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Assembly of the Alu Domain of the Signal Recognition Particle (SRP): Dimerization of the Two Protein Components Is Required for Efficient Binding to SRP RNA

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The signal recognition particle (SRP), a cytoplasmic ribonucleoprotein, plays an essential role in targeting secretory proteins to the rough endoplasmic reticulum membrane. In addition to the targeting function, SRP contains an elongation arrest or pausing function. This function is carried out by the Alu domain, which consists of two proteins, SRP9 and SRP14, and the portion of SRP (7SL) RNA which is homologous to the Alu family of repetitive sequences. To study the assembly pathway of the components in the Alu domain, we have isolated a cDNA clone of SRP9, in addition to a previously obtained cDNA clone of SRP14. We show that neither SRP9 nor SRP14 alone interacts specifically with SRP RNA. Rather, the presence of both proteins is required for the formation of a stable RNA-protein complex. Furthermore, heterodimerization of SRP9 and SRP14 occurs in the absence of SRP RNA. Since a partially reconstituted SRP lacking SRP9 and SRP14 [SRP(−9/14)] is deficient in the elongation arrest function, it follows from our results that both proteins are required to assemble a functional domain. In addition, SRP9 and SRP14 synthesized in vitro from synthetic mRNAs derived from their cDNA clones restore elongation arrest activity to SRP(−9/14).

The signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein that has a crucial role in targeting secretory proteins to the rough endoplasmic reticulum membrane (for reviews, see references 26 and 31). The particle consists of six polypeptides and one molecule of SRP (7SL) RNA and can be cleaved into two subparticles by micrococcal nuclease (7, 24). One of these subparticles, SRP(S), consists of four proteins and the central portion of SRP RNA and was found to be essential for the signal recognition and targeting activity of SRP (25). The other subparticle, which includes the Alu domain, comprises two proteins, SRP9 and SRP14, and the sequences of SRP RNA that are homologous to the mammalian Alu family of repetitive sequences (7).

The "Alu domain" of the particle has been demonstrated to contain the elongation arrest or pausing function (24), which effects a specific inhibition of full-length presecretory polypeptide synthesis in vitro (13, 18, 30, 33). A partially reconstituted SRP, SRP(−9/14), which lacks SRP9 and SRP14, can no longer arrest translation of preprolactin in a wheat germ translation system. In addition, a particle lacking SRP54, and hence the signal recognition function of SRP, has no elongation arrest activity (25). Thus, the SRP9 and SRP14 proteins are essential but not sufficient for the elongation arrest activity. The components of SRP can be fractionated into five homogeneous subfractions (24). Since SRP9 and SRP14 cofractionate in this procedure, it has so far not been possible to examine the role of each of these proteins in binding to SRP RNA and in mediating elongation arrest.

We have studied the requirements for the assembly of a functional elongation arrest domain. To this end, we isolated a cDNA clone encoding SRP9. We used the SRP9 and the SRP14 (26a) cDNA clones to elucidate the RNA-binding properties of both proteins. We found that the formation of a heterodimer of the two proteins is a prerequisite for efficient binding to SRP RNA. We discuss this interesting feature for RNA-binding proteins and its implication for the elongation arrest function. Furthermore, we provide evidence that the translation products of the SRP9 and the SRP14 cDNA clones are functional in complementing the elongation arrest activity of SRP(−9/14).

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MATERIALS AND METHODS

Materials. SP6 RNA polymerase, the plasmids pSP64 and pGEM2, and the restriction enzyme *Aha*III were obtained from Promega Biotec, Madison, Wis. All other restriction enzymes, T4 polynucleotide kinase, the pUC118 plasmid, and DNA ligase were purchased from New England BioLabs, Inc., Beverly, Mass. Calf liver tRNA was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and ribonucleoside triphosphates were from Pharmacia LKB Biotechnology, Inc., Piscataway, N.J. Protein A-Sepharose and all other reagents were obtained from Sigma Chemical Co., St. Louis, Mo. [γ-^{32}P]ATP (7000 Ci/mmol) and [^{35}S]methionine (1,500 Ci/mmol) were from Amersham Corp., Arlington Heights, Ill.

Purification and sequence analysis of SRP9. The SRP was purified to homogeneity from canine pancreas (29), and the six proteins were separated on a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel. The SRP9 protein was eluted from the gel slice (8), further purified by C4 reverse-phase high-pressure liquid chromatography and sequenced by sequential Edman degradation in a gas phase sequenator.

Isolation and sequencing of the SRP9 cDNA clones. About 10^5 λ bacteriophages of a gt10 library containing cDNA inserts derived from poly(A)$^+$ RNA of MDCK cells (11) were screened by standard techniques (14) with 20 pmol of ^{32}P-end-labeled oligonucleotide. The hybridization was carried out for 16 h at 42°C in 5× NET (1× NET is 50 mM Tris hydrochloride [pH 8], 150 mM NaCl, and 1 mM EDTA)–7% SDS–30% formamide for oligonucleotide O9-1 and in 5× NET–7% SDS–20% formamide for O9-2. The filters were

washed three times in 1× NET–0.2% SDS at 42°C for 20 min and then once at the same temperature in 0.2× NET for oligonucleotide O9-1 and 0.5× NET for O9-2. Standard recombinant DNA techniques were used (14) for the preparation of λ DNA followed by restriction enzyme digests and recloning of the cDNA inserts as *Eco*RI fragments into pUC118. The inserts were sequenced from restriction enzyme sites in the polylinker and in the cDNA inserts by the procedure of Maxam and Gilbert (16) and with an internal primer by using the chain termination method (23).

In vitro expression of the SRP9 and the SRP14 proteins. A DNA fragment generated with the restriction enzymes *Nla*IV and *Aha*III (which cut at positions 44 and 562 in the SRP9 cDNA sequence, respectively) was isolated from SRP9 cDNA insert 9.2 and subcloned into the *Sma*I site of plasmid SP64. The insert was rescued with a *Bam*HI-*Eco*RI digest and transferred into plasmid SP64T (kindly given to us by D. Melton, Harvard University, Boston, Mass.) which had been linearized with *Bgl*II and *Eco*RI. In this construct (pSP6T9) the untranslated leader sequence of the SRP9 cDNA is replaced by the untranslated leader sequence of the *Xenopus laevis* β-globin gene, which results in a higher yield of SRP9 protein in the translation reactions. The cDNA insert of the mouse SRP14 protein (26a) was cloned into the *Eco*RI site of plasmid pGEM2, resulting in plasmid pG14. The clone containing the human SRP19 cDNA insert (pG19) has been described previously (12). The plasmids pSP6T9, pG14, and pG19 were linearized with *Eco*RI, *Xba*I, and *Hin*dIII, respectively, and 2-μg aliquots were transcribed with SP6 RNA polymerase in a 20-μl reaction for 1 h at 40°C as described previously (17), except that each reaction contained 0.5 mM UTP, ATP, and CTP and the dinucleotide G(5′)ppp(5′)G and 0.1 mM GTP. The RNA was extracted with phenol and phenol-chloroform, precipitated with ethanol, and suspended in 40 μl of water. To program the translation reactions, the synthetic mRNAs of SRP9, SRP14, and SRP19 were diluted 1:10, 1:3 and 1:2, respectively. A 10-μl translation reaction consisted of 2.4 μl of wheat germ extract (5); 1 μl of EGS (10 mM ATP and GTP, 80 mM creatine phosphate, 80 μg of creatine phosphokinase per ml, 250 μM all amino acids except methionine, 2.40 mg of calf liver tRNA per ml, 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethylsulfonic acid [HEPES]-KOH [pH 8], and 100 U of RNasin per ml); 1 μl of a buffer containing 50 mM HEPES-KOH (pH 8), 0.5 M potassium acetate, 5 mM magnesium acetate, and 0.01% Nikkol; 0.5 μl of compensation buffer (16 μM *S*-adenosylmethionine, 4 mM spermidine, 0.4 M potassium acetate, and 14 mM magnesium acetate to yield final concentrations of 95 mM potassium acetate and 2.5 mM magnesium acetate in the translation reaction); 1 μl of [^{35}S]methionine (10 μCi/μl); 1 μl of RNA; and 3.1 μl of H_2O. Incubations were carried out at 26°C.

RNA-binding experiments. Translation reactions (20 μl each) were programmed with synthetic SRP9 or SRP14 mRNA or both. After incubation at 26°C for 1 h, each sample was split into three 6-μl aliquots, and to each aliquot was added 6 μl of a translation reaction mixture primed with synthetic SRP19 mRNA. One aliquot of each set of three samples was precipitated with trichloroacetic acid (TCA) and later displayed as an input control in SDS-polyacrylamide gel electrophoresis (PAGE). The salt concentration of the other two aliquots of each set was adjusted to 0.5 M potassium acetate and to 5 mM magnesium acetate; 1 μl of 13 μM canine SRP RNA (purified from SRP as described in reference 28) or 1 μl of 13 μM *Escherichia coli* tRNA was added, and the samples were incubated for 12 min at 4°C and for 12 min at 37°C. The samples were diluted to a final volume of 80 μl and to a salt concentration of 250 mM potassium acetate and applied to 40-μl DEAE-Sepharose columns each contained in a 100-μl siliconized glass capillary and preequilibrated with equilibration buffer (250 mM potassium acetate, 5 mM magnesium acetate, 50 mM HEPES-KOH [pH 8], 1 mM dithiothreitol, and 0.01% Nikkol). The columns were each washed with 80 μl of equilibration buffer followed by 160 μl of wash buffer (the same as equilibration buffer but with 350 mM potassium acetate) and 160 μl of elution buffer (the same as equilibration buffer but with 1 M potassium acetate). The proteins in the three different fractions were precipitated with TCA and analyzed by SDS-PAGE.

Production of an antiserum against SRP9. The *Rsa*I fragment from the SRP9 cDNA which contains the entire SRP9 coding sequence except for the amino-terminal 4 amino acids, was ligated into the *Sma*I site of the vector pRIT2T (Pharmacia). In this construct, SRP9 is fused to the carboxy terminus of the cytoplasmic version of *Staphylococcus aureus* protein A. The fusion protein was expressed as described previously (20) and extracted from the bacterial cells by four 1-min sonications in lysis buffer (50 mM HEPES-KOH [pH 8], 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). The fusion protein was purified by affinity chromatography with immunoglobulin G Sepharose as specified by the manufacturer's instructions. The antiserum against SRP9 was obtained by immunizing a rabbit with the purified protein A-SRP9 fusion protein. The antiserum was shown to recognize the SRP9 protein on Western immunoblots (data not shown).

Immunoprecipitation experiments. For the immunoprecipitation reactions the protein A beads were loaded with antibodies from the rabbit anti-SRP9 antiserum or from the preimmune serum and incubated with the products of a 20-μl translation reaction primed with the appropriate synthetic mRNA(s). The beads were washed five times with buffer (150 mM NaCl, 50 mM Tris hydrochloride [pH 8], 0.05% Nonidet P-40). The proteins in the supernatant and the first wash fractions were combined, precipitated with TCA, and analyzed by SDS-PAGE together with the immunoprecipitated proteins.

Complementation experiments. SRP was purified by the method of Walter and Blobel (29) and was frozen after the DEAE concentration step. The separation of SRP into five homogeneous fractions and reconstitution of partial or complete SRP was carried out as described previously (24). Plasmid pSPBP4, which generates synthetic preprolactin mRNA, has been described previously (25). In vitro translation of the synthetic preprolactin and total rabbit reticulocyte mRNAs was carried out as described above, except that the ionic conditions were kept at 120 mM potassium acetate and 1.5 mM magnesium acetate. The translation products were analyzed by SDS-PAGE and were quantitated by densitometric scanning of the autoradiograph.

RESULTS

Isolation of an SRP9 cDNA. To isolate a cDNA clone encoding SRP9, we first determined the N-terminal amino acid sequence of the protein (see Materials and Methods). With the exception of amino acids 1 and 6, 27 amino acids could be determined unambiguously (Fig. 1A). Two unique oligonucleotides, which overlap by 10 nucleotides (Fig. 1A), were designed based on the amino acid sequence information. Since no amino acid derivative was detected in cycle 6,

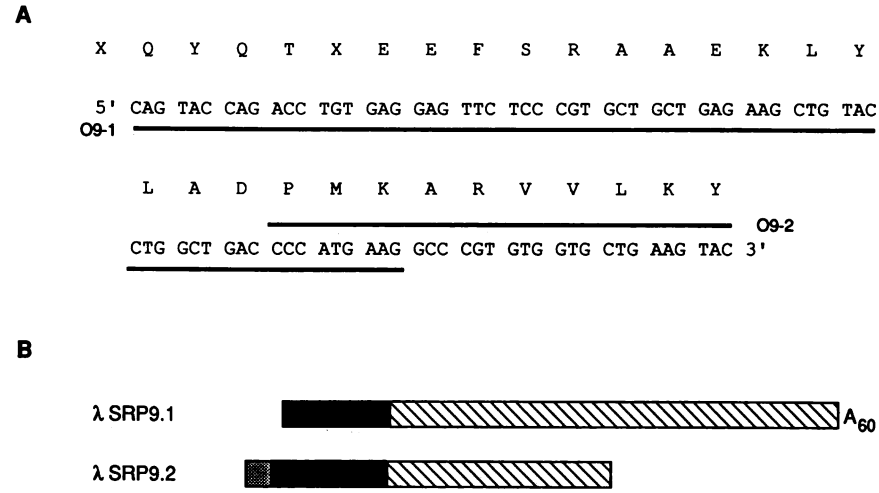

FIG. 1. Cloning of the SRP9 cDNA. (A) The amino-terminal amino acid sequence analysis of the SRP9 protein is shown above the nucleotide sequence derived from it. Symbols: X, unidentified amino acid residues; —, antisense oligonucleotide O9-1 (bar beneath sequence) and mRNA-like oligonucleotide O9-2 (bar above sequence). (B) Schematic outline of the two SRP9 cDNA inserts isolated from a canine λgt10 cDNA library. The open reading frame of the SRP9.1 cDNA insert lacks the initiator methionine and the following amino acid residue. Symbol: ■, SRP9 coding sequence; ▨, 5′ untranslated sequence; ▧, 3′ untranslated sequence; A_{60}, poly(A) tract of 60 nucleotides.

we assumed that it was a cysteine. The 66-nucleotide oligonucleotide O9-1 was used to screen a λgt10 library containing cDNA inserts derived from poly(A)$^+$ RNA of Madin-Darby canine kidney (MDCK) cells (11). Phages that hybridized with probe O9-1 in the primary screen were rescreened with the oligonucleotides O9-1 and O9-2 on separate filters. Two λ phages hybridized with both oligonucleotides, and their inserts were subcloned and sequenced.

The cDNA insert of one clone, λSRP9.2, is 794 base pairs (bp) long and has a 58-bp untranslated leader sequence followed by an open reading frame of 86 amino acids (molecular mass, 10,017 daltons [Da]), shown diagrammatically in Fig. 1B. The putative initiator methionine of SRP9 is followed by an alanine and an amino acid sequence identical to that determined from the protein sequence analysis (underlined in Fig. 2). The cDNA insert of the clone λ SRP9.1 is 1,288 bp long and has a 60-bp poly(A) tail. The sequences of the two cDNAs are identical over the entire length of the shorter insert, except that the SRP9.1 insert lacks the leader sequence, the putative initiator methionine, and the amino terminal amino acid (Fig. 1B). Instead, it contains at the 5′ end a short sequence of 7 bp of unknown origin which is unrelated to the λ SRP9.2 insert. The sequence identity in the overlapping portion of the two cDNA clones strongly suggests that the two cDNAs originated from the same gene.

The composite sequence for the SRP9 cDNA as derived from the two λ clones is shown in Fig. 2. From the electrophoretic mobility of canine SRP9 in an SDS-polyacrylamide gel, its size was determined to be approximately 9 kDa. We conclude that this cDNA encodes SRP9, because the protein predicted by the cDNA sequence is the correct size and has the correct amino-terminal sequence. The major transcript in MDCK poly(A)$^+$ RNA, which hybridizes to the radiolabeled SRP9.2 insert, is approximately 1,300 bases long (data not shown). The cDNA sequence as shown in Fig. 2 can therefore roughly account for the full length of the major transcript. The proposed initiator methionine is located in a favorable context for translation initiation (9).

The presence of both SRP9 and SRP14 is required for efficient binding to SRP RNA. To investigate the RNA-binding properties of the two proteins, we synthesized them in vitro by using a wheat germ translation extract. To this end, we cloned the portion of the SRP9.2 cDNA insert encoding canine SRP9 and the entire SRP14.1 cDNA insert encoding mouse SRP14 (26a) into a vector that allowed us to generate synthetic transcripts with SP6 RNA polymerase. The two ^{35}S-labeled translation products derived from the synthetic mRNAs have an electrophoretic mobility identical to the SRP9 and the SRP14 proteins isolated from canine pancreas (data not shown).

The ability of the translation products of SRP9 and SRP14 synthetic mRNAs to bind to SRP RNA was examined by using ^{35}S-labeled SRP19 (12) as a positive control. SRP19 has previously been shown to bind SRP RNA (12, 28). The translation reactions were incubated with canine SRP RNA or as a control with *E. coli* tRNA under reconstitution conditions (28). Note that both reactions already contained a high concentration of calf liver tRNA (240 μg/ml), which was added previously as part of the in vitro translation reaction. The amount of the proteins bound to SRP RNA was determined by fractionating the reactions on DEAE-Sepharose columns (12, 28). The SRP proteins bound to SRP RNA elute at higher salt concentrations than the SRP proteins alone because SRP RNA has a high affinity for the DEAE resin. Thus, the binding of the SRP proteins to the SRP RNA can be monitored by following their elution pattern. Four column volumes were collected: both the flowthrough fraction (0.25 M potassium acetate) and the wash fraction (0.35 M potassium acetate) are expected to contain unbound SRP proteins, and the eluate fraction (1 M potassium acetate) is expected to contain SRP proteins which were bound to SRP RNA when the sample was loaded onto the column. We have not determined whether, at high salt concentration, the protein-RNA complex elutes from the DEAE column or whether the proteins dissociate from the RNA. However, this does not change the interpretation of the elution profiles.

The results of such experiments are shown in Fig. 3. The control protein, SRP19, shows a different elution profile in the presence and in the absence of SRP RNA. As expected, all of SRP19 eluted in the 1 M salt fraction in the presence of

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GCCCACCTACCACCTACCTCGGGCGGCCAGAAACCGATGCGGGGGGCCCAGCGGCAAG

  1                                  10
Met Ala Gln Tyr Gln Thr Trp Glu Glu Phe Ser Arg Ala Ala Glu
ATG GCG CAG TAC CAG ACT TGG GAG GAG TTC AGC CGC GCG GCC GAG

                  20                                      30
Lys Leu Tyr Leu Ala Asp Pro Met Lys Ala Arg Val Val Leu Lys
AAA CTC TAC CTC GCC GAC CCT ATG AAG GCA CGT GTG GTT CTC AAA

                                     40
Tyr Arg His Ser Asp Gly Ser Leu Cys Ile Lys Val Thr Asp Asp
TAT AGG CAT TCT GAT GGG AGT TTG TGT ATT AAA GTA ACA GAT GAT

                  50                                      60
Leu Val Cys Leu Val Tyr Arg Thr Asp Gln Ala Gln Asp Val Lys
TTA GTT TGT TTG GTG TAT AGA ACA GAC CAA GCC CAA GAT GTA AAG

                                     70
Lys Ile Glu Lys Phe His Ser Gln Leu Met Arg Leu Met Val Ala
AAG ATT GAG AAA TTC CAC AGT CAA CTA ATG CGA CTC ATG GTA GCC

                  80                        86
Lys Glu Ser Arg Ser Val Ala Met Glu Thr Asp OP
AAG GAA TCC CGC AGT GTT GCC ATG GAA ACG GAC TGA CGGGTTTGAAAT

GAAGATCCTTCATGTTCTTAGGAGTAAATATCTTTTGAATCAGAAAAAGTGTTGGGAAAG

AAAATATGTAACTAAGTGGGCTCTTCAGAAGTGGGGAGATCATTTTTTGTACTTTGTTTT

TTAATGTTTACTTTAGAGAGCTAGGAACGTACATGCTTTCGGTGAAAGCCTTTATTTATT

TTTGGAAATTCAGTAAAAGGCAGTTCTTCCTTAAATTTAGTTAATCTGTCTTTAAAAGAA

AATTAAATTTAACCATTTTGCTGGATTGTTGTATTTCTTTTGGAGCATAAAATTTGTGCT

ATTGATGACCAACAAACAAACATAAAATATAGTAATTGGAATTACCTGTGCACAGCAGTG

TACCTATGTATAATATAGTAATTAGTCTCAGTTCTATCTAAAAGTAATCATGGAAATGAG

TATGCTTTACCTAAAACTTTTCCAAACTTAAACTGTATTTTTGAATGTAAGGAATTTGTA

GTATCGTTAGCTTGTTGAGCAGGGACTTGCTTTAATCTAGTTTCCAGTGCTCAAAAACAA

CTGCATTTACTTGAAGTGCATGAACAGATGATCACTAGTGGACTGAACCACCATATTACG

CAAGTATTTGCCTGCAGATTTCCCATCTATATTTTCTCAGAAGGGCTAAAGATTATTTGA

ACTGTTAAATCTTTGCCATATGTCTGTGCCACTCCTGCCTGTTTCTCCCTGTACTTAACC

AAGGTGTTGAACATGACTGTCACAACTGTTAGTTAAATCTTTGCATATGTCTGTGCCACT

CCTGCCTGTTTCTCCCTGTACTTAACCAAGGTGTTGAACATGACTGTCACAACTGTTATT

TTTTTCATTAAGTCAGAAGGATATCATTTGATATTTATCATATAATTGTAACCTCAGTTT

TACCATCTCAATGTAATGTTCACATGTTGTTCCTACATTAAAATATTTTTTGTTAGGpolyA
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FIG. 2. cDNA and protein sequences of canine SRP9. The composite cDNA sequence shown here is derived from the two SRP9 cDNA inserts described in Fig. 1. The amino acid sequence known from the protein sequence analysis is underlined. The amino acid sequence predicts a molecular mass of 10,017 Da for the SRP9 protein. Note that the mature protein lacks the initiator methionine.

the SRP RNA, whereas a large portion of SRP19 was found in the 0.25 M salt fraction in the absence of SRP RNA (Fig. 3A and B). In contrast, the elution profiles of SRP9 and SRP14 remained identical in the presence and absence of SRP RNA (Fig. 3A and B). Almost all of the protein was found in the flowthrough fraction. Only a very minor and variable portion of the two proteins (less than 5%) eluted at higher salt concentration, whether or not SRP RNA was present (for SRP9, see Fig. 3A and C; for SRP14, see Fig. 3B and C). A similar small fraction of SRP19 always eluted in the 0.35 M and 1 M salt fractions. Since the proteins are highly basic (pI_{SRP9} = 9.4; pI_{SRP14} = 11.05; pI_{SRP19} = 11.0), it is conceivable that small amounts of the proteins bind nonspecifically to some RNA molecule in the translation reaction. Alternatively, the binding of SRP9 and SRP14 to the DEAE resin in the absence of canine SRP RNA could be

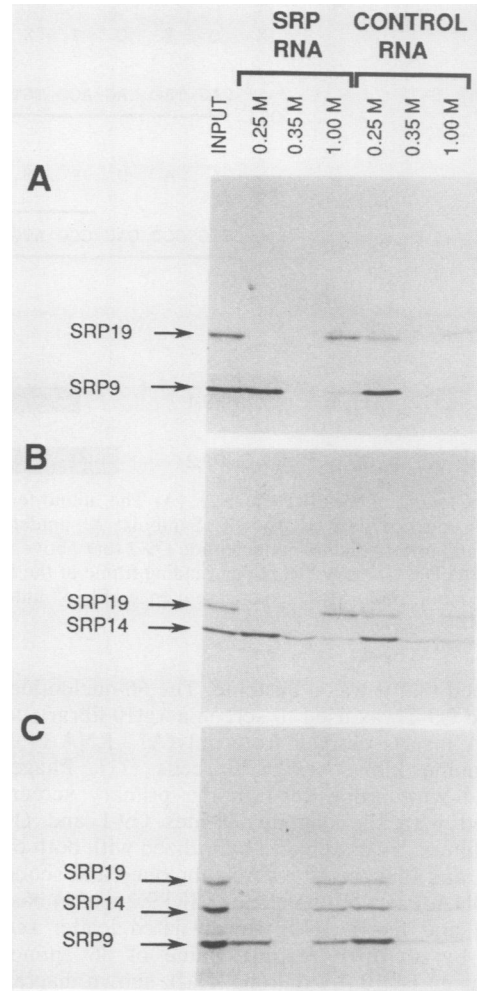

FIG. 3. RNA-binding characteristics of SRP9 and SRP14. (A) Translation reactions were programmed with synthetic SRP9 and SRP19 mRNAs. Aliquots thereof (input lane) were incubated with either a final concentration of 1 μM SRP RNA (left) or, as a negative control, 1 μM tRNA (in addition to the tRNA contribution from the translation cocktail) (right) under reconstitution conditions. The total amount of ^{35}S-labeled proteins present in each aliquot is shown in the input lanes. SRP9 and SRP19 binding to SRP RNA was assessed by DEAE-Sepharose chromatography. The protein content of the different fractions was analyzed by SDS-PAGE. The potassium acetate concentrations of the different fractions (flowthrough fraction, 0.25 M; wash fraction, 0.35 M; eluate fraction, 1.0 M) are indicated at the top of each lane. The magnesium acetate concentration was kept constant at 5 mM. (B and C) Same as above, but translation reactions were programmed either with synthetic SRP14 and SRP19 mRNAs or with all three synthetic mRNAs, respectively.

explained by the presence of a small amount of endogenous free wheat germ SRP RNA in the translation reaction. The fractionation patterns of SRP9 and SRP14 in the presence or absence of SRP RNA demonstrate that neither of the two proteins by itself binds efficiently to SRP RNA, although we cannot formally exclude the possibility that each protein individually binds to the RNA specifically, yet with low affinity.

In subsequent experiments we tested whether the two proteins could bind SRP RNA efficiently when synthesized simultaneously in a wheat germ translation system. The

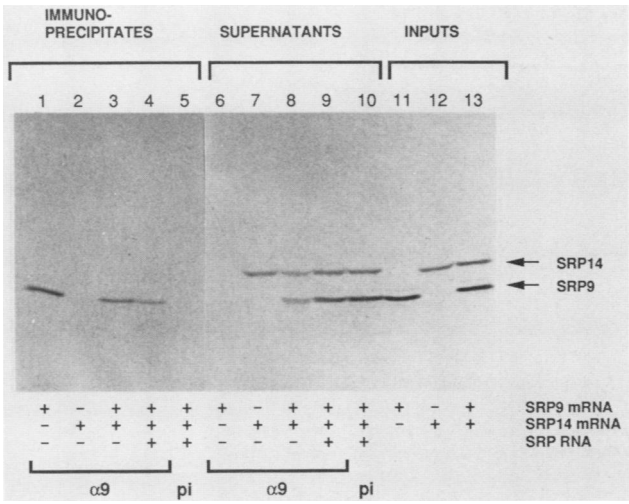

FIG. 4. The immunoprecipitation efficiency of the SRP9 protein decreases in the presence of the SRP14 protein. In vitro-synthesized SRP9 or SRP14 or SRP9 and SRP14 (protein inputs shown in lanes 11, 12, and 13) were immunoprecipitated with immobilized antibodies from an anti-SRP9 antiserum or with antibodies from a preimmune serum. The proteins in the immunoprecipitates and in the supernatants were visualized by autoradiography following SDS-PAGE. Lanes: 1 and 6, SRP9 alone; 2 and 7, SRP14 alone; 3 and 8, SRP9 and SRP14; 4 and 9, SRP9 and SRP14 in the presence of SRP RNA; 5 and 10, immunoprecipitate and supernatant, respectively, of the immunoprecipitation reaction with the preimmune serum. Abbreviations: α9, anti-SRP9 serum; pi, preimmune serum.

result of such an experiment is shown in Fig. 3C. The fractionation profiles of SRP9 and SRP14 resembled the one found for SRP19, the positive control (Fig. 3C). All of SRP14 and SRP19 and about half of SRP9 eluted in the 1 M salt fraction. Taking into account the fact that SRP9 and SRP14 contain 5 and 3 methionines, respectively, we synthesized SRP9 in excess over SRP14. This excess accounts for the SRP9 found in the 0.25 M salt fraction. In the absence of SRP RNA, all of the proteins were predominantly found in the 0.25 M salt fraction. As expected, SRP9 and SRP14 also bound to SRP RNA in the absence of the internal standard SRP19 (data not shown). These results demonstrate that both proteins together form a stable complex with the SRP RNA. Similar experiments using synthetic SRP RNA obtained with T7 RNA polymerase confirmed these results (data not shown).

The SRP9 and the SRP14 proteins form a complex in the absence of SRP RNA. To further characterize the binding of SRP9 and SRP14 to SRP RNA, we tested whether the proteins would form a complex in the absence of SRP RNA. SRP9 and SRP14 were synthesized in vitro either separately or together and subsequently incubated with Sepharose-coupled antibodies raised against SRP9 (see Materials and Methods). The proteins in the immunoprecipitates and in the supernatants were analyzed by SDS-PAGE (Fig. 4). The antibodies specifically and quantitatively precipitated all SRP9 synthesized by itself (lanes 1 and 6) but not SRP14 (lane 2), which was found in the supernatant (lane 7). Preimmune serum precipitated neither protein (lane 5; supernatant lane 10). Surprisingly, we found that the presence of SRP14 decreased the immunoprecipitation efficiency of SRP9 in the absence (lane 3) as well as in the presence (lane 4) of SRP RNA. Possible explanations for this finding are (i) that the antibodies recognize an epitope(s) of SRP9 which is specific for the uncomplexed conformation of SRP9 and (ii) that the epitope is located in the domain of SRP9 which interacts with SRP14. In either case the antibodies would not recognize SRP9 in the complex and we would therefore expect to find SRP9 and SRP14 in equimolar amounts in the supernatant of the immunoprecipitation reactions. The data shown in Fig. 4, lanes 8 and 9, demonstrate that this was indeed observed, although complex formation seems to occur slightly less efficiently in the absence of SRP RNA. Since we obtained qualitatively identical results in the presence and absence of the SRP RNA, it seemed likely that SRP9 and SRP14 assemble into a complex independently of SRP RNA.

In subsequent experiments glutaraldehyde-mediated cross-linking was used to test directly for complex formation between the two proteins. Recently, this method has been successfully used to show dimerization of a transcription factor (10). One of its advantages lies in the fact that even small increases in molecular mass due to dimerization of relatively small proteins can readily be detected in a denaturing protein gel after cross-linking has occurred. Four different translation reactions were carried out with synthetic mRNA of SRP9 and SRP14 alone or of both proteins in the presence or absence of SRP RNA. The proteins in the translation extract were incubated with glutaraldehyde at room temperature for various times, and the proteins were subsequently separated on SDS-PAGE. When each protein was translated separately, no specific cross-linking product was seen even after an 8-min incubation with glutaraldehyde (Fig. 5, center left and far left). In contrast, in the presence of both proteins a time-dependent appearance of a single additional component with an apparent molecular mass of 23 kDa was observed (Fig. 5, center right). The molecular mass of 23 kDa is roughly the correct size for a heterodimer between SRP9 and SRP14. Most importantly, the formation of this cross-linked product was independent of the presence of SRP RNA (Fig. 5, center right and far right). The results of this experiment directly demonstrate that SRP9 and SRP14 form a complex in the absence of SRP RNA. The decrease in the relative amounts of the SRP9 and SRP14 over the incubation time can be partially attributed to nonspecific cross-linking of the two proteins to components in the extract. This interpretation is supported by the observed increase of large aggregates of labeled products which did not enter the protein gel.

Results obtained by velocity sedimentation analysis in sucrose gradients further corroborated the finding that the two proteins form a heterodimer. SRP9 and SRP14 shifted into fractions of higher density when both proteins were allowed to interact (data not shown).

The proteins generated in vitro from the SRP9 and SRP14 cDNA clones restore elongation arrest activity. We wanted to determine whether in vitro-synthesized SRP9 and SRP14 would restore elongation arrest activity upon binding to a partially reconstituted SRP, SRP(−9/14), which lacks SRP9 and SRP14. To assemble complete SRP and SRP(−9/14), we first purified the proteins and SRP RNA individually (24) and then recombined them accordingly. The partially reconstituted SRP or the complete SRP were added to a wheat germ translation system which, when appropriate, was programmed with synthetic SRP9 and SRP14 mRNAs. Following a 1-h incubation at 26°C, the translational arrest capacity of the reconstituted particles was assayed by the addition of fresh translation extract containing synthetic preprolactin mRNA and globin mRNA. After an additional 20 min of incubation the ^{35}S-labeled proteins were subjected to analy-

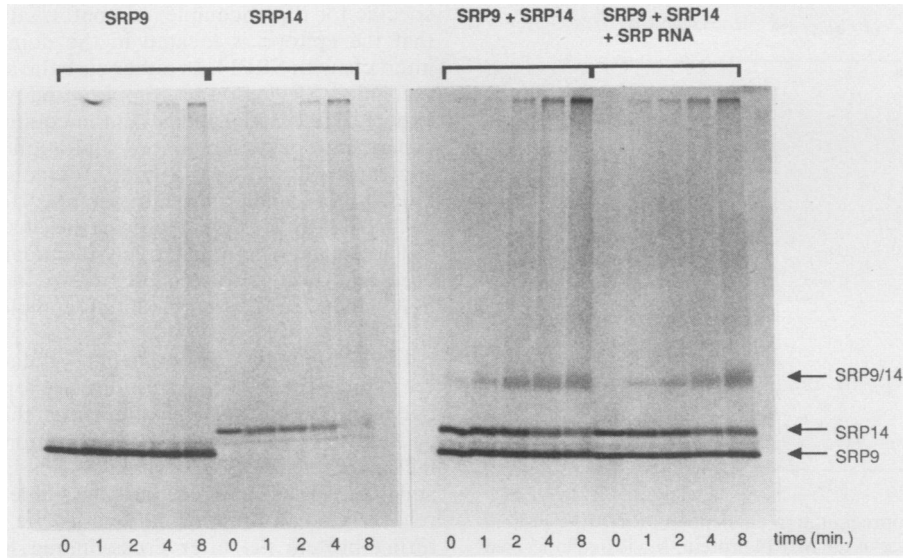

FIG. 5. Cross-linking between SRP9 and SRP14. SRP9 (far left) and SRP14 (center left) were synthesized in a 20-μl translation reaction either alone or together in the absence (center right) or in the presence (far right) of SRP RNA. The salt concentration of the translation reactions was subsequently adjusted to 0.5 M potassium acetate and 5 mM magnesium acetate, and the ribosomes were removed by centrifugation through a sucrose cushion (32). The total "soluble" proteins (consisting of the supernatant and two-thirds of the cushion) were incubated with a final concentration of 0.1% glutaraldehyde at room temperature. Portions were removed at the five time points indicated, and the reactions were stopped with 0.1 M Tris buffer. The proteins were precipitated with TCA and analyzed by SDS-PAGE followed by autoradiography. The small amount of complex formation observed at the 0 time point reflects the progression in the cross-linking reaction in the time span required to add the quenching reagent.

sis by SDS-PAGE followed by autoradiography (Fig. 6A). The relative amounts of preprolactin and globin synthesized were determined by densitometry, and the specific inhibition of preprolactin synthesis as compared with globin synthesis was calculated (Fig. 6B). In the presence of intact SRP the amount of preprolactin synthesis was specifically reduced by 80% (Fig. 6A and B, lane 1) when compared with a translation which lacked SRP (lane 5). In contrast, SRP(−9/14) or SRP9 and SRP14 alone had no significant effect on the amount of preprolactin synthesized (lanes 3 and 4). However, when SRP(−9/14) was complemented with in vitro-synthesized SRP9 and SRP14, preprolactin synthesis was reduced by 50%. This result demonstrates that the SRP9 and the SRP14 proteins, when expressed from their corresponding cDNA clones, confer upon SRP(−9/14) the elongation arrest activity of SRP.

DISCUSSION

We have described the isolation of a canine cDNA clone for SRP9 which, together with SRP14 and the Alu portion of the SRP RNA, constitutes the elongation arrest domain of SRP (24). We have now used this and a cDNA clone for SRP14 (Strub and Walter, in press) to study the RNA-binding characteristics of both proteins. Using several approaches, we have demonstrated that the two proteins form a complex in the absence of SRP RNA. This finding is consistent with the previous observation that SRP9 and SRP14 isolated from canine pancreas cofractionate in several separation procedures (24). Interestingly, the complex of the two proteins, rather than either of the proteins by itself, appears to be required for SRP RNA binding. In addition, we could complement the elongation arrest function of SRP by using proteins derived from their corresponding cDNA clones.

General structural features and primary sequences of

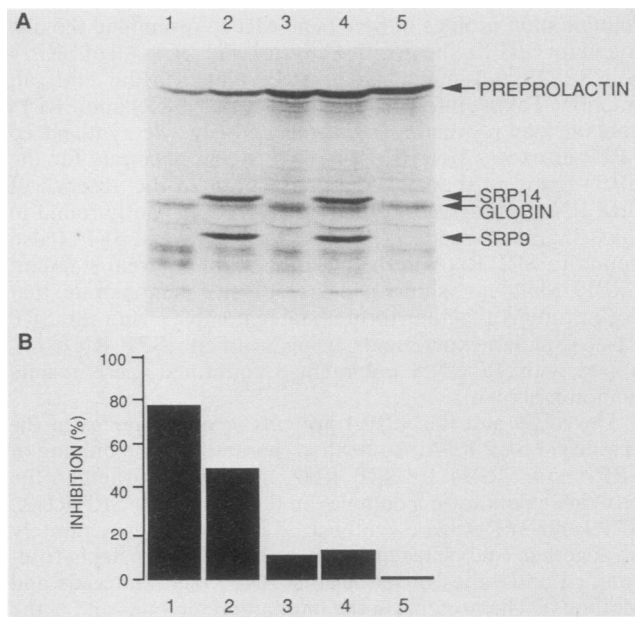

FIG. 6. In vitro expression of SRP9 and SRP14 from their cDNA clones restores elongation arrest activity of SRP(−9/14). Completely reconstituted SRP (lane 1) or SRP(−9/14) (lanes 2 and 3) was added at a final concentration of 25 nM to 20-μl translation reactions primed with synthetic SRP9 and SRP14 mRNAs (lanes 2 and 4) or with no RNA (lanes 1, 3, and 5). After incubation for 1 h at 26°C, 10 μl of a fresh translation reaction programmed with synthetic preprolactin mRNA and total reticulocyte mRNA was added to each sample. After an additional 25 min, the proteins in the five samples were then precipitated with TCA and visualized by SDS-PAGE (A). The elongation arrest activity was quantitated as described previously (24) (B).

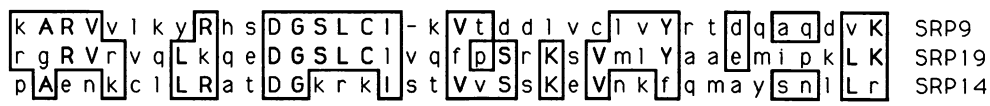

FIG. 7. Sequence homology between SRP proteins. The protein sequences of SRP9 (amino acids 24 to 60), SRP19 (amino acids 79 to 116), and SRP14 (amino acids 51 to 88) are aligned. Amino acids with identical or similar chemical properties (4) are boxed. Boldface capital letters indicate sequence identity.

proteins that confer the ability to bind RNA have so far been only poorly defined. As a result of a sequence comparison between several RNA-binding proteins, a very highly conserved octapeptide (RNP-CS) was discovered (1). Recently, it has been demonstrated experimentally that this conserved motif is the core of an RNA-binding domain comprising about 90 amino acid residues (21; for reviews, see also references 2 and 15). SRP9 and SRP14 lack the RNP-CS motif and, in contrast to the RNP-CS-type proteins, form heterodimers most probably before binding specifically to SRP RNA. Thus, they represent another class of RNA-binding proteins. It has previously been reported that two ribosomal proteins, S6 and S18, are mutually dependent for their binding to 16S rRNA, but it was not determined whether they form a heterodimer in the absence of 16S rRNA (19). It was also observed that an RNA-free 6S particle which contains the small nuclear ribonucleoprotein peptides D, E, F, and G is an intermediate in the assembly of U snRNPs (6). However, the individual RNA-binding properties of these proteins have not been determined, and it is therefore unknown whether the assembly of the 6S particle is a prerequisite for binding to U RNAs. The exact nature of the interactions between the heterodimer SRP9/14 and SRP RNA still remains to be established. The dimerization could simply bring defined regions of the two proteins in close physical proximity and thereby generate an RNA-binding domain. Alternatively, the formation of an RNA-binding domain, consisting of both or only one of the proteins, could result from a conformational change induced by the dimerization. As yet, we cannot distinguish between these possibilities. It was previously observed that the cysteines in both proteins are protected from *N*-ethylmaleimide modification when bound to SRP RNA (25), which suggests that both proteins are in close contact with the RNA.

The RNA-binding properties of SRP9 and SRP14 are reminiscent of the DNA-binding properties of a group of transcription activators which have recently been characterized. The members of this group have been shown to require the formation of homo- or heterodimers before binding with high affinity to specific DNA sequences (for reviews, see reference 27 and references therein). The common structural theme of such proteins consists of an α-helical motif, termed the leucine zipper, which is essential for dimerization of the protein(s), and an adjacent basic sequence which is involved in DNA binding. However, SRP9 and SRP14, which form heterodimers exclusively, do not contain a similar structural motif.

SRP9, SRP14, and SRP54 display a very high (95 to 99%) primary sequence conservation between the dog and the mouse proteins (3, 22, 26a; Strub and Walter, unpublished results). The finding that mouse SRP14 can restore the RNA binding and the elongation arrest function of SRP together with canine SRP RNA and SRP9 might therefore not be surprising. Nevertheless, we can conclude from this result that the amino acid residues which differ between the canine and mouse SRP14 can functionally replace each other.

The elongation arrest activity was first observed by using a wheat germ translation system (30) and canine SRP. Recently, it has also been demonstrated that endogenous SRP in the reticulocyte extract causes a pause in the translation of a presecretory protein (33). The role of this function in vivo, possibly as a means of ensuring that protein translocation will occur cotranslationally (and thus efficiently) or as part of a regulatory mechanism, still remains to be established. The proteins in the Alu domain have previously been shown to be essential for the elongation arrest activity in vitro (25). We do not yet know whether one or both proteins are directly involved in functional interactions with the ribosome. However, our experiments show that the presence of both proteins is required to assemble a functional domain. As a result, the inhibition of the biosynthesis or the modification of one protein alone would be expected to abolish the translational control function of SRP.

SRP9 and SRP14 are highly charged. They consist of 19 and 24% basic and 15 and 10% acidic amino acid residues, respectively (26a). Both proteins lack significant homology to any other protein in the Dayhoff data bank, except for SRP9, which shares a short stretch of sequence identity with the SRP19 protein (Fig. 7), flanked by additional conserved amino acids. In SRP14, this motif is only partially conserved; however, it has additional sequence similarity to SRP19 in the same region. In all three proteins this motif is predicted to be located in a turn between two α-helices or two short β-sheets followed by α-helices. Potentially, this domain could play a role in one of the two functional characteristics shared by the three proteins: in complex formation with another protein (SRP19 mediates SRP54 binding to SRP RNA [28]) and/or in SRP RNA binding. An additional sequence similarity between SRP19 and SRP14 is their highly basic C-terminal sequence. In SRP19 and SRP14, 7 of 9 and 9 of 16 amino acid residues, respectively, at the carboxy terminus are either lysines or arginines.

SRP9 and SRP14 potentially harbor three different functions: the dimerization function, the RNA-binding function, and the ability to confer elongation arrest activity to SRP(−9/14). With the SRP9 and SRP14 cDNAs and assays for all three functions at hand, it will now be possible to define in detail the structure-function relationship in this SRP domain.

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