardized to its relative accumulation in the presence of RC(–9/14) (Materials and Methods).

In the positive control, SRP9/14 conferred maximal elongation arrest activity to the particle at equimolar concentrations of the protein, thereby confirming the biological activity of the purified heterodimeric protein (Fig. 2A and C). Increasing concentrations of SRP9/14 had no additional effect on preprolactin synthesis. Furthermore, this effect was specific for reconstituted SRP, since in parallel control experiments, in which SRP9/14 was added to the translation reactions in the absence of RC(–9/14), the ratio between preprolactin and cyclin synthesis remained the same (results not shown). In contrast to SRP9/14, SRP9/14–20C failed to complement RC(–9/14) for its lack of elongation arrest activity, even in the presence of a 10-fold excess of the heterodimeric protein over the other SRP components (Fig. 2B and D). In some experiments we observed an increase in cyclin and preprolactin synthesis upon addition of SRP9/14–20C or of a large excess of SRP9/14 (Fig. 2A and B). However, since the ratio of cyclin to preprolactin synthesis was unchanged, it remained within the 10% error limit, as for RC(–9/14) (Fig. 2D), this effect did not interfere with interpretation of our results.

In competition experiments, SRP components, including SRP9/14, were combined at an equimolar ratio in the presence of increasing SRP9/14–20C concentrations. The SRP samples were then added to translation reactions programed with synthetic preprolactin and cyclin transcripts. We found that increasing concentrations of the competitor, SRP9/14–20C, resulted in diminished elongation arrest activity of the particle (Fig. 3). This effect was specific for reconstituted SRP, since increasing concentrations of SRP9/14–20C in the absence of RC(9/14) during translation had no effect on synthesis of full-length preprolactin and cyclin (results not shown). Hence, these results provide evidence that assembly of SRP9/14–20C into the particle caused the defect in elongation arrest function of the particle. The competition experiments also confirmed the capacity of SRP9/14–20C to bind to the RNA, albeit at a slightly reduced efficiency as compared with SRP9/14. A 5-fold excess of SRP9/14–20C over SRP9/14 was required to reduce elongation arrest activity of the particle 2-fold (Fig. 3, lanes 1 and 4). However, because of the uncertainty associated with the concentrations of the heterodimers, the exact difference in affinity was not quantified.

RC(9/14–20C) is functional in translocation and in direct, signal sequence-independent ribosome binding

Next, we wanted to examine whether particles containing SRP14–20C, RC(9/14–20C), exclusively lack elongation arrest activity or whether they have other functional defects. The three other functions of SRP that can be studied *in vitro* are the signal recognition and targeting functions and the capacity of SRP to bind to ribosomes directly in a signal sequence- and nascent chain-independent fashion.

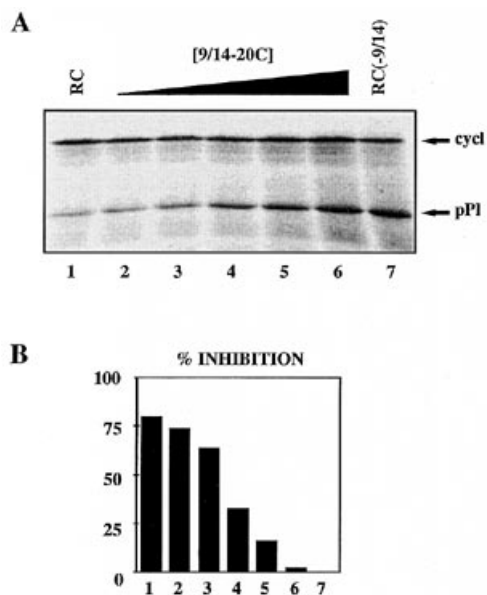


Figure 3. Heterodimer SRP9/14-20C competes efficiently with wild-type SRP9/14 for assembly into SRP and concomitantly abolishes elongation arrest activity of the particle. (A) All SRP components, including SRP9/14, were combined at 0.5 μ M each in the presence of increasing concentration of SRP9/14-20C: 0 (lane 1), 0.5 (lane 2), 1.25 (lane 3), 2.5 (lane 4), 3.75 (lane 5) and 5 (lane 6) μ M. The negative control, RC(-9/14), is shown in lane 7. The final concentration of the reconstituted particles in the translation reactions was 50 nM. (B) Relative inhibition in accumulation of preprolactin as compared with cyclin. RC(-9/14) in lane 7 was taken as a standard for 0% inhibition.

To examine the signal recognition and targeting activities of RC(9/14-20C), we determined its capacity to promote translocation. SRP(S), which comprises the S portion of SRP RNA, SRP19, SRP54 and SRP68/72, and RC(-9/14) have both been shown to be functional in signal recognition and targeting and to translocate secretory proteins into the ER co-translationally, albeit at an efficiency 50% lower than that of complete canine SRP (5,6,12). If RC(9/14-20C) was functional in signal recognition and targeting, we would expect it to promote co-translational translocation at least as efficiently as RC(-9/14). To determine its capacity to promote translocation, different SRP samples were reconstituted *in vitro* and added individually to translation reactions programmed with preprolactin and cyclin synthetic mRNAs and complemented with SRP-depleted canine microsomes. SRP-depleted canine microsomes are translocation incompetent in the absence of exogenously added SRP. The products of the translation/translocation experiments were analyzed by SDS-PAGE. The translocation efficiencies of the different samples were compared with the translocation efficiency of canine SRP (Fig. 4). The positive control, canine SRP, promoted translocation of preprolactin at an efficiency of 75%. As previously observed (25), fully reconstituted SRP, RC(9/14), was slightly less effective in promoting translocation of preprolactin than canine SRP. Notably, RC(9/14-20C) had the same translocation capacity as RC(-9/14), demonstrating that these particles are functional in signal recognition and in targeting of nascent chain-ribosome complexes (Fig. 4, lanes 2 and 4). In addition, this result confirmed the presence of SRP68/72 within the particle, since in its absence only elongation-arrested nascent chains are translocated (6).

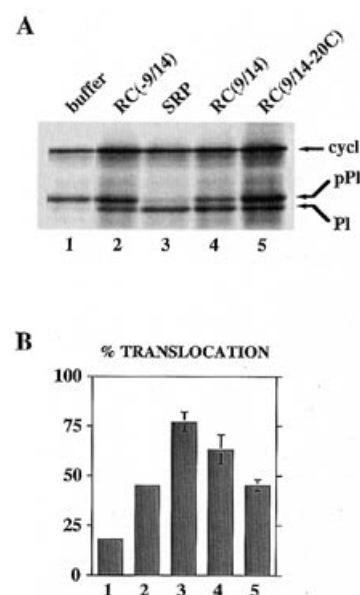


Figure 4. Signal recognition and targeting functions are intact in RC(9/14-20C). (A) Translocation of preprolactin was assayed in the presence of the different reconstituted samples as indicated at the top of each lane. SRP-depleted canine microsomes were present in all samples at 0.15 equivalents/ μ l. (B) The preprolactin (pPI) to prolactin (PI) ratio was determined as described in Materials and Methods. Buffer, SRP sample was replaced by buffer; RC(-9/14), particles lacking SRP9/14; SRP, canine SRP; RC(9/14) and RC(9/14-20C), particles reconstituted with SRP9/14 and SRP9/14-20C respectively.

Two modes of interaction between SRP and ribosomes can be distinguished by their sensitivities to salt concentration. The high salt-resistant interactions reflect signal sequence-dependent binding of SRP to ribosome-nascent chain complexes. Ribosome binding of SRP at physiological or lower salt concentrations is independent of nascent chains (10) and reflects direct interactions between the ribosome and SRP. SRP binding to ribosomes at low salt was found to be very sensitive to omission of single SRP subunits, since it required the presence of all SRP components (10). Based on these results, SRP binding to ribosomes at low salt concentrations provides a very sensitive assay to detect changes in the molecular interactions of SRP and ribosomes.

Hence, we examined the capacity of RC(9/14-20C) to bind in a nascent chain-independent fashion to ribosomes. As an alternative for a secretory protein we used plasminogen activator inhibitor-2 with an altered signal sequence, PAI-2-IgI₁₄. This protein has previously been shown to translocate efficiently *in vitro* (21). Cyclin was used as a cytoplasmic protein. The elongation-arrested nascent chains of PAI-2-IgI₁₄ and cyclin comprised 143 and 130 amino acid residues respectively. Wheat germ ribosomes alone and associated with nascent chains of PAI-2-IgI₁₄ and cyclin were purified by centrifugation through a high salt sucrose cushion. This procedure removes the nascent polypeptide-associated complex (26) as well as non-ribosomal wheat germ proteins from the ribosomes. The nascent polypeptide-associated complex has been found to modulate signal sequence-independent interactions between SRP and the ribosome (10). The supernatants of the centrifugation (Fig. 5, S1) were included in the analysis described below. The ribosomes and the nascent chain-ribosome complexes found in the pellet were resuspended

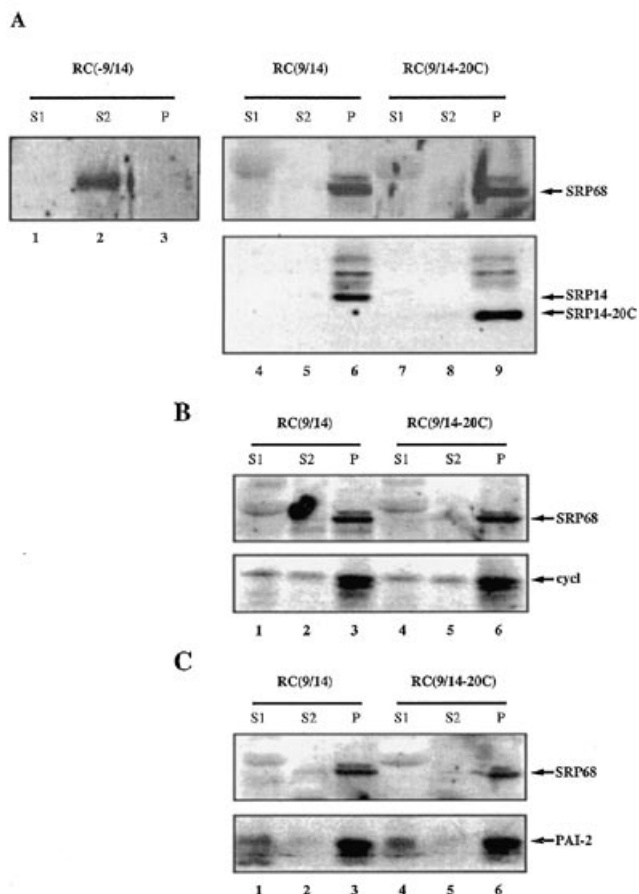


Figure 5. RC(9/14-20C) preserved its capacity to bind directly in a nascent chain-independent fashion to ribosomes. Ribosomes alone (A) and associated with truncated cyclin (B) and truncated PAI-2-IgI₄ (C) were sedimented through high salt sucrose cushions to remove NAC. Binding of different particles to salt-washed ribosomes was assayed by sedimenting the complexes through sucrose cushions at a salt concentration of 70 mM potassium acetate (Materials and Methods). S1 and S2, supernatants of first and second centrifugations; P, ribosomal pellet; Cycl and PAI-2-IgI₄, ³⁵S-labeled nascent chains of a cytosolic and a secretory protein respectively. SRP68 and SRP14 proteins were revealed by immunoblotting. RC(9/14) and RC(9/14-20C), reconstituted particles comprising SRP9/14 and SRP9/14-20C proteins respectively; RC(-9/14), particles lacking SRP9/14.

and incubated with RC(9/14) and with RC(9/14-20C) at a final salt concentration of 70 mM potassium acetate. As a negative control, we also included RC(-9/14) in the binding experiments with ribosomes alone. Binding of the different reconstituted particles to the ribosomes was monitored by centrifugation of the complexes through a sucrose cushion. Bound particles would sediment with the ribosomes into the pellet (Fig. 5, P), whereas unbound particles would be found in the low density fractions (Fig. 5, S2). Sedimentation of the ribosomes is revealed by the presence of elongation-arrested nascent chains in the pellet (Fig. 5B and C).

The different samples were displayed by SDS-PAGE and analyzed by autoradiography and by immunoblotting with anti-SRP68 and anti-SRP14 antibodies. Immunoblotting with anti-SRP68 antiserum indicated that the positive control sample, RC(9/14), and the experimental sample, RC(9/14-20C), sedimented together with the ribosomes in both the absence and presence of

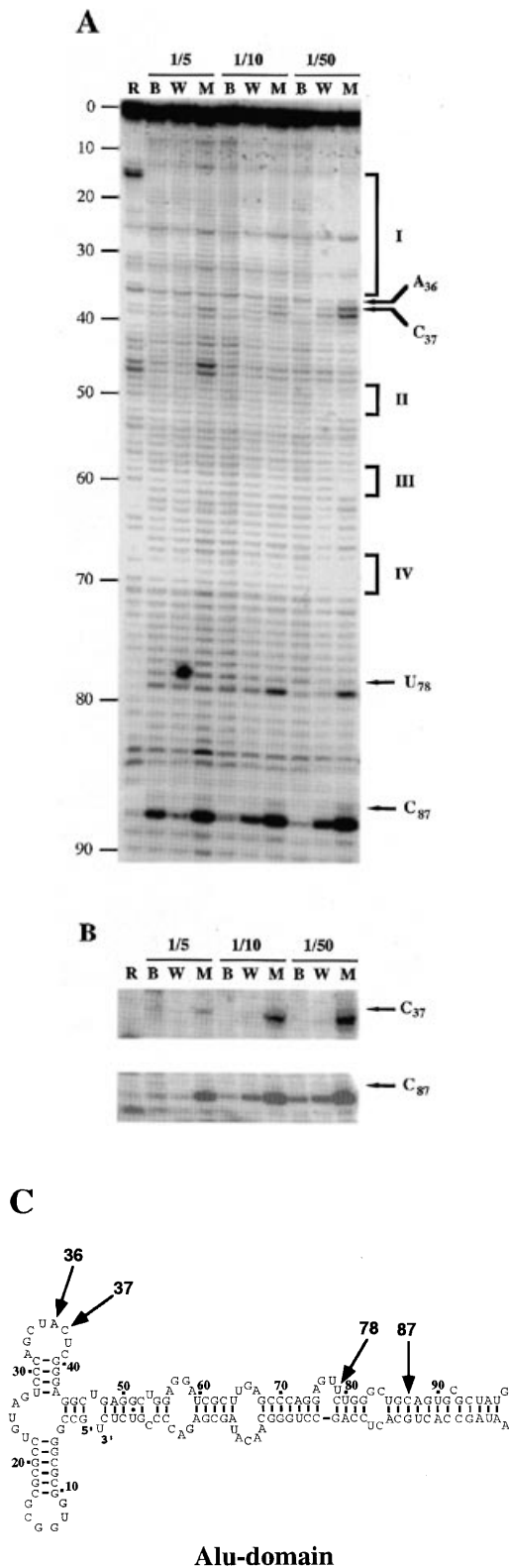
nascent chains (Fig. 5A-C). The anti-SRP68 antiserum showed cross-reactivity with a slightly larger protein in wheat germ extract (Fig. 5, S1). However, the cross-reactive protein did not sediment with the ribosomes and therefore did not interfere with the analysis. Association of the particles with the ribosomes as well as the presence of the SRP14 proteins within the particles were confirmed using affinity purified anti-SRP14 antibodies in the Western blot analysis shown in Figure 5A. The negative control, RC(-9/14), was defective in ribosome binding and was found in the supernatant of the gradient (Fig. 5A), in agreement with previous results (10).

These results demonstrated that RC(9/14-20C) and RC(9/14) have a comparable capacity to bind directly to ribosomes in a signal sequence-independent fashion. Thus, the removed region is not critical for SRP binding to ribosomes; its absence specifically impairs interactions with the ribosome that are required to effect elongation arrest.

Hydroxyl radical probing of SRP RNA complexes with SRP9/14 and SRP9/14-20C

The slightly different affinities of the two heterodimers for SRP RNA in the competition experiments suggested that subtle changes in protein-RNA interactions exist. RNA cleavage by hydroxyl radicals has previously been used to study the canine SRP9/14-SRP RNA complex (19). The hydroxyl radical cleavage reagent, which attacks the ribose backbone, is a valuable tool to probe the tertiary structure and protein binding sites in RNA (27,28), since it cleaves RNA independently of its secondary structure.

SRP9/14 and SRP9/14-20C and, as a negative control, the same amount of bovine serum albumin were incubated with SRP RNA and exposed to the hydroxyl radical cleavage reagent (Materials and Methods). The cleaved products were analyzed by primer extension with reverse transcriptase. Comparison of the cleavage patterns revealed that four sites in the Alu domain of SRP RNA became more sensitive to hydroxyl radical cleavage in the SRP9/14-20C-RNA complex as compared with the SRP9/14-RNA complex (Fig. 6A). These sites are represented by the fragments extending to positions C37/U38, C79 and A88. Hence, the nucleotides preceding the extended products, A36/C37, U78 and C87, became more sensitive to attack by hydroxyl radicals. These differences were highly reproducible and clearly visible at low protein concentration at positions C37, U78 and C87. Higher protein:RNA ratios increased the sensitivity at the same sites and revealed an additional hypersensitive nucleotide, A36. To confirm that these sites were hypersensitive to cleavage by hydroxyl radicals, we repeated the cleavage reactions choosing milder reaction conditions. Under the modified conditions, the reactivity was strongly diminished at all positions except at the hypersensitive nucleotides C37 and C87 (Fig. 6B). Again, the increased sensitivity at position A36 was exclusively observed at higher protein:RNA ratios. The U78 position was not hypersensitive to cleavage under milder conditions. We reproducibly observed a considerable background in the reverse transcription of uncleaved SRP RNA (Fig. 6A, R). Such a background has been observed for many RNA species and is commonly thought to result from premature termination of the reverse transcriptase within regions with highly stable secondary structures (28,29). However, the background does not interfere with the interpretation of our data because: (i) it relies on comparing signals of cleaved



Compared with the negative control, bovine serum albumin, the regions protected from hydroxyl radical cleavage in the presence of SRP9/14 and of SRP9/14-20C were the same, thus confirming that SRP9/14-20C binds efficiently to SRP RNA. The protected regions were also similar to those reported previously for canine SRP9/14 (19). Region I [corresponding to region II in Strub *et al.* (19)] could be observed at low protein:RNA ratios, whereas regions II and III (regions III and IV in the previous paper) could only be detected at higher protein:RNA ratios. Furthermore, we observed a protected region around nt 66-70 in SRP RNA at very high protein concentrations which has previously not been detected at lower protein:RNA ratios. The requirement for high protein:RNA ratios for detection of the protected regions may be explained by the different composition of the RNA-protein complexes. Previously, we used authentic canine SRP RNA and SRP9/14, whereas in the experiments here we used *in vitro* synthesized SRP RNA and mouse SRP9/14. In addition, hydroxyl radical cleavage reactions were carried out at a 10-fold lower complex concentration than the elongation arrest assays. Thus, a difference in the affinity of the murine and canine proteins for SRP RNA could explain the observation that higher concentrations of murine protein are required for efficient binding to the RNA. Furthermore, we reproducibly observed a high premature termination rate of reverse transcriptase in the region comprising nt 40-60 in SRP RNA, which makes it more difficult to reveal protection patterns.

In summary, we observed similar protection patterns for murine SRP9/14 and SRP9/14-20C, as expected from their RNA binding activities. However, significant changes in protein-RNA interactions were uncovered by the appearance of hypersensitive sites in the second stem-loop structure and in the central stem of SRP RNA in the SRP9/14-20C-RNA complex (Fig. 6C). The same sites lack sensitivity to the reagent in the absence of the protein, suggesting that SRP9/14-20C induces a more open conformation at these positions in SRP RNA.

DISCUSSION

We generated an altered heterodimeric subunit of SRP, SRP9/14-20C, which, when assembled into the particle, specifically lacks the capacity to confer elongation arrest activity to the particle. The results of a functional analysis of reconstituted particles comprising SRP9/14-20C, RC(9/14-20C), provide conclusive evidence that direct interactions between the components of the Alu domain of SRP and the ribosome are required to effect elongation arrest. The loss of elongation arrest activity of RC(9/14-20C) arises upon removing a conserved region in the

RNA in the presence of different proteins; (ii) the observed hypersensitive sites do not coincide with strong termination sites of reverse transcriptase. In contrast, the strong signal observed at position 46 and 47 (lane M) is most likely explained by premature termination.

Figure 6. Alterations in the hydroxyl radical cleavage pattern of SRP RNA in the presence of SRP9/14 and SRP9/14-20C heterodimeric proteins. (A) The primer extension products of the cleavage reactions were displayed on an 8 M urea-6% polyacrylamide gel. Black arrows indicate the hypersensitive sites exclusively observed in the SRP9/14-20C complex. Numbers to the left indicate the position of SRP RNA as deduced from a sequencing reaction run in parallel. The hypersensitive nucleotides precede the extension products. R, SRP RNA; B, bovine serum albumin; W, SRP9/14; M, SRP9/14-20C. Regions numbered I-IV are protected in the presence of the proteins. Regions I-III correspond to regions II-IV in Strub *et al.* (19). Region IV has not previously been detected. (B) Cleavage pattern in the 30-40 nt region in SRP RNA using milder hydroxyl radical cleavage conditions. (C) Secondary structure model of the Alu portion of SRP RNA. Arrows indicate hypersensitive sites.

C-terminal part of SRP14 comprising amino acid residues 90–100 and correlates with a conformational change in the Alu portion of SRP RNA, as indicated by the appearance of hypersensitive cleavage sites. Based on these results, we suggest that truncation of SRP14 abolishes elongation arrest activity by changing the tertiary structure of the Alu portion of SRP RNA, thus revealing a critical role of the RNA moiety in the elongation arrest function.

Elongation arrest activity of SRP requires the presence of all SRP components. A role for the Alu domain and the heterodimer SRP9/14 in elongation arrest has previously been suggested by the absence of elongation arrest activity in reconstituted particles lacking the complete Alu domain or SRP9/14 (12,30). Later experiments demonstrated that the same particles were also defective in binding directly, in a nascent chain-independent fashion, to ribosomes (6,10). Their elongation arrest-negative phenotype could therefore indirectly result from a more general defect of the particle, such as the loss of its capacity to bind directly to ribosomes.

We have previously shown that the removal of 10 amino acid residues at the C-termini of SRP14 or SRP9 failed to interfere with the RNA binding capacity of the heterodimer and with the elongation arrest function of reconstituted particles (22). SRP14–20C, which lacks 10 more amino acid residues at the C-terminus, forms a heterodimeric complex with SRP9 that can be purified from bacterial extracts and the heterodimer binds with a similar, albeit slightly reduced, affinity to SRP RNA. The particles comprising SRP9/14–20C, RC(9/14–20C), have intact signal recognition and targeting functions and bind with a similar efficiency to ribosomes as particles reconstituted with wild-type SRP9/14. Based on these results, the complete loss of elongation arrest activity of RC(9/14–20C) cannot be explained by a strongly diminished affinity for the ribosome or by major conformational changes within the particle. Rather, it results from loss of specific interactions between SRP and the ribosome that are necessary to effect elongation arrest.

An important functional role for the C-terminal region in SRP14 is also supported by the high conservation of its amino acid sequence in mammalian, plant and yeast SRP14 proteins (Fig. 1). In the regions corresponding to amino acids 90–100 in murine SRP14, five amino acid residues are completely conserved between all known SRP14 proteins and conservative substitutions of amino acids with similar physicochemical properties are found at three additional positions. Thus, eight out of 10 amino acid residues are conserved within this region, whereas the overall conservation of amino acid residues between the murine and the other SRP14 proteins are 50, 40 and 30% for *Arabidopsis thaliana*, *Oryza sativa* (rice) and *Sassharomyces cerevisiae* (yeast) respectively. The identity of the amino acids which play a direct or indirect role in the elongation arrest function remains to be determined.

How does the absence of this region interfere with elongation arrest activity of the particle? We favor a model in which truncation of the SRP14 protein abolishes the elongation arrest function of the reconstituted particles through changes in the tertiary structure of the Alu portion of SRP RNA. This model is supported by our experimental finding that the conformation of SRP RNA is different in the SRP9/14–20C–RNA complex as compared with the wild-type complex. Furthermore, the slightly reduced affinity of SRP9/14–20C for SRP RNA is consistent with a direct or indirect role of the C-terminal domain of SRP14 in

protein–RNA interaction. Minor changes in protein–RNA interactions may result in small tertiary structure changes in SRP RNA. The model is also indirectly supported by the structure of the heterodimeric protein complex (31). This region has been found to contribute to the dimer interface of the heterodimeric protein. Specifically, Gly93 and Lys95 form hydrogen bonds with amino acid residues of SRP9 and Leu94 is part of the hydrophobic core between the two proteins. It is therefore very unlikely that this region also makes direct contacts with the ribosome to effect elongation arrest.

At this moment we cannot distinguish whether the subtle changes in protein–RNA interaction result from the loss of a direct contact between the C-terminal domain of SRP14 and SRP RNA or from conformational changes in the heterodimer that indirectly interfere with RNA–protein contacts. As mentioned before, amino acid residues 93–95 contribute to direct interactions between SRP9 and SRP14 and it is feasible that their removal changes the conformation of the heterodimer without greatly reducing its stability and the stability of the RNA–protein complex.

The conformational change in SRP RNA in the SRP9/14–20C–RNA complex is indicated by the appearance of hypersensitive sites. They are located outside the protected regions and are specifically induced by binding of SRP9/14–20C to SRP RNA, since they are absent in naked RNA and in the SRP9/14–RNA complex. The structure of the RNA and the RNA–protein complex are as yet unknown and the observed hypersensitivity can therefore not be interpreted in exact structural terms. However, the increased accessibility to the reagent most likely reflects a more open structure of the RNA in the specific regions. The nucleotides A36 and C37 are in the loop of the second stem–loop structure. The potential for base pairing between the loops of the first and the second stem–loop structures (Fig. 6C) has been conserved in evolution, suggesting the existence of a pseudoknot structure formed between the two loops (32). In fact, it has been hypothesized that base pairing between the two loops may result in formation of a tRNA-like structure lacking the anticodon stem and that this structure would then interact with the ribosome to effect elongation arrest (33,34). Interference of SRP9/14–20C binding with the formation of a putative tRNA-like structure is certainly an attractive hypothesis to explain the negative effect of the more open structure in this region on elongation arrest function.

Our results provide conclusive evidence that the components of the Alu domain of SRP interact directly with the ribosome to effect elongation arrest and support a crucial role of the RNA moiety in this function. The intimate relationship between the structure of the RNA and the structure of the protein will make it a challenging task to determine unequivocally whether the RNA alone or the RNA and the proteins together make direct contact(s) with the ribosome. Maybe the interacting partners in the ribosome will have to be identified first to answer this question.

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