**Assembly of the 68- and 72-kD Proteins of Signal Recognition Particle with 7S RNA**

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**Abstract.** Signal recognition particle (SRP), the cytoplasmic ribonucleoprotein particle that mediates the targeting of proteins to the ER, consists of a 7S RNA and six different proteins. The 68- (SRP68) and 72- (SRP72) kD proteins of SRP are bound to the 7S RNA of SRP as a heterodimeric complex (SRP68/72). Here we describe the primary structure of SRP72 and the assembly of SRP68, SRP72 and 7S RNA into a ribonucleoprotein particle.

The amino acid sequence deduced from the cDNA of SRP72 reveals a basic protein of 671 amino acids which shares no sequence similarity with any protein in the sequence data libraries. Assembly of SRP72 into a ribonucleoprotein particle required the presence of 7S RNA and SRP68. In contrast, SRP68 alone specifically bound to 7S RNA. SRP68 contacts the 7S RNA via its NH2-terminal half while COOH-terminal portions of SRP68 and SRP72 are in contact with each other in SRP. SRP68 thus serves as a link between 7S RNA and SRP72. As a large NH2-terminal domain of SRP72 is exposed on SRP it may be a site of contact to other molecules involved in the SRP cycle between the ribosome and the ER membrane.
of SRP68 resulted in a ~30-kD fragment which remained bound to the proteolyzed particle (Scoulica et al., 1987).

The use of cDNAs coding for the four smallest SRP proteins has facilitated a detailed analysis of their assembly with the 7S RNA (Lingelbach et al., 1988; Strub and Walter, 1990; Römisch et al., 1990; Zopf et al., 1990). In the case of SRP54, the analysis of its amino acid sequence has furthermore yielded important clues to its signal sequence binding function and to a GTP-dependent function: SRP54 was predicted to contain a GTP binding site (Römisch et al., 1989) and later experimentally demonstrated (Zopf et al., 1990; High and Dobberstein, 1991) by using mutant SRP54 proteins expressed from transcribed cDNAs (Lütcke et al., 1992).

The least well characterized subunits of SRP are the heterodimeric SRP68 and SRP72 proteins. It has not yet been possible to disassemble them under native conditions and test their individual functions. Likewise, a detailed analysis of the assembly of SRP68 and SRP72 with 7S RNA has not been possible due to the lack of a cDNA coding for SRP72. Here we describe the primary structure of canine SRP72. Domains necessary for the association of the two proteins with each other and for binding SRP68/72 to 7S RNA were identified by making use of the cDNAs of SRP68 (Herz et al., 1990) and SRP72.

Materials and Methods

Isolation and Sequencing of cDNA Clones Encoding SRP72

SRP72 was purified from canine SRP and digested with trypsin. Eight resulting peptides were N-terminally sequenced as described previously for SRP68 (Herz et al., 1990). The amino acid sequence of one tryptic peptide (ELYGGV... ) was used to synthesize a 1:256 degenerate oligonucleotide as a probe for screening. Approximately 200,000 colonies of a cDNA library which has been constructed from MDCK cell poly(A)+ RNA in a pTEX vector (Herz et al., 1990) were screened by colony hybridization. The probe for the probe was the 5’-GAG-HindIII fragment (720 bp) of pSRP68-16 (Herz et al., 1990). Conditions for generating the probe and screening were as recommended by Boehringer Mannheim. Four independent genomic clones were sequenced. Invariably they encoded nine glycine residues instead of 12 or 15.

A cDNA encoding SRP68 with a stretch of nine glycine residues was cloned in plusscript as follows: plusscript(Str) was digested with AccI and HindIII and ligated with (a) a HindII complementary sequence (in primer 5’amp-I) which was derived from one of the canine genomic DNA clones that contained the first exon of the SRP68 gene and (b) an EcoRl-HindIII fragment containing the remainder (~2.3 kb) of the cDNA insert of the published clone encoding SRP68, pGem-6816 (Herz et al., 1990).

Expression of a cDNA Encoding Modified SRP68

As heterogeneity had been observed in the GGC-rich glycine-encoding region of our previously published cDNA clones of SRP68 we sequenced the corresponding genomic region. Approximately 2 x 10⁶ plaques of a canine genomic library in XDH (kindly provided by Dr. G. A. Scheele, M.D., Beth Israel Hospital, Boston, MA) were screened with a randomly primed, digoxigenated (Boehringer Mannheim Corp.) probe. The template for the probe was the 5’-GAG-HindIII fragment (720 bp) of pSRP68-16 (Herz et al., 1990). Conditions for generating the probe and screening were as recommended by Boehringer Mannheim. Four independent genomic clones were sequenced. Invariably they encoded nine glycine residues instead of 12 or 15.

A pSRP68 transcript with a stretch of nine glycine residues was cloned in plusscript as follows: plusscript(Str) was digested with AccI and HindIII and ligated with (a) a HindII complementary sequence (in primer 5’amp-I) which was derived from one of the canine genomic DNA clones that contained the first exon of the SRP68 gene and (b) an EcoRl-HindIII fragment containing the remaining (~2.3 kb) of the cDNA insert of the published clone encoding SRP68, pGem-6816 (Herz et al., 1990).

Cell-Free Transcription and Translation and 7S RNA Binding Assays

gGem-72, pCITE-72, and pBS-SRP68(G9) were linearized with the appropriate restriction enzymes to give either the full-length (BamHI, NotI, or HindIII, respectively) or truncated (restriction enzymes indicated in the figure legends) transcripts. Transcripts were made with T7 RNA polymerase and translated in a reticulocyte lysate system.

Translations of pGem-72 and pBS-SRP68(G9) transcripts in rabbit reticulocyte lysate (SalSelect; Promega) were done without added Mg²⁺ (final concentration of 1.8 mM) but with added 30 mM KCl for pGem-72 and pBS-SRP68(G9) transcripts and with added 110 mM KCl for pCITE-72 transcripts. SRP72 obtained from translation of the pCITE-72 transcripts had the same size as SRP72 from SRP (not shown).

When the assembly of SRP68 and SRP72 was tested the translation products were mixed as indicated in the figure legends and adjusted to 2 mM puromycin, 0.5 M KOAc and 5 mM MgOAc₂. After an incubation for 30 min at 30°C in the presence or absence of added 7S RNA (~4 ug, isolated from canine pancreas SRP) the samples were loaded onto sucrose gradients.

Sucrose Gradient Analysis of SRP and Subparticles

Cleavage of SRP with elastase was done as described by Scoulica et al. (1987). SRP cleaved with 10 µg/ml elastase (0.5 ml, 1.5 A₂₈₀U) was centrifuged through a sucrose gradient (4 ml, otherwise as described below). Elastase cleaved or in vitro-synthesized samples (30-50 µl) were loaded onto sucrose gradients (5-20%, ~4.3 ml, 20 mM Hepes, pH 7.5, 0.5 M KOAc, 5 mM MgOAc₂) and centrifuged for 15 h at 4°C and 45,000 rpm (273,000 g) in an SW60 rotor. 10-11 fractions (~430 µl) were collected automatically from the top (Auto Densi-flow II, Buchler Instruments, Lenexa, Kansas).

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Protein Sample Preparation and Analysis

For the analysis of proteins separated by sucrose gradients equal portions of the fractions were precipitated with 10% TCA with 1 μl wheat germ lysate as carrier. The pellets were washed with cold acetone and dissolved in SDS sample buffer. Samples were analyzed in 10-15% SDS/polyacrylamide gels (Laemmli, 1970). For fluorography, gels were treated with Enhance (DuPont de Nemours, Bad Homburg, Germany) as recommended. For immunodetection, protein was blotted onto nitrocellulose filter (Schleicher & Schüll, Dassel, Germany) and probed with the indicated antisera essentially as described (Towbin et al., 1979). Second antibodies, goat anti-rabbit IgG (Dianova, Hamburg, Germany) coupled to either alkaline phosphatase (see Fig. 5) or to peroxidase (see Fig. 6), were visualized by nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate or the ECL system (Amersham Buchler GmbH, Braunschweig, Germany), respectively.

Rabbit Antisera

The polyclonal rabbit antisera α-72 has been described previously (Scoulica et al., 1987). The following antisera were raised in rabbits (rabbit numbers in parentheses) against synthetic peptides (first and last amino acid [aa] in the respective protein in square brackets) derived from SRP72 and SRP68 which had been coupled to keyhole limpet hemocyanine: α72-1 (162)[aa 10-24], α72-2 (163)[aa 112-126], α72-3 (164)[aa 561-576], α72-4 (165)[aa 657-671], α68-1 (160)[aa 1-15], α68-2 (143)[aa 232-246] and α68-4 (42)[aa 608-622]. The locations of the different peptides within SRP72 and SRP68 are indicated in Fig. 7. The identification of the peptides by amino acid positions is according to Fig. 1 b (SRP72 peptides) and Herz et al. (1990) (SRP68 peptides). For Fig. 6, antibodies were affinity purified (Harlow and Lane, 1988) on Affi-Gel (BioRad Labs., Hercules, CA) columns to which the peptides used in the immunization had been coupled as recommended.

Results

Isolation and Sequence Analysis of a cDNA Encoding SRP72

Canine SRP72 and tryptic fragments of it were isolated (see Materials and Methods) and NH2-terminally sequenced. Intact SRP72 yielded no sequence, suggesting that the NH2 terminus is blocked. Several peptide sequences of fragments of SRP72 were used to design oligonucleotides as probes for screening a cDNA library made from MDCK cell poly(A)+ RNA (Herz et al., 1990). Two positive clones with the longest inserts were sequenced. Amino acid sequences of eight tryptic peptides of SRP72 could be identified in the deduced amino acid sequence (underlined in Fig. 1 b).

Fig. 1 shows a schematic representation of pGem-72 (a) and the complete nucleotide and deduced amino acid sequence of SRP72 (b). Since none of the cDNA clones contained the initiation codon, the missing nucleotides were obtained by applying the protocol for RACE (Frohman et al., 1988) to poly(A)+ RNA from MDCK cells (see Materials and Methods for details). The nucleotides obtained by directly sequencing the amplified cDNA end were appended to the 5' end of the longest SRP72 cDNA insert and subcloned into pGem-72 (Fig. 1 a) as described in Materials and Methods. The amplified DNA was also cloned and sequenced. The extended nucleotide sequence of the SRP72 cDNA is shown in Fig. 1 b.

The single large open reading frame of pGem-72 encodes a protein with a predicted molecular mass of 74 kD which agrees fairly well with the apparent molecular mass of SRP72 in SDS-PAGE. SRP72 has an overall basic character with a calculated pI of 10.0. Positively charged amino acids cluster in the COOH-terminal 19 kD of SRP72 displaying a pI of 11. By contrast, the NH2-terminal 55 kD contain mixed charges resulting in a slightly acidic pI (6.8) of this segment.

In Vitro Expression of SRP72 and SRP68

To further characterize SRP72 and its interactions with...
SRP68 we transcribed their cDNAs with T7 RNA polymerase and translated the resulting mRNAs in a reticulocyte lysate translation system.

Sequencing of several SRP68 clones (Herz et al., 1990) had revealed discrepancies in the number of GGC repeats which encode the glycine-rich region near the NH₂ terminus of SRP68. This may be due to allelic variability as has been found in other genes with GGC repeats (Fu et al., 1991; Korge et al., 1992) or due to cloning artifacts during the construction of the cDNA library. To verify the SRP68 cDNA we sequenced four independent clones from a canine genomic DNA library (kindly provided by Dr. G. A. Scheele). Invariably these clones encoded nine glycine residues instead of 12 or 15 as in the previously published clones (Herz et al., 1990). Using the genomic sequence we constructed pBS-SRP68(G9) (see Materials and Methods) and used this plasmid for all in vitro expressions of SRP68.

**Binding of SRP72 to 7S RNA**

The monomeric SRP proteins, SRP19 and SRP54, were both found to bind to RNA (Lingelbach et al., 1988; Römsch et al., 1990). In contrast, neither SRP9 nor SRP14 alone bound to 7S RNA, but their dimerization was needed for binding to occur (Strub and Walter, 1990). SRP72 and SRP68 could be detached from the 7S RNA as a heterodimer in solutions of high ionic strength (Scoulica et al., 1987; Walter and Blobel, 1983). Whether they bind to the particle as monomers or as an oligomeric complex is not known.

To investigate the binding of SRP72 and SRP68 to 7S RNA, we synthesized each of these proteins in a reticulocyte lysate programmed with in vitro transcripts of pGem72 or pBS-SRP68(G9). SRP72 and SRP68 alone or in combination were incubated in the presence or absence of added 7S RNA to allow assembly to occur. Assembled particles were then analyzed by centrifugation through a 5-20% sucrose gradient as described previously (Scoulica et al., 1987). SRP protein associated with 7S RNA would be expected to migrate as an 8-9S particle, while unassembled proteins would either remain at the top of the gradient in their monomeric form or move deeper into the gradient due to either aggregation or attachment to other molecules. The result of such an experiment is shown in Fig. 2.

SRP72 alone remained near the top of the gradient (Fig. 2, left panels, lanes 2-4) irrespective of the presence (top) or absence (bottom) of added 7S RNA. This indicates that SRP72 alone remains a free protein and does not bind by itself to 7S RNA. In contrast, SRP68 alone did associate with added 7S RNA as it co-migrated with added 7S RNA in a sucrose gradient (Fig. 2, middle panels, lanes 6 and 7). When no 7S RNA was added, SRP68 sedimented to the bottom of the gradient (Fig. 2, lower middle panel, lane 11). This indicates that free SRP68 either aggregated or interacted with a large component.

When lysate containing SRP72 was combined with SRP68, a proportion of SRP72 comigrated with SRP68 as an oligomeric complex of ~9S. The migration in the gradient was the same irrespective of the addition of 7S RNA (Fig. 2, right panels, lanes 7 and 8). This indicates that SRP72 associated with SRP68 in vitro and that this association was independent of the addition of exogenous 7S RNA. Since reticulocyte lysate is known to contain SRP, it was likely that SRP72 and SRP68 had associated with endogenous 7S RNA or partially assembled SRP or had exchanged with the endogenous SRP68/72 heterodimer. The notion of a partially assembled endogenous SRP is supported by the finding that SRP54 made in reticulocyte lysate also migrated at ~9S, while SRP19 stayed at the top of the gradient (H. Lütcke and B. Dobberstein, unpublished observations).

To test for the presence of partially assembled SRP, the translation mixtures of SRP72 and SRP68 were freed of endogenous 7S RNA. Resulting complexes were analyzed by SDS-PAGE and fluorography. The positions of SRP72 and SRP68 are indicated on the right. Added 7S RNA was found mainly in fractions 5-7 while RNA migrated in fractions 3 and 4 of the gradient in the upper right panel.

Figure 2. Assembly of SRP72 into a ~9S particle requires the presence of SRP68. SRP72 and SRP68 were synthesized separately in a reticulocyte lysate cell-free system. After a further incubation of the separate or combined proteins with (top) or without (bottom) added 7S RNA the samples were centrifuged through a 5-20% sucrose gradient. Fractions were collected from top (lane 1) to bottom (lanes 10 or 11) and were analyzed by SDS-PAGE and fluorography. The positions of SRP72 and SRP68 are indicated on the right. Added 7S RNA was found mainly in fractions 5-7 while RNA migrated in fractions 3 and 4 of the gradient in the upper right panel.

Figure 3. Assembly of SRP72 with SRP68 into a ~9S particle requires the presence of 7S RNA. In vitro synthesized SRP72 and SRP68* (shortened from the COOH-terminus by 24 amino acids; see Fig. 4 b) were separated by centrifugation through sucrose gradients as in Fig. 2 (lower left and middle panels) and recovered from fractions 2-4 (SRP72) or the pellet fraction (SRP68*). Subsequently they were mixed with reticulocyte lysate which had been absorbed twice to an equal volume of DEAE-Sepharose and incubated in the presence (upper panel) or absence (lower panel) of purified 7S RNA. Resulting complexes were analyzed by sucrose gradient centrifugation as in Fig. 2.
A COOH-terminal Region of SRP72 Is Required for Binding to the SRP68/7S RNA Complex

To identify regions of SRP72 that interact with SRP68, COOH-terminally truncated SRP72 proteins were incubated with SRP68 and 7S RNA, and oligomeric complexes were separated by sucrose gradient centrifugation. Plasmids pCITE72 or pGem72 were linearized at several positions inside the coding region, and COOH-terminally truncated SRP72 molecules were synthesized from the different run-off transcripts (Fig. 4a). After an incubation with full-length SRP68 and 7S RNA, complexes were centrifuged through a sucrose gradient as before (Fig. 2). Results depicted and summarized schematically in Fig. 4a show that a 5%10) NH2-terminal portion of SRP72 can still interact with the SRP68/7S RNA complex whereas a 42-kD fragment no longer can.

A COOH-terminal Region of SRP68 Is Required for Binding to SRP72

To identify the region of SRP68 which is required for tethering SRP72 to the particle, COOH-terminally truncated versions of SRP68 were generated. Plasmid pBS-SRP68(G9) was cut at several sites within the coding region and transcribed. Truncated SRP68 molecules were synthesized and allowed to form complexes with 7S RNA and SRP72 which were analyzed on sucrose gradients.

SRP72 formed a 9S oligomeric complex with SRP68 lacking the 24 COOH-terminal amino acids (Fig. 4b, SRP68/XhoI, 67.2 kD). However, removal of only 28 more amino acids e.g. (early quitter). The calculated molecular masses of SRP72, SRP68, and their deletion mutants are shown on the right. Association of SRP72 mutants with full-length SRP68/7S RNA and association of SRP68 mutants with 7S RNA or full-length SRP72 is indicated by +, failure to do so by -.
acids abolished the ability of SRP72 to form a complex with the truncated SRP68/7S RNA (Fig. 4 b, SRP68/ApaLI, 50.7 kD). All further truncated versions of SRP68 no longer associated with SRP72 (Fig. 4 b, SRP68/BglIII, and summary below). Thus, amino acid residues important for the association with SRP72 lie close to the COOH terminus of SRP68.

An NH₂-terminal Region of SRP68 Is Sufficient for Binding to 7S RNA

To determine the region of SRP68 that binds to 7S RNA we made again use of COOH-terminally truncated proteins. Like the full-length protein, all examined truncated versions of SRP68 were found to comigrate with 7S RNA (see Fig. 4 b). Thus, the 7S RNA binding region of SRP68 lies close to the NH₂ terminus.

Proteolysis of SRP72 Results in Two Major Fragments Both of which Are Released from SRP

Proteolysis of SRP with elastase had identified a 55-kD fragment of SRP72 which was released from SRP upon such treatment (Scoulica et al., 1987). To locate this and the other elastolytic fragments within the SRP72 molecule we raised antisera against four synthetic peptides derived from the positions of SRP72 depicted in Fig. 5 (top, hatched: 72-1 to 72-4; see Fig. 1 b). SRP was digested with increasing concentrations of elastase, separated by SDS-PAGE, blotted onto nitrocellulose and probed by Western analysis with the anti-SRP72 peptide antisera.

All antisera recognized the 72-kD protein of undigested SRP (Fig. 5, lanes 0, arrow 72). Upon proteolysis with 2.5 μg/ml elastase SRP72 lost ~4 kD from its COOH terminus. This is deduced from the fact that antiserum α72-4 which had been raised against the COOH-terminal peptide no longer recognized the first degradation product of ~68 kD (Fig. 5, 72-4, lane 2.5) while all other antisera still did (Fig. 5, lanes 2.5). The same and higher concentrations of elastase degraded SRP72 further to a fragment of ~56 kD which at higher elastase concentrations was further digested to a fragment of ~53 kD. As both fragments were recognized by antiserum α72-1, they must include the NH₂ terminus of SRP72.

Figure 5. Identification of SRP72 domains with anti-peptide antibodies. DEAE-Sepharose-purified SRP (0.2 A280/lane) was digested for 30 min at 30°C with 0, 2.5, 10, and 25 μg/ml elastase as indicated below the lanes and TCA precipitated. Samples were analyzed by SDS-PAGE and immunoblotting with polyclonal antiserum raised against SRP72 (α72; Scoulica et al., 1987), and antisera raised against peptides 72-1 to 72-4 from SRP72 as indicated above the blots. The positions of the peptides are indicated on the schematic diagram of SRP72 and specified in Fig. 1 b. The marked bands recognized by the antiserum α72-3 (α) are unrelated to SRP72 because they were not recognized by antibodies that had been affinity purified on immobilized peptide 72-3 (see Fig. 6, a and b). Numbers on the right indicate relative molecular masses of marker proteins. Dashed elascase cleavage sites are indicated by arrowheads on the outline of SRP72.

Figure 6. All detected elastase-cleaved fragments of SRP72 are released from SRP while a fragment near the NH₂ terminus of SRP68 remains bound to SRP. SRP was left intact (a) or digested as in Fig. 5 with 10 μg/ml elastase (b–e) and centrifuged through a 5–20% sucrose gradient. Fractions were collected from the top (fractions 1) and analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. The antibodies had been raised against and affinity-purified on peptides derived from SRP72 (α–c) (see Fig. 7 a) and SRP68 (d and e) (see Fig. 7 b). Molecular masses of marker proteins are indicated on the right. The weak reactivity seen in lane 4 of e is due to incomplete stripping of antibody α72-1 with which the blot had been immunodecorated before.
Figure 7. Schematic representations of SRP72 (a) and SRP68 (b) and the positions of the peptides used to raise antisera. Positions of sites cleaved by elastase are indicated by solid arrow heads. Elastase fragments detected in Fig. 6 are shown underneath the sequences, and their estimated molecular masses are indicated. Re-lease of the fragments from SRP is represented by stippled bars, retention in SRP by solid bars. The RNA binding domain of SRP68 and regions important for the assembly of SRP68 and SRP72 as derived from the combined results of Figs. 4 and 6 are indicated by lines above the sequences.

To identify free and particle-bound COOH-terminal fragments of SRP72, SRP was digested with elastase at 10 μg/ml, separated on a sucrose gradient, and fragments were analyzed by SDS-PAGE and Western blotting as before. Undigested SRP (11S) migrated mainly in fractions 8 and 9 as identified by immunodecoration of SRP72 with the affinity-purified antibody α2-3 (Fig. 6 a). (Affinity purification cleared the antiserum α72-3 of antibodies against a non-related protein seen in Fig. 5.) Antibody α2-3 detected two ~14-kD fragments of elastase-digested SRP (Fig. 6 b). While the smaller of the two fragments was found only in the upper three fractions of the sucrose gradient (Fig. 6 b, lanes 1–3), the slightly larger one moved further into the gradient (Fig. 6 b, lanes 1–6). The increased velocity of sedimentation might be due to the interaction with another protein. The NH2-terminal ~53-kD fragment of SRP72 was, as expected (Scollica et al., 1987) found near the top of the gradient and therefore had been released from the particle (Fig. 6 c).

The NH2 Terminus of SRP68 Is Not Required for Binding to 7S RNA

Sucrose gradient analysis of elastase digested SRP was also used to further characterize the RNA binding domain of SRP68. Fragments generated from SRP68 by elastase cleavage were identified by antibodies raised against and affinity-purified on peptides derived from the predicted amino acid sequence (Fig. 7 b). At 10 μg/ml elastase fragments of 24 and 31 kD were recognized by the antibody α68-2 (Figs. 6 e and 7 b). The 31-kD fragment was recognized also by the antibody α68-1 (Figs. 6 d and 7 b) and therefore contains the NH2 terminus of SRP68. As the 24-kD fragment was not recognized by antibody α68-1, it lacks the NH2-terminus of SRP68. Both fragments migrated in fractions 7–9 (Fig. 6, d and e) and were thus retained in the digested SRP. This result is consistent with the findings described above that the RNA binding region of SRP68 is contained in the NH2-terminal 21 kD (Fig. 4 b). In addition it suggests that the NH2-terminal 7 kD are not part of the 7S RNA binding domain of SRP68.

Discussion

Sequence Analysis of SRP72 and SRP68

We have cloned a cDNA encoding canine SRP72. The authenticity of the clone was verified by the following three independent criteria: (a) the sequences of tryptic peptides derived from gel-purified SRP72 were located in the deduced amino acid sequence; (b) the protein expressed from the cDNA migrated at 72 kD in SDS-PAGE, formed a ribonucleoprotein particle with SRP68 and 7S RNA and was recognized by an antiserum raised against SRP72 from canine pancreas; and (c) canine SRP72 was immunodecorated by antibodies raised against four peptides which had been synthesized according to the amino acid sequence deduced from the cDNA.

The initiation codon was assigned on the following grounds: (a) the single large open reading frame encodes a protein of 72 kD as determined by SDS-PAGE and of 74 kD as calculated from its amino acid sequence; (b) sequences obtained by primer extension and PCR contained only the assigned ATG; and (c) a downstream initiation site is excluded because initiation from any downstream ATG codon would abolish the expression of peptide 72-1 against which an SRP72-specific antiserum was raised (see Fig. 1 b).

The amino acid sequence of SRP72 is not similar to any sequence in the PIR or Swissprot databases (as of January 1993). Secondary structure prediction with the programs Robson, Segment 83, GOR, and Levin indicates SRP72 to be ~50% in helical and only to 4–16% in extended conformation. The calculated pl of the whole protein is 10.0. A very conspicuous feature is the uneven charge distribution: mixed charges in the NH2-terminal 53 kD of SRP72 result in a calculated pl of 6.7. By contrast, the COOH-terminal remainder of the protein is predominantly positively charged (pl = 11) and contains some long stretches of lysine residues (n ~< 8). In this region two putative nuclear localization signals (NLS) were predicted by the program "motifs" (GCG package, University of Wisconsin, Madison, WI). Whether the predicted NLSs are actually used remains to be determined. However, preliminary data indicate that SRP72 over-expressed in BHK cells is localized in the cytoplasm (H. Lütcke and B. Dobberstein, unpublished observations).

The available cDNAs encoding SRP68 differ in their numbers of GGC repeats near the 5' end. This could be due to allelic variation which has been observed in other genes featuring GGC repeats (Fu et al., 1991; Korge et al., 1992). However, it is also conceivable that the high GC content could have resulted in artifacts during the cDNA cloning procedure. We therefore sequenced genomic clones which were found to invariably encode nine (instead of 12 or 15) consecutive glycine residues. Since the sources of the cDNA and the genomic clones were different (MDCK cells vs. one mongrel dog) we cannot distinguish between the two possibilities. However, the number of glycine residues had no
influence on the RNA binding capability of in vitro-made SRP68 (not shown).

Like SRP72, SRP68 has an uneven charge distribution: positive charges cluster in a region of \(\sim 14\) kDa (pI = 11) which lies 7 kDa from the NH\(_2\) terminus, whereas the remainder of SRP68 is weakly acidic (pI around 6).

**Assembly of SRP72 and SRP68 with 7S RNA**

SRP can be disassembled into its RNA and protein components and efficiently be reconstituted from these components into a functional particle (Walter and Blobel, 1983). Even cell-free synthesized SRP proteins can combine with 7S RNA. While such a reconstitution is efficient for the monomeric SRP19 and SRP54 (Römisch et al., 1990) it is inefficient for in vitro synthesized SRP9 and SRP14 (Strub and Walter, 1990). Neither SRP9 nor SRP14 alone interact specifically with 7S RNA. Rather, they need to heterodimerize to form a stable RNP with 7S RNA. Heterodimerization occurs in the absence of 7S RNA and appears to be a prerequisite for RNA binding (Strub and Walter, 1990).

SRP68 and SRP72 are known to form a rather stable heterodimeric complex when bound to 7S RNA (Scoullica et al., 1987). The complex is released from the 7S RNA in 2 M KCl but does not dissociate into the monomeric proteins under these conditions. In contrast to SRP9 and SRP14, SRP68 and SRP72 do not, or do only very inefficiently, form a heterodimer in the absence of 7S RNA (Fig. 3). Binding of SRP68 to the 7S RNA appears to be a prerequisite for its stable interaction with SRP72. The tight interaction with SRP72 could be mediated by a conformational change which SRP68 undergoes upon binding to the 7S RNA.

The affinity of SRP68 for 7S RNA appears to be higher in the presence of SRP72 than in its absence. This is concluded from the finding that SRP68 alone binds very inefficiently to the endogenous 7S RNA, while efficient binding was observed in the presence of SRP72 (Fig. 2). Different binding affinities of SRP68 and the SRP68/72 heterodimer to the 7S RNA are also suggested by absorption to DEAE-Sepharose. SRP68/72 remained largely bound to the 7S RNA whereas SRP68 alone detached from the RNA in the presence of DEAE-Sepharose (H. Lütcke and B. Dobberstein, unpublished observations).

The RNA binding domain of SRP68 is located in the highly positively charged region near the NH\(_2\) terminus of SRP68. This is concluded from binding COOH-terminally truncated SRP68 proteins to 7S RNA (Fig. 4 b) and limited proteolysis of SRP (Fig. 6, d and e). No primary sequence similarities with RNA binding proteins or known RNA binding consensus motifs were detected. The positive charges could mediate the binding of SRP68 to 7S RNA consistent with the fact that SRP68/72 can be detached from 7S RNA by solutions of high ionic strength (Scoullica et al., 1987). The domain's affinity for 7S RNA may be increased by the association of SRP68 with SRP72. Alternatively, SRP72 and SRP68 together could form a second RNA binding domain once they associate with each other. We favor the former view which implies that SRP72 is connected to SRP only by its interaction with SRP68 because all fragments of SRP72 were released from SRP upon digestion with elastase (Fig. 6, b and c).

The finding that free SRP68 sediments in a complex (Fig. 2) indicates that it either aggregates or associates with a large component. For this reason, a batch absorption and sedimentation assay which has been used previously to study the assembly of SRP19 and SRP54 with 7S RNA (Lingelbach et al., 1988; Römisch et al., 1990) was inappropriate in this study. The finding that SRP68 could be recovered from the complex in the presence of 7S RNA (Figs. 2 and 3) may indicate that it is kept assembly competent by its association with the yet unknown large component. Preliminary experiments suggest that this component sediments at \(\sim 40S\).

**Sites of Interaction between SRP72 and SRP68/7S RNA**

By deletion analysis a region within the COOH-terminal half of SRP72 was found to be essential for the interaction with the SRP68/7S RNA complex (Fig. 4 a). In SRP68, a region of 3 kDa near the COOH terminus and distinct from the RNA binding domain was found essential for the interaction with SRP72 (Fig. 4 b; see Fig. 7). We propose that the two COOH-terminal domains of SRP72 and SRP68 directly associate with each other. In support of this, a 14-kD COOH-terminal fragment of SRP72 was found associated with a larger component which most likely is a COOH-terminal fragment of SRP68 (Fig. 6 b). As 75% of the amino acid residues spanning the 3-kDa region of SRP68 are hydrophobic, this would indicate a hydrophobic interaction between SRP68 and SRP72. This is consistent with the previous observation that SRP68 and SRP72 cannot be separated under non-denaturing conditions.

**Implications for the Biogenesis of SRP**

Nothing is known about the steps and cellular sites of assembly of SRP from its RNA and protein components. Assembly may occur in the nucleus or in the cytoplasm. It is also conceivable that some of the SRP proteins are transported into the nucleus where they bind to 7S RNA, whereas others may bind to the partially assembled SRP in the cytoplasm. 7S RNA is transcribed by RNA polymerase III (PolIII), and it has been suggested for other PolIII transcripts that their nuclear export is mediated by attached proteins (Zapp, 1992). Our analysis of partially assembled SRP strongly suggests that assembly of SRP68 and SRP72 with 7S RNA occurs in the cytoplasm: reticulocyte lysate was found to contain SRP with binding sites available for SRP54, SRP68, and SRP72 but not SRP19 (H. Lütcke and B. Dobberstein, unpublished observations).

With cDNAs available for SRP68 and SRP72, the stages in the assembly of SRP can be further characterized. Reconstitution of SRP from cloned constituents will also allow to determine the function of SRP68 and SRP72 in the SRP-mediated targeting of secretory proteins to the ER membrane.

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