

The 68 kDa protein of signal recognition particle contains a glycine-rich region also found in certain RNA-binding proteins

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Signal recognition particle (SRP) interacts with the signal sequence in nascent secretory and membrane proteins and directs them to the membrane of the endoplasmic reticulum. Membrane targeting is mediated by the 68 and the 72 kDa proteins of SRP. We have cloned and sequenced cDNA encoding the 68 kDa protein of canine signal recognition particle (SRP68). SRP68 is a basic protein comprised of 622 amino acid residues. Close to the amino terminus there is a glycine-rich region which SRP68 has in common with some RNA-binding proteins. SRP68 shares no detectable similarity to any of the proteins in data libraries.

Signal recognition particle; Translocation; Ribonucleoprotein particle; Glycine-rich

1. INTRODUCTION

The signal recognition particle (SRP) is a small ribonucleoprotein particle that is involved in the targeting of presecretory and membrane proteins to the endoplasmic reticulum (ER) membrane [1,2]. During the targeting SRP interacts with the signal sequence of nascent preproteins and arrests their further elongation [3,4]. Elongation resumes after SRP has made contact with the docking protein (DP) or SRP receptor in the ER membrane [5,6]. The nascent polypeptide is then translocated across the membrane. Thus at least three different functions of SRP can be distinguished: (i) interaction with the signal sequence, (ii) translation arrest and (iii) interaction with the DP at the ER membrane. Structural components in SRP that are involved in these three functions have been identified. SRP consists of one molecule of 7S RNA (SRP7S or 7SL) and 6 different polypeptides of 9, 14, 19, 54, 68, and 72 kDa [7]. SRP9 and SRP14 can be released from the particle as a heterodimeric complex [8,9]. Their presence in the particle is required for the elongation arrest function [10,11]. SRP19 directly contacts the RNA and its presence is required for the binding of SRP54 to the particle [1,11,12]. Crosslinking experiments have shown that SRP54 interacts with the signal sequence of

nascent polypeptides [13,14]. The SRP68 and SRP72 proteins form a heterodimeric complex (SRP68/72) which interacts with the central rod of SRP7S RNA [15]. Inactivation of SRP68/72 by alkylation with *N*-ethylmaleimide leads to a SRP that has lost the ability to promote translocation but which can still arrest elongation [1,11]. Thus SRP68/72 might directly interact with the DP in the ER membrane [11]. Protease mapping studies revealed that a 55 kDa domain of SRP72 could be liberated from the particle whereas no detectable fragment of SRP68 was found to be released. It was therefore suggested that SRP68 interacts with the RNA while SRP72 is linked to the particle through its interaction with SRP68 [9].

In order to further characterize the structure and function of the SRP68/72 complex we have isolated the cDNA encoding SRP68.

2. MATERIALS AND METHODS

2.1. Purification and sequence analysis of SRP68

SRP was purified from canine pancreas [9] and the 6 proteins of SRP were separated on a SDS polyacrylamide gel. The band corresponding to the SRP68 protein (approximately 400 pmol) was excised from the gel, cut into pieces and incubated in water for 16 h with frequent changes. The washed gel pieces were then immersed in 0.1 M ammonium hydrogen carbonate pH 8.0 containing trypsin at a 1:3 enzyme to protein ratio. Following incubation at 37°C for 3 h the generated protein fragments were eluted from the gel by shaking the pieces twice for 3 h in 0.1% trifluoroacetic acid. Residual water and peptide were extracted from the gel matrix by treatment with acetonitrile. The eluates were combined, concentrated and extracted twice with a 1:4 mixture of isomyl alcohol/heptane [16] to remove traces of SDS. Separation of the tryptic peptides was performed by reversed phase HPLC (high-performance liquid chromatography) on a Vydac 218TP5 column (1.6 × 250 mm). The peptides were sequenced in a gas phase sequencer [33].

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2.2. Library construction

Poly(A)⁺ RNA was prepared from MDCK cells by standard techniques [17]. cDNA was synthesized by oligo dT priming and cloned essentially as described [18], with the following modifications:

The adaptors used for the ligation of the cDNA into the vector had the following sequence (encoded amino acids are shown below in italics):

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(Bam HI)  Kpn I      Sph I  Not I
GA.TCC.GGT.ACC.AGC.ATG.CGG.CCG.C
G.CCA.TGG.TCG.TAC.GCC.GGC.G
G      T      S      M      R      P
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The presence of a *NotI* site immediately adjacent to the cDNA insert is advantageous for excising large inserts as a single fragment.

The cloning vector pTEX was constructed by cloning a double stranded oligonucleotide encoding the bacteriophage T7 promoter and 3 restriction sites (5'-*XhoI*, *EcoRI*, *SmaI*-3') into the *SmaI*-site of pUEX1 [19]. The restriction enzyme sites in pTEX are in the same reading frame as in pUEX1 between the *BamHI* and the *HindIII* sites. This vector allows the expression of a β -galactosidase fusion protein under the control of the lambda P_R promoter in *E. coli*, or the synthesis of transcripts from the T7 promoter in vitro. The latter may be used for in vitro expression of a cloned cDNA without the amino(N)-terminal portion of β -galactosidase. The library thus obtained consisted of approx. 450 000 independent clones.

2.3. Isolation and sequencing of SRP68 cDNA clones

Approximately 300 000 colonies were screened by colony hybridization [17]. Two degenerate oligonucleotides derived from the amino acid sequences of two different tryptic peptides (peptide 1: ala-phe-thr-glu-glu-gln-ala-val-leu-tyr-asn-gln; peptide 2: tyr-glu-ala-tyr-asn-ile-gly-asp-gln) were end-labeled using polynucleotide kinase to a specific activity of 10⁹ cpm/ μ g. Filters were hybridized over night with a mixture of both oligos at 10⁶ cpm/ml in 6 \times SSC, 5 \times Denhardt's, 0.1% SDS at 42°C. After washing in 1 \times SSC, 0.1% SDS at 42°C the filters were exposed to Kodak XAR film for 4 h at -70°C. Positive colonies were picked and rescreened separately with both oligonucleotide probes. Plasmid DNA was prepared from clones hybridizing with both probes and one insert sequenced using the Sequenase kit (USB, Cleveland, OH, USA). Using the insert DNA from one of the identified clones the library was rescreened and 25 more clones identified (pTEX-SRP68-II.1 to 25) The 5' ends of the longest cDNA clones (6, 13, 15 and 23) were sequenced.

2.4. Subcloning into pGEM vectors

pTEX-SRP68-II.6, -II.13, -II.15 and II.23 were cut with *NotI*, the inserts isolated and the ends filled in with Klenow enzyme. pGEM 3 and 4 plasmids (Promega, Madison, WI, USA) were cut with *XbaI*, phosphatase treated and overhangs filled in with Klenow enzyme. Inserts and vectors were ligated and orientations of the inserts determined by restriction enzyme analysis.

2.5. Transcription/translation and immunoprecipitation

pGEM plasmids containing inserts SRP68-II.6, -II.13, II.15 or II.23 were transcribed with either SP6 or T7 RNA polymerase [20] and the transcripts translated in the wheat germ cell-free system [21] and where indicated immunoprecipitated with an anti SRP68 antibody [9]. An aliquot of the translation mixture or the immunoprecipitate was separated by SDS-PAGE and labeled proteins visualized by autoradiography.

2.6. Northern blot analysis

Cytoplasmic poly(A)⁺ RNA of MDCK cells and ribosomal RNA of canine pancreas and *E. coli* were separated on a 1% agarose-formaldehyde gel and transferred to gene screen plus membrane (NEN) [17]. The *NotI* insert fragment of pTEX-SRP68-II.6 was labelled by random priming and hybridized according to Church and Gilbert [22]. Filters were washed at 65°C in 1 mM EDTA, 40 mM

NaHPO₄ (pH 7.2) and 1% SDS. Ribosomal RNA from dog pancreas and from *E. coli* was used to calculate the approximate size of SRP68 mRNA (ribosomal RNAs: 28 S: 4800 bases (b), 18 S: 1900 b, 23 S: 2900 b, 16 S: 1540 b).

3. RESULTS

3.1. Sequence of SRP68

Canine SRP68 and tryptic fragments of it were isolated (see section 2) and subjected to N-terminal sequence analysis. No sequence was obtained from intact SRP68 suggesting that its N-terminus is not accessible for the chemical degradation used in the sequence analysis. Several peptide sequences of SRP68 fragments were obtained. Based on the amino acid sequences of two of these peptides degenerate oligonucleotides were designed and used to screen a MDCK (Madin Darby Canine Kidney) cell cDNA library (see section 2). One clone hybridized to both oligonucleotides and the insert in this clone was therefore used to rescreen the library. 25 more positive clones were found. The cDNA insert in one of these clones (pTEX-SRP68-II.6) was sequenced. A schematic outline of pTEX-SRP68-II.6 is shown in Fig. 1A and the sequence of the cDNA insert in Fig. 1B. It comprises 2485 bp and has one large open reading frame encoding 622 amino acid residues and one small open reading frame close to the polyA tail in the 3' end. The calculated *M_r* of the protein encoded by the large open reading frame is 70.2 kDa and thus in good agreement with the apparent MW of 68 kDa as deduced for SRP68 from SDS-PAGE. The amino acid sequences of 7 peptides of the SRP68 had been determined by sequence analysis. The sequences of these peptides could be identified in the amino acid sequence deduced from the cDNA (Fig. 1B) showing that the isolated cDNA encodes authentic SRP68.

Using Northern blot analysis on mRNA from MDCK cells we estimated the size of the full length mRNA for SRP68 to be about 2.5 kb (Fig. 2). Thus pTEX-SRP68-II.6 contains a SRP68 cDNA of nearly full length.

3.2. Expression of SRP68 from pGEM-SRP68 cDNA clones

In order to obtain more information about the 5' end of SRP68 cDNA and the ATG initiation codon we sequenced the 5' ends of the longest SRP68 clones. Sequences at the 5' ends of these clones are shown in Fig. 3A. Three independent groups of cDNAs were found: SRP68-II.13 has 5 more bp at its 5' end whereas SRP68-II.15 and II.23 lack 13 and 14 bp respectively. SRP68-II.15 and -II.23 also lack the first ATG codon. As the first ATG codon could be the initiating methionine codon we tested whether all or only the two longest of these clones encode full length SRP68. pGEM-SRP68 clones were transcribed from either the SP6 or the T7 promoter located at the 5' side of the respective cDNA insert. As shown in Fig. 3B (lanes 6 and 13) only the

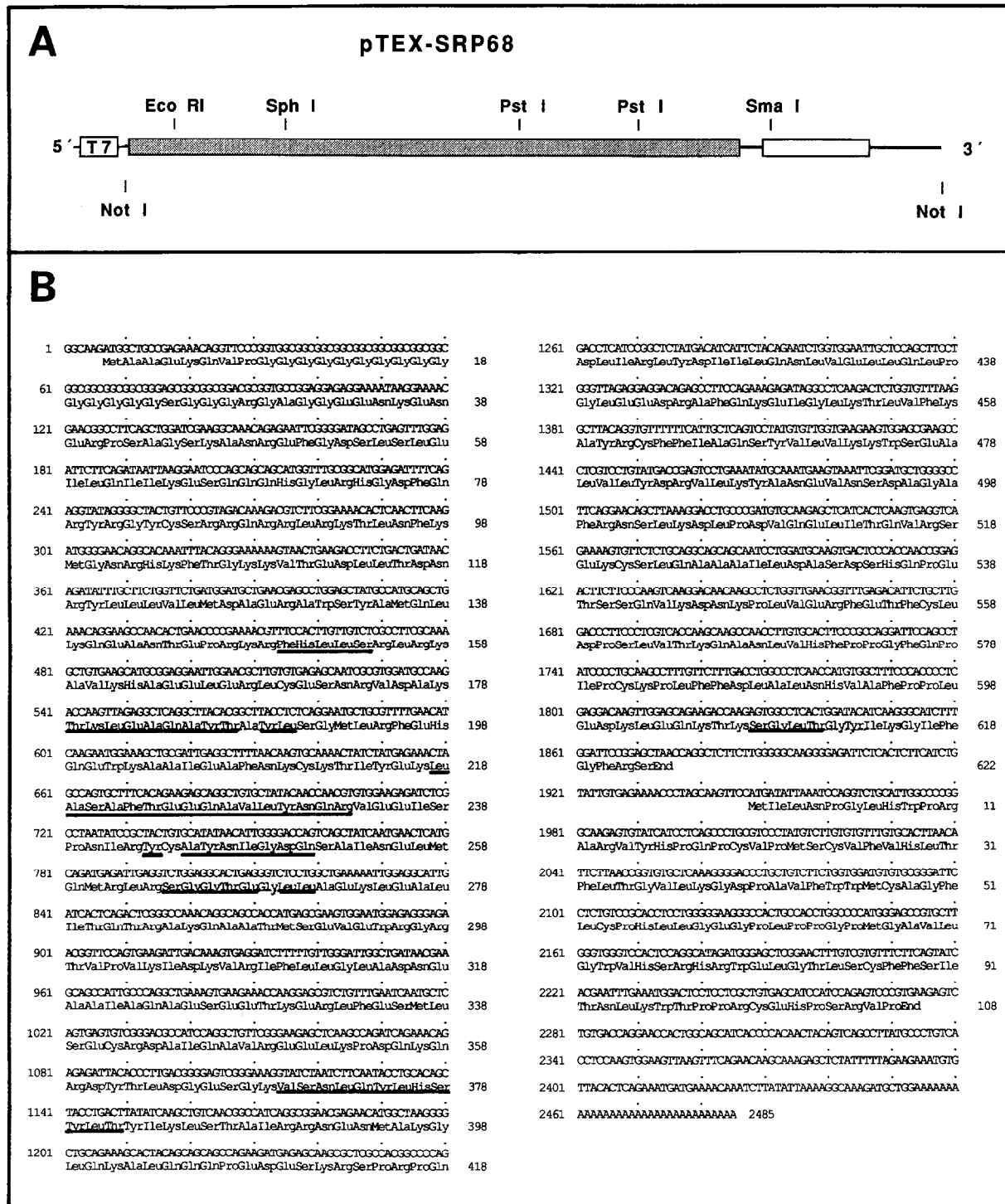


Fig. 1 Outline and sequence of cDNA pSRP68-II.6 coding for the 68 kDa protein of SRP (SRP68) (A) Outline and partial restriction map of pSRP68-II.6. The grey box indicates the coding region of the cDNA for SRP68, the white box a short open reading frame at the 3' end of pSRP68. T7: indicates the T7 promoter. The following restriction sites are indicated: *NotI*, *EcoRI*, *SphI*, *PstI* and *SmaI*. (B) DNA sequence and deduced amino acid sequence of the insert in pTEX-SRP68-II.6. Amino acid sequences determined by peptide sequence analysis are underlined. Gaps indicate amino acids that were not assigned by the direct sequencing of peptides derived from the SRP68.

clones which contain the first ATG codon (pSRP68-II.6 and -II.13) also result in the synthesis of a 68 kDa protein. Smaller proteins are synthesized from clones pSRP68-II.15 and -II.23 suggesting that some initiation

has occurred at internal codons (Fig. 3B, lanes 15 and 23). The 68 kDa protein resulting from transcription/translation of clones pSRP68-II.6 and -II.13 can specifically be immunoprecipitated with an antiserum

SRP68 interacts with the SRP72 protein in authentic SRP [9,11]. This became evident from disassembly of SRP into its RNA and protein components. SRP68 and the SRP72 can be released from SRP as a heterodimeric complex (SRP68/72) under two different conditions: in 2 M KCl and in the presence of EDTA and DE53 cellulose resin [9,11]. Thus the SRP68 must interact with the SRP72. The SRP68/72 complex can directly interact with SRP7S RNA and fragments generated by cleavage of the SRP68 with elastase were not released from the particle [9,11]. It therefore has been tentatively concluded, that the SRP68 anchors the SRP68/72 complex to the particle and that the SRP68 might directly interact with SRP7S RNA [9]. Thus two functions of the SRP68 can be distinguished, binding to the SRP72 protein and possibly to SRP7S RNA [9]. Methods for binding of in vitro synthesized SRP proteins to SRP7S RNA have been established [12,27,28]. Attempts to bind in vitro synthesized SRP68 to SRP7S RNA by a previously described method [27] failed (Römisch, unpublished). This could indicate that the formation of the SRP68/72 heterodimer is a prerequisite for binding to SRP7S RNA. Dimer formation between the SRP9 and SRP14 protein was found to be required for their interaction with SRP7S RNA [28]. Alternatively a modification of the SRP68 could be necessary that is not obtained in the in vitro cell-free system.

Only a few sequence elements that interact with RNA have been characterized to date. The best characterized is the so called RNP consensus sequence (RNP-CS) [29,30]. This is a conserved stretch of 8 amino acids within a putative RNA binding domain of 80–90 amino acid residues [29]. No such region is found in the sequence of the SRP68. A detailed functional analysis of this protein will require the isolation of cDNA coding for the SRP72 protein with which SRP68 interacts in the SRP and with which it may form a functional complex.

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