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Disassembly and domain structure of the proteins in the signal-recognition particle

Efstathia SCOULICA, Elke KRAUSE, Klaus MEESE and Bernhard DOBBERSTEIN

European Molecular Biology Laboratory, Heidelberg

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The signal-recognition particle (SRP) is a ribonucleoprotein (RNP) complex consisting of six different polypeptide chains and a 7SL RNA. It participates in initiating the translocation of proteins across the membrane of the endoplasmic reticulum.

SRP was disassembled in 2 M KCl into three components, one RNP composed of 7SL RNA and the 54-kDa and 19-kDa proteins, and two heterodimers consisting of the 72/68-kDa and the 14/9-kDa proteins respectively. The 54-kDa protein could be released from the RNP subparticle by chromatography on DEAE-Sepharose in Mg^{2+}-depleted buffer, while the 19-kDa protein remained bound to the 7SL RNA.

The domain structure of SRP proteins was probed by using mild elastase treatment and protein-specific antibodies. It was found that the 72, 68, 54 and 19-kDa SRP proteins were proteolytically processed in distinct steps. Most remarkably a protein fragment of 55-kDa, generated from the 72-kDa SRP protein, and a 35-kDa fragment from the 54-kDa SRP protein were both released from the RNP particle. Fragments generated from the 68-kDa protein and detectable with the anti-(68-kDa protein) antibody remained associated with the RNP particle. Cleavage of the SRP proteins by elastase at 2.5 μg/ml resulted in partial loss of activity, while 10 μg/ml caused complete inactivation of the particle. Neither the elongation arrest of IgG light chain nor its translocation across SRP-depleted microsomal membranes was promoted. The implications of these results on the possible interaction between the SRP subunits are discussed.

Secretory and membrane proteins are translocated across or inserted into the membrane of the endoplasmic reticulum (ER) [1]. This process is mediated by the signal-recognition particle (SRP) and docking protein, the receptor for SRP in the endoplasmic reticulum membrane [2–5].

Details of the functions of SRP and docking protein have been elucidated by using the wheat germ cell-free system and microsomal membranes derived from dog pancreas [4, 6, 7]. It has been found that SRP interacts with the signal sequence in nascent secretory proteins and can arrest polypeptide chain elongation after about 70 and more amino acids have been polymerized [4, 7, 8]. When the arrested complex binds to the docking protein in the endoplasmic reticulum membrane, translation resumes and translocation of the nascent polypeptide chain across the membrane is initiated. According to these results, SRP would have at least three functional interactions: (a) recognition of nascent secretory polypeptides, (b) arresting elongation, and (c) interacting with the docking protein. This complex set of functions is probably reflected in the structural complexity of SRP.

SRP is a rod-shaped RNP particle composed of six polypeptides with molecular masses of 72, 68, 54, 19, 14 and 9 kDa and one 7SL RNA molecule of 300 bases [9–11]. The 7SL RNA can be divided into three distinct segments, the central 'S fragment', which contains a unique sequence of about 155 nucleotides, and the two flanking regions which show high homology to human Alu sequences [12–14]. By treatment of SRP with micrococcal nuclease two subparticles can be generated, one containing the central S fragment and the 72, 68, 54 and 19-kDa proteins and one containig the hydrogen-bonded 5′ and 3′ flanking Alu-like segments with the 14-kDa and 9-kDa proteins [14]. It has been proposed that the subparticle containing the S fragment plays an essential role in translocation, whereas the part containing the Alu sequence is involved in the elongation-arrest function [15, 16].

Different approaches have been used to elucidate functions of the individual SRP proteins and of the 7SL RNA. Specific sequences in the 7SL RNA have been proposed to function in the elongation arrest of nascent secretory proteins [17] and the interaction with the docking protein [18]. Using a novel crosslinking approach it has been shown that the 54-kDa protein of SRP is located close to the signal sequence and might actually bind to it [8]. Walter and Blobel showed that SRP can be disassembled into its RNA and protein components and reassembled subsequently into a functional particle [19].

In order to obtain further insight into the structure of SRP, we disassembled it in successive steps. We describe antibodies directed against the four larger SRP proteins and their use in the analysis of protein domains released from the SRP particle upon mild treatment with elastase.

MATERIALS AND METHODS

[^{35}S]Methionine was purchased from Amersham Buchler; elastase from Merck & Co. (Darmstadt, FRG); Trasylol from Boehringer (Mannheim, FRG); Ponceau S from Serva (Heidelberg, FRG).

Correspondence to B. Dobberstein, EMBL, Postfach 102209, D-6900 Heidelberg, Federal Republic of Germany

Abbreviations. SRP, signal-recognition particle; RM-K, rough microsomes extracted with 0.5 M KOAc; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; $NaCl/P_i$, phosphate-buffered saline.

Purification of SRP

Rough microsomes were prepared as described by Meyer et al. [4] with the following modifications. A dog pancreas homogenate was centrifuged for 10 min at 6000 rpm at 4 °C in a Sorvall SS34 rotor. From the supernatant microsomes were pelleted by centrifugation for 40 min at 20000 rpm at 4 °C in a Sorvall SS34 rotor. Microsomes were resuspended in 0.25 M sucrose, 50 mM KOAc, 1 mM dithiothreitol and pelleted again by centrifugation for 40 min at 20000 rpm, 4 °C. The washed microsomes were extracted with buffer containing 0.5 M KOAc as previously described [6]. From the salt extract, SRP was purified by chromatography on ω-aminopentyl-agarose, DEAE-Sepharose and sucrose gradient centrifugation [10]. The buffer composition of the 5–17% sucrose gradient was: 600 mM KOAc, 20 mM Hepes/KOH, pH 7.5, 5 mM $Mg(OAc)_2$, 1 mM dithiothreitol.

Disassembly of SRP in 2 M KCl

Gradient-purified SRP (1 A_{280}/ml) was dialysed at 4 °C for 5 h against 1000 volumes of 500 mM KOAc, 50 mM Hepes/KOH, pH 7.5, 5 mM $Mg(OAc)_2$, 1 mM dithiothreitol. To 250 µl dialysed SRP, 250 µl 4 M KCl, 20 mM Hepes/KOH, pH 7.5, was added and the mixture incubated for 1 h on ice. It was then layered onto a 4 ml 5–20% sucrose gradient in 2 M KCl, 5 mM $Mg(OAc)_2$, 50 mM Hepes/KOH, pH 7.5, 1 mM dithiothreitol. After centrifugation in a SW60 Beckman rotor for 15 h at 45000 rpm, 4 °C, eleven fractions were collected. An aliquot from each of the fractions was characterized for its protein content by SDS-PAGE and silver staining and for RNA by electrophoresis in polyacrylamide gels containing 8 M urea [12].

Release of the 54-kDa protein from a SRP subparticle

SRP, disassembled in 2 M KCl, was separated by sucrose gradient centrifugation (see above). Fractions containing the 54-kDa and 19-kDa proteins and the 7SL RNA were collected. Two portions of 0.5 ml each were diluted with 10 volumes of 20 mM Hepes/KOH, pH 7.5, 1 mM dithiothreitol. To one portion EDTA was added to 1 mM and to the other $Mg(OAc)_2$ to 5 mM. The samples were passed through DEAE-Sepharose columns (1 ml) bed volume), equilibrated in 20 mM KOAc, 20 mM Hepes/KOH, pH 7.5, 1 mM dithiothreitol containing 1 mM EDTA or 5 mM $Mg(OAc)_2$ respectively. The columns were washed with five volumes of 250 mM KOAc, 20 mM Hepes/KOH, pH 7.5, 1 mM dithiothreitol containing either 1 mM EDTA or 5 mM $Mg(OAc)_2$. Elution of bound material was with 800 mM KOAc, 20 mM Hepes/KOH, pH 7.5, 1 mM dithiothreitol, 5 mM $Mg(OAc)_2$. Proteins were characterized by SDS-PAGE and silver staining.

Preparation of antibodies against SDS-denatured SRP proteins

Proteins from gradient-purified SRP were separated by preparative SDS-PAGE. Protein bands were visualized by incubating the gel in 0.5 M KOAc in the cold, and bands corresponding to the SRP proteins were cut out of the gel. Protein was eluted either by diffusion or by electrophoretic elution [20]. Eluates were Cl_3AcOH-precipitated and solubilized in $NaCl/P_i$ containing 0.1% SDS.

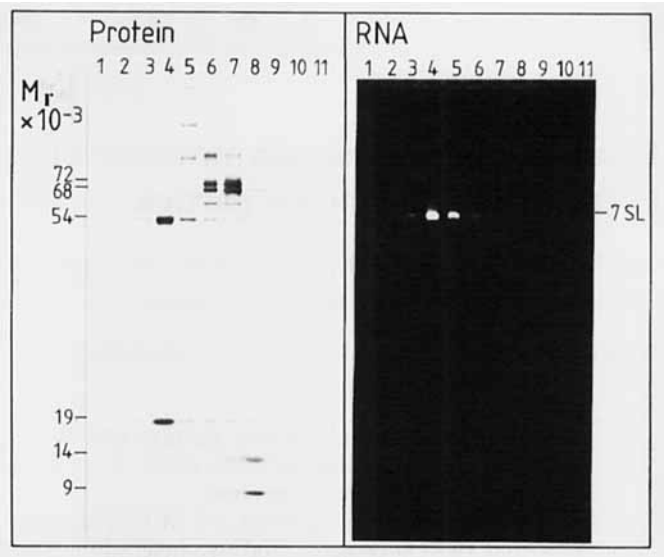

Fig. 1. *Disassembly of SRP in 2 M KCl.* SRP was incubated in a buffer containing 2 M KCl and centrifuged in a 5–20% sucrose gradient in the same buffer. The gradient was fractionated from the bottom and the proteins in each fraction were separated by SDS-PAGE. Fraction numbers are indicated on top of the gels. Fraction 1 is the bottom fraction. Protein bands were visualized by silver staining (left panel). RNA from each fraction was characterized by electrophoresis in an polyacrylamide gel containing 8 M urea and by ethidium bromide staining (right panel). Control SRP, separated in a sucrose gradient containing 0.5 M KOAc and analysed as above, is shown in Figs 5 and 7

Rabbit polyclonal antibodies

In order to raise antibodies in rabbits, 50–100 µg of each of the isolated SRP proteins in complete Freund's adjuvant were injected into the lymph nodes of white New Zealand rabbits. Rabbits were boosted twice at 10-day intervals with the same amount of antigen. Development of antibody titer was tested by Western blot analysis of SRP proteins separated by SDS-PAGE.

Mouse monoclonal antibodies

Female Balb/c mice were immunized at 4-week intervals with 200 µg purified 54-kDa SRP protein. The antigen was emulsified in complete Freund's adjuvant for the first subcutaneous injections. For the second injection, antigen was administered in incomplete Freund's adjuvant. The serum antibody response was assayed by immunoblotting 8 weeks after the initial immunization. The third injection with antigen in $NaCl/P_i$ was given intraperitoneally 3 days before fusion. Spleen cells were fused with mouse P3X63-Ag8.653 myeloma cells, a non-secreting variant of P3X63-Ag8 [21] using poly(ethyleneglycol) 4000 [22]. Cells were distributed into ten 24-well plates. The supernatants of microcultures were screened by immunoadsorption to sucrose-gradient-purified SRP proteins spotted in a concentration of about 0.5 mg/ml onto nitrocellulose filters. Bound antibody was revealed with peroxidase-labeled rabbit antibody to mouse light chains. Cells, from which positive supernatants were obtained, were subcloned and supernatants further tested by Western blot analysis on SRP proteins separated by SDS-PAGE. One antibody, which remained positive for the 54-kDa SRP protein throughout all tests, was called mAb 54.1. Its immunoglobulin

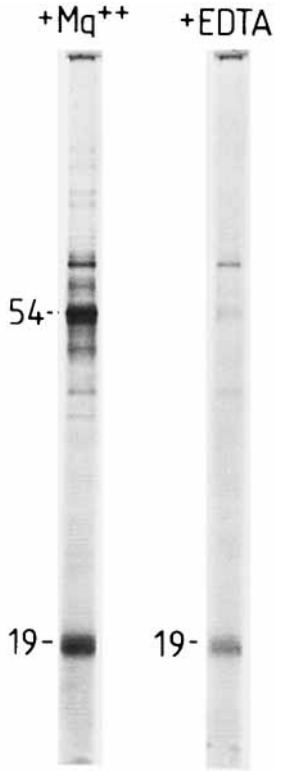

Fig. 2. *Chromatography of a SRP subparticle on DEAE-Sepharose.* The SRP subparticle, containing the 19-kDa and 54-kDa proteins and the 7SL RNA (7SL-19/54) (see fraction 4 from a sucrose gradient as shown in Fig. 1), was chromatographed on DEAE-Sepharose 6B in buffer containing either Mg^{2+} or EDTA. Material which bound to the DEAE-Sepharose and eluted at 800 mM KOAc was separated by SDS-PAGE and proteins were visualized by silver staining

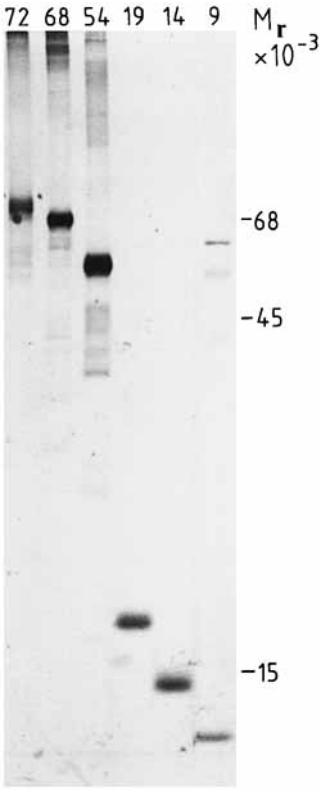

Fig. 3. *Isolation of individual SRP proteins.* Individual SRP proteins were isolated as described in Materials and Methods and characterized by SDS-PAGE. The size of each of the polypeptides is indicated above each lane

species and class were determined to be IgGγ2b. The light chain is of the κ type. Cells secreting mAb 54.1 were grown as an ascites tumor.

Protein and RNA gel electrophoresis and immunoblotting

Proteins were separated on 10–15% Laemmli-type SDS/polyacrylamide gels [23]. Silver staining was done as described by Ansorge [24].

For immunoblotting essentially the protocol of Towbin [25] was followed. For detection of total protein, the blots were stained with Ponceau S. Rabbit. Immunserum was used in the following dilutions: for the anti-(72-kDa protein) serum 1:400, the anti-(68-kDa protein) 1:400, the anti-(54-kDa protein) 1:100 and the anti-(19-kDa protein) 1:400. Ascites fluid with mAb 54.1 was used at a 1:10000 dilution. For rabbit antibodies a 1:5000 dilution of goat anti-(rabbit IgG)-peroxidase conjugate (Dianova, Hamburg, FRG) was used and for mouse antibodies a 1:5000 dilution of peroxidase-labeled sheep anti-(mouse IgG light chains) antibodies (Dianova, Hamburg, FRG). 7SL RNA was separated on polyacrylamide gels containing 8 M urea as described [12].

Digestion of SRP with elastase

To 10 µl gradient-purified SRP (0.6 A_{280} unit/ml), 2 µl elastase were added to give a final concentration of 2.5, 10 and 25 µg/ml elastase respectively and 500 mM KOAc, 20 mM Hepes/KOH, pH 7.5, 5 mM $Mg(OAc)_2$, 1 mM dithiothreitol. The elastase stock solution (5 mg/ml) contained 5 mg/ml Trasylol in order to inhibit non-elastolytic activity. Digestions were for 30 min at the temperatures indicated in the figure legends and were stopped by the addition of $PhMeSO_2F$ to 20 µg/ml.

SRP activity assays

Messenger RNA from rough microsomes of MOPC41 tumors, coding primarily for IgG light chain, and mRNA coding for rabbit globin were translated in a wheat germ cell-free system in the presence or absence of SRP [4, 7]. Protein synthesis was synchronized after 2 min by the addition of the cap analog 7-methylguanosine 5′-monophosphate to 4 mM. Peptide elongation was allowed to proceed for 30 min. 5-µl aliquots of the translation mixture were precipitated with Cl_3AcOH and characterized by SDS-PAGE. The gel was fluorographed and bands visualized by autoradiography. The amount of label in the pre-IgG light chain and rabbit globin were quantified by densitometric scanning. The percentage of pre-IgG light chain inhibition was calculated by taking rabbit globin as an internal standard [7]. In order to determine the ability of SRP to promote translocation of pre-IgG light chain across microsomal membranes, MOPC 41 mRNA was translated in the presence of SRP and salt-washed microsomal membranes (RM-K) [4]. The translation products, pre-IgG light chain and processed light chain, were quantified as described above. Processing of pre-IgG light chain to light chain by signal peptidase was taken as a measure for protein

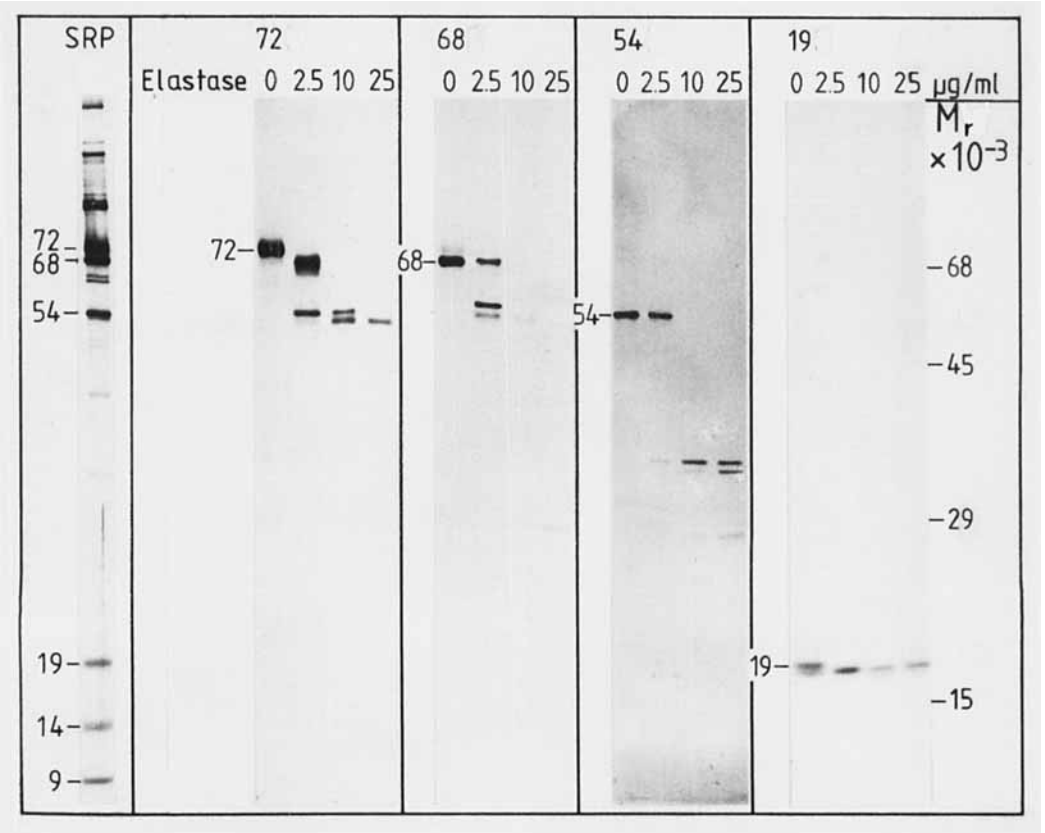

Fig. 4. *Characterization of elastase cleavage products by specific polyclonal antibodies.* Sucrose-gradient-purified SRP (0.6 A_{280}) was digested for 30 min at 30 °C with 0, 2.5, 10 and 25 µg/ml elastase as indicated on top of the lanes. Proteins were then separated by SDS-PAGE and individual proteins and their proteolytic fragments characterized by immunoblotting using antisera against the 72, 68, 54 and 19-kDa SRP proteins. The positions of intact 72, 68, 54, 19, 14 and 9-kDa SRP proteins are indicated in the left lane. This lane (SRP) shows the SRP proteins used in the assay after silver staining. The positions of protein standards are indicated on the right. These are bovine serum albumin, 68-kDa; ovalbumin, 45-kDa; chymotrypsinogen, 25-kDa; lysozyme, 15-kDa

translocation, as these chains were also protected against exogenously added protease (data not shown).

Gradient centrifugation of elastase-digested SRP

Gradient-purified SRP was dialyzed against 600 mM KOAc, 20 mM Hepes/KOH, pH 7.5, 5 mM $Mg(OAc)_2$, 1 mM dithiothreitol and adjusted to an A_{280} of 0.6. Elastase and buffer were added to final concentrations of 2.5 µg/ml and 10 µg/ml elastase, 500 mM KOAc, 20 mM Hepes/KOH, pH 7.5, 5 mM $Mg(OAc)_2$, 1 mM dithiothreitol. Digestion was for 30 min at 37 °C. Elastolytic activity was then stopped by adding $PhMeSO_2F$ to 20 µg/ml. The mixture (0.5 ml) was layered on top of 4-ml 5–20% sucrose gradients in 500 mM KOAc, 20 mM Hepes/KOH, pH 7.5, 5 mM $Mg(OAc)_2$ and 1 mM dithiothreitol. After centrifugation in a SW60 Beckmann rotor for 15 h at 40000 rpm, 4 °C, the gradients were fractionated and the proteins in each of the fractions analysed by SDS-PAGE, silver staining and Western blot analysis.

RESULTS

Disassembly of proteins from the SRP

Walter and Blobel disassembled SRP into its protein and RNA components by removal of Mg^{2+} with EDTA and subsequent fractionation of the individual components on DEAE-cellulose [13]. We describe here the stepwise removal of proteins from the SRP.

SRP was isolated by chromatography on ω-aminopentyl-agarose, DEAE-Sepharose and sucrose gradient centrifugation in 0.5 M KOAc [10]. Under these conditions SRP is known to sediment as a single 11S particle [10] (and Fig. 7A). However, when SRP is resuspended in 2 M KCl and analysed on a sucrose gradient containing 2 M KCl, it is found disassembled into three components (Fig. 1). One contains the 54-kDa and 19-kDa proteins (fraction 4), one the 72-kDa and 68-kDa proteins (fractions 6 and 7) and one the 14-kDa and 9-kDa proteins (fraction 8). When gradient fractions are analysed for their RNA content, the 7SL RNA is found in the fractions containing the 54-kDa and 19-kDa proteins (Fig. 1, lane 4). As the 54-kDa and 19-kDa proteins alone would sediment on the top of the gradient, it can be concluded that they bind to 7SL RNA and form a RNP particle of 10 S. The fact that the 72/68-kDa and 14/9-kDa proteins cofractionate is consistent with the previous findings by Walter and Blobel that these proteins form heterodimers [19].

The RNP subparticle (Fig. 1, fraction 4) was further disassembled by chromatography in Mg^{2+}-depleted buffer on DEAE-Sepharose. Only the 7SL RNA and the 19-kDa protein were retained on the DEAE-Sepharose (Fig. 2). Since, under these conditions, the 19-kDa protein alone does not bind to the DEAE-Sepharose [19], it can be concluded that the 19-kDa protein remains bound to the 7SL RNA and thus is indirectly retained by the DEAE-Sepharose.

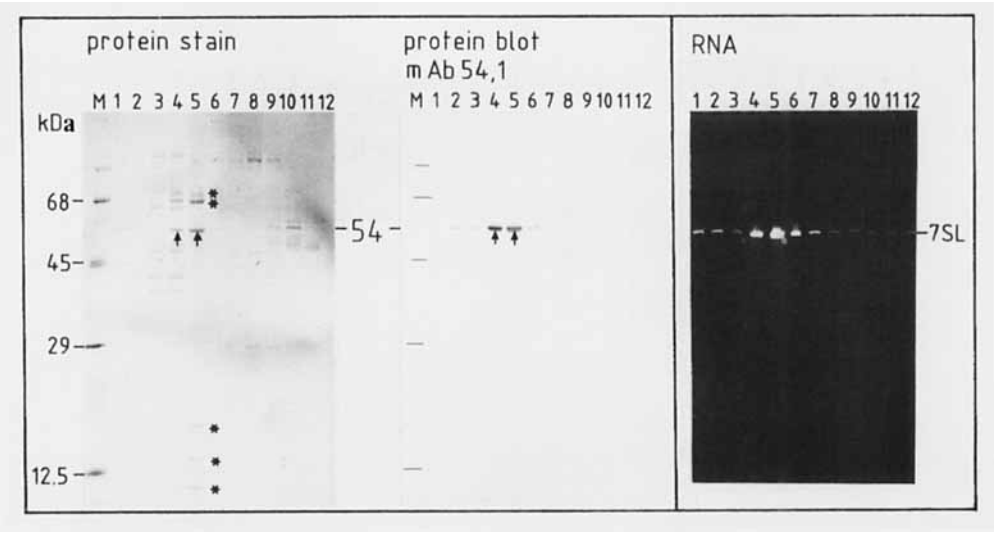

Fig. 5. *Characterization of the 54-kDa SRP protein by monoclonal antibody (mAb) 54.1.* SRP, purified by chromatography on ω-aminopentyl-agarose and DEAE-Sepharose, was separated on a 5–20% sucrose gradient. Fraction 1 is the bottom fraction. Proteins from each of the gradient fractions were separated by SDS-PAGE and either silver-stained (left panel) or tested with mAb 54.1 by Western blot analysis (middle panel). RNA of the gradient fractions was separated on a polyacrylamide gel containing 8 M urea and stained with ethidium bromide (right panel)

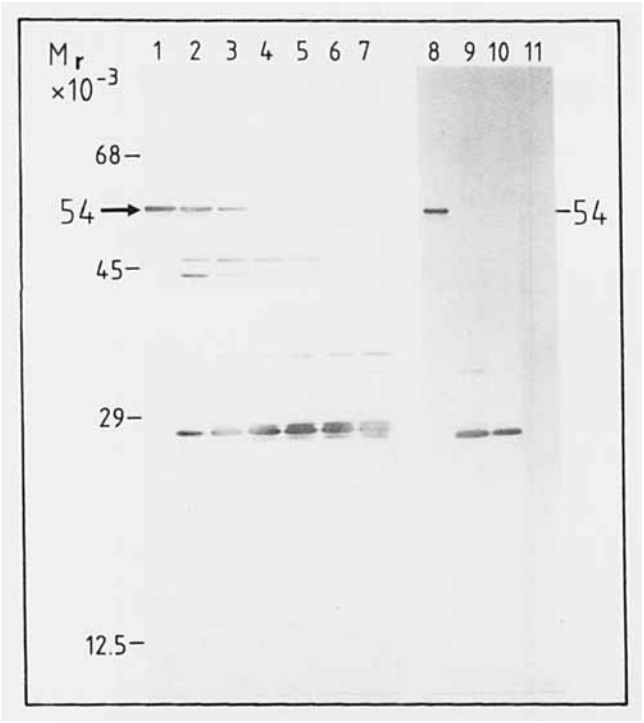

Fig. 6. *Idenfication of elastase cleavage products of the 54-kDa SRP protein with mAb 54.1.* A SRP-enriched fraction, after chromatography on ω-aminopentyl agarose (0.6 A_{280} ml), was digested with no (lanes 1 and 8), 2.5 (lane 2), 4 (lane 3), 5.5 (lane 4), 7 (lane 5), 8.5 (lane 6), 10 (lane 7), 100 (lane 9), 200 μg/ml (lane 10) elastase for 30 min at 37 °C. Lane 11 contains the same amount of elastase as lane 10 but no SRP. Protein was characterized by SDS-PAGE and Western blotting using mAb 54.1

Antibodies to SRP proteins

In order to be able to characterize individual SRP proteins and their proteolytic fragments, we raised antibodies against SRP proteins in rabbits. SRP was purified and proteins were separated by SDS-PAGE. Bands containing the individual proteins were cut out from the gel; the proteins were eluted and used for the immunization of rabbits or mice. The purity of the proteins was tested by SDS-PAGE and silver staining (Fig. 3). Polyclonal antibodies against the 72, 68, 54 and 19-kDa proteins were raised in rabbits and a monoclonal antibody (mAb) against the 54-kDa protein (mAb 54.1) was obtained in mice. The specificity of all antibodies was tested by Western blot analysis on salt-extracted proteins from rough microsomes and on gradient-purified SRP proteins separated by SDS-PAGE.

Fig. 4 shows that each of the polyclonal antibodies is monospecific for an individual SRP protein and Fig. 5 shows the same for the monoclonal antibody. Silver-stained SRP after SDS-PAGE is shown in Fig. 4 under 'SRP'. The lanes 0 in each panel show immunoblots of SRP proteins tested with the antibodies indicated on top of the lanes. In each case the expected antigen is recognized by the respective antibody. Fig. 5 shows the immunoblot analysis with mAb 54.1. The left panel shows Ponceau S staining of proteins transferred onto nitrocellulose. The middle panel in Fig. 5 shows that mAb 54.1 reveals a single protein in fractions 4 and 5 with a molecular mass of 54-kDa. In the right panel RNA in sucrose gradient fractions was analysed on polyacrylamide gels containing 8 M urea. 7S RNA was found in fractions 4 and 5 which also contained the SRP proteins.

Elastase treatment generates discrete domains of SRP proteins

Proteases such as elastase and papain have been used successfully to characterize protein domains in large protein complexes or in single monomeric proteins [26, 27]. We probed the domain structure of SRP proteins by digesting SRP with 2.5, 10 and 25 μg/ml elastase for 30 min at 30 °C. The resulting protein fragments were separated by SDS-PAGE and identified by Western blotting using monospecific antibodies against SRP proteins.

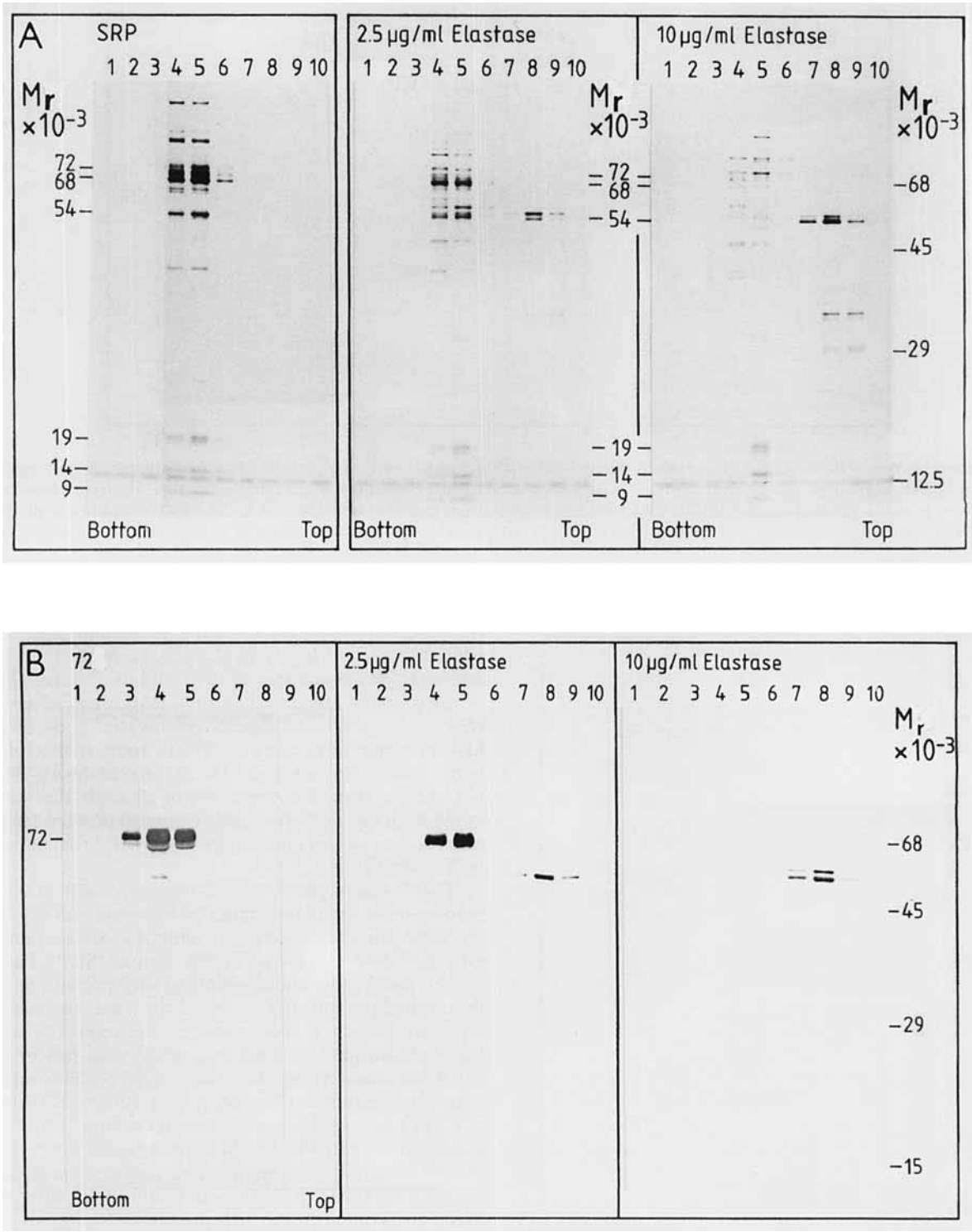

Fig. 7. *Sucrose gradient centrifugation of SRP before and after treatment with 2.5 and 10 µg/ml elastase.* Gradient-purified SRP (0.6 A_{280}/ml) was treated with no, 2.5 µg/ml or 10 µg/ml elastase for 30 min at 37°C. The components were separated by sucrose gradient centrifugation and, after fractionation from the bottom, proteins from each fraction were separated by SDS-PAGE. Either proteins were silver-stained (A) or individual SRP proteins were characterized by Western blotting with anti-(72-kDa protein) (B), anti-(68-kDa protein) (C) and anti-(54-kDa protein) (D) polyclonal antibodies. Positions of intact SRP proteins are indicated on the left, positions of molecular mass marker proteins on the right. Numbers above the lanes correspond to fraction numbers

The results in Fig. 4 show that treatment of SRP with 2.5, 10 and 25 µg/ml elastase degrades the 72, 68, 54 and 19-kDa proteins in discrete steps. The resulting fragments were characterized using the polyclonal antibodies. The 72-kDa protein is converted to 68, 55 and 53-kDa fragments; the 68-kDa protein to 55, 54 and 30-kDa fragments and the 54-kDa protein to 35-kDa and 27-kDa fragments. Elastase digests of SRP were also tested with mAb 54.1 revealing fragments of 45,

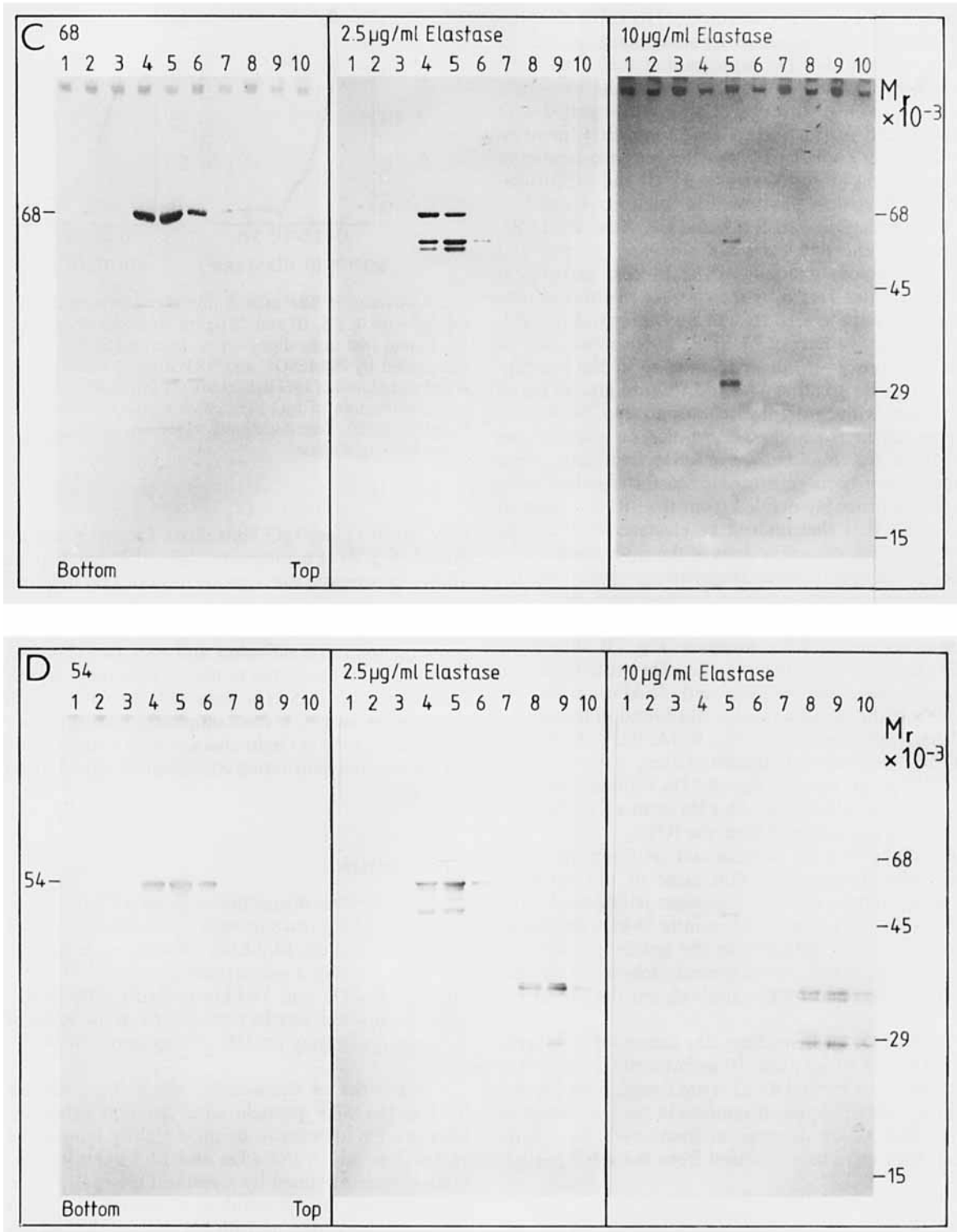

Fig. 7

35 and 27-kDa (Figs 5 and 6). The 45-kDa fragment cannot always be detected. It seems to be a rapidly converted intermediate (cf. Figs 4, 6 and 7D).

At elastase concentrations of 100 µg/ml and 200 µg/ml the 72-kDa and 68-kDa proteins became undetectable with our antibodies. In contrast a 27-kDa domain of the 54-kDa SRP protein remained resistant to treatment with elastase at 100 µg/ml and 200 µg/ml. This domain was recognized by mAb 54.1 (Fig. 6).

Protein domains released from SRP upon elastase treatment

The proteins in the SRP could be bound to the 7SL RNA either directly or indirectly via protein–protein interaction. In order to detect protein domains which can be released from the RNP particle, we digested SRP with 2.5 µg/ml and 10 µg/ml elastase and separated the resulting SRP subparticle from the released fragments on sucrose density gradients. The gradient was fractionated from the bottom and proteins and

RNA from each fraction were characterized by gel electrophoresis. Total protein was visualized by silver staining and the individual 72, 68 and 54-kDa proteins and their fragments were detected by immunoblot analysis using the respective polyclonal antisera (Fig. 7). Control SRP, not digested with elastase, was found in fractions 4 and 5 (see SRP proteins as indicated in Fig. 7A left panel). After elastase treatment (2.5 μg/ml and 10 μg/ml for 30 min at 37°C) and centrifugation, the SRP subparticle was found in fractions 4 and 5 as judged by the presence of 7SL RNA and the three small SRP proteins (data on the RNA are not shown). The size and position in the sucrose gradient of the 14-kDa and 9-kDa proteins remained unaffected by the elastase treatments. The 19-kDa protein was processed to a 18.5-kDa form (Fig. 7A).

In the gradient with control SRP, no proteins can be detected in fractions 7–10 close to the top of the gradient (Fig. 7, 'SRP'). After treatment with 2.5 μg/ml and 10 μg/ml elastase, proteins with molecular masses around 54, 35 and 30 kDa appeared in fractions 7–9. Since no protein was present in these fractions before elastase treatment, these peptides must have been generated from faster-sedimenting material and are probably derived from the SRP proteins. It should be noted that the amount of elastase used for the digestion is below the detection limit of the silver stains.

In order to identify protein fragments generated by the elastase treatment and the gradient fractionation, as shown in Fig. 7A, antibodies against the 72, 68 and 54-kDa proteins were used in an immunoblot analysis. Fig. 7B shows the immunoblot analysis using the anti-(72-kDa protein) serum. With 2.5 μg elastase the 68-kDa and 55-kDa forms are generated. While the 68-kDa form is still found in fractions 4 and 5 and therefore bound to the 7SL RNA, the 55-kDa form is released and found largely in fraction 8 close to the top of the gradient. At 10 μg/ml elastase the 68-kDa form disappeared completely and in addition to the 55-kDa form a 53-kDa form appears, which is also released from the RNA.

Analysis of the 68-kDa protein and its fragments after gradient centrifugation reveals that none of the antigenic fragments generated by elastase treatment is liberated from the 7SL RNA (Fig. 7C). The 56, 54 and the 30-kDa fragments are all detected by the antibody in the gradient fraction 5 containing the 7SL RNA. No fragments related to the 68-kDa protein are detected by the antibody on the top of the gradient.

From the 54-kDa protein three size classes of fragments are generated with 2.5 μg/ml and 10 μg/ml elastase (Fig. 7D). Fragments with sizes around 45-kDa are found in fractions 4 and 5 with the 7SL RNA, but fragments in the size ranges of 35-kDa and 28-kDa are detected in fractions 8 and 10 indicating that they have been released from the RNP particle (Fig. 7D).

Effect of elastase treatment on SRP activity

After having established conditions for proteolytically generating defined fragments from SRP proteins, we tested SRP digested with 2.5, 10 and 25 μg/ml elastase for its ability to arrest elongation of IgG light chain or to promote translocation of IgG light chains across salt-washed microsomal membranes (RM-K). When mRNA from MOPC 41 tumors was translated in the absence of SRP in the wheat germ cell-free system, the major translation product is the pre-IgG light chain. When SRP is included in the translation, pre-IgG light chain synthesis is arrested by about 70% (4 and Fig. 8). When SRP, digested with 2.5 μg/ml elastase, is added to the translation, arrest of pre-IgG light chain synthesis is drastically reduced (Fig. 8). Furthermore, this SRP has lost most of its ability to promote the translocation of IgG light chain across endoplasmic reticulum membranes. Note that at an elastase concentration of 2.5 μg/ml some of the 68-kDa and 54-kDa proteins are still unaffected and that the 72-kDa protein is quantitatively converted to the 68-kDa form and the 19-kDa protein to the 18.5 kDa form. SRP treated with 10 μg/ml and 25 μg/ml elastase has completely lost its ability to arrest synthesis of pre-IgG light chain as well as to promote translocation of light chain across endoplasmic reticulum membranes (Fig. 8).

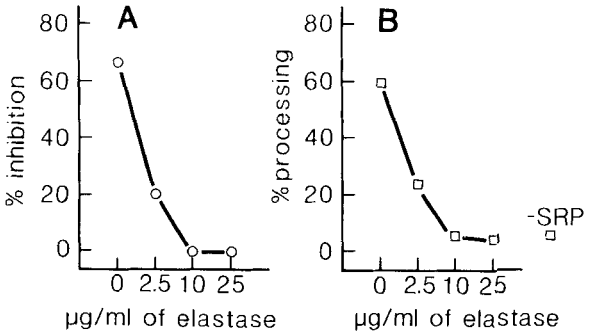

Fig. 8. *Activity of SRP after limited digestion with elastase.* SRP was treated with 0, 2.5, 10 and 25 μg/ml elastase and incubated at 30°C for 30 min (the same digestion as described in Fig. 4). Elastase was inactivated by PhMeSOF and SRP directly tested for its ability to arrest elongation of IgG light chain (% inhibition) (A) or for promoting translocation of IgG light chain across salt-washed microsomal membranes (B). Translocation is expressed as percentage processing of pre-IgG light chain

DISCUSSION

Proteins were disassembled from SRP in two consecutive steps. In 2 M KCl two protein heterodimers, consisting of the 72/68-kDa and the 14/9-kDa subunits, were dissociated from the SRP, leaving a subparticle composed of the 7SL RNA and the 54-kDa and 19-kDa proteins. The 54-kDa protein could then selectively be removed from the RNA subparticle by chromatography on DEAE-Sepharose in Mg^{2+}-free buffer.

This order of disassembly shows that the proteins are held in the SRP particle with different affinities. The 19-kDa protein appears to be most tightly bound to the RNA. Heterodimers of 72/68-kDa and 14/9-kDa protein have previously been obtained by a method using EDTA for the disassembly and DEAE-cellulose as matrix. Reconstitution experiments by Walter and Blobel showed that all SRP protein subunits, except for the 54-kDa protein, can bind directly to the 7SL RNA. The 54-kDa protein requires the presence of the 19-kDa protein on the 7SL RNA in order to bind [19].

The domain structure of the four large SRP proteins was characaterized by treatment of SRP with elastase, followed by sucrose gradient centrifugation and identification of the proteolytic fragments by specific antibodies. The sizes of the fragments obtained are summarized in Fig. 9. The figure also indicates which of the fragments remain bound to the RNP particle (encircled) and which are released. A 55-kDa fragment is released from the 72-kDa protein and fragments in the range of 35 kDa from the 54-kDa protein.

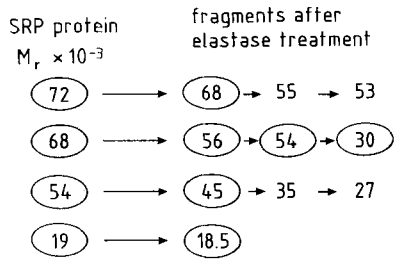

Fig. 9. *Summary of fragment sizes produced from SRP proteins upon cleavage with elastase.* Apparent molecular masses are estimated from the migration on SDS/polyacrylamide gels using the molecular mass markers described in Fig. 4. Proteins or fragments cosedimenting with 7SL RNA are encircled

Many proteins have been shown to be organized in several domains of which some perform a distinct function. Proteases have been used as tools for probing the domain structure of a number of proteins. Among them are the docking protein [26], the class I histocompatibility antigens [28] and clathrin, the major surface protein of coated vesicles [27]. Papain, for instance, cleaves the class I antigens into two segments, one responsible for anchoring the protein in the membrane and one that extends to the outside of the cell and interacts with a receptor on T cells. It is quite conceivable that the proteoloytic fragments generated from the 72-kDa and 54-kDa SRP proteins represent functionally distinct domains. It is likely, for instance, that the 72-kDa protein contains a small domain that retains this protein in the RNP particle. We find that the 72-kDa SRP protein at the lowest concentration of elastase is converted into a 68-kDa form, which still binds to the RNP particle. However, the 55-kDa fragment generated from the 72-kDa protein at higher elastase concentration is released from the SRP. Thus a protein segment of about 13-kDa may contain that region of the 72-kDa SRP protein, which retains the protein in the RNP particle. As the 72-kDa SRP protein forms a heterodimer with the 68-kDa protein, it is likely that the 13-kDa segment of the 72-kDa protein interacts with the 68-kDa protein and that the 72-kDa protein does not directly interact with RNA. The 68-kDa protein would then be responsible for binding the 72/68-kDa dimer to the 7SL RNA. This view is supported by the finding that all fragments generated by the elastase treatment from the 68-kDa protein are found associated with the RNP particle.

The 54-kDa protein requires for its binding to the 7SL RNA the presence of the 19-kDa protein [19]. It is still unclear whether the 54-kDa protein interacts with the 19-kDa protein or whether the binding of the 19-kDa protein changes the conformation of the 7SL RNA such that the 54-kDa protein is then able to bind to the RNA. The 35-kDa domains of the 54-kDa protein, generated by elastase treatment, are released from the RNP particle. A 45-kDa form of the 54-kDa protein was still retained on the RNP particle. From this it can be concluded that a segment of about 10 kDa might be responsible for binding the 54-kDa protein to the RNP particle. It has recently been shown that the 54-kDa protein can be crosslinked to the signal sequence of nascent preprolactin [8]. It is conceivable that this binding occurs to the 35-kDa or 28-kDa domains identified here by the elastase digestion.

SRP digested with 2.5 μg/ml elastase retained about 40% of its ability to arrest pre-IgG light chain synthesis and about the same percentage of its ability to promote translocation across endoplasmic reticulum membranes. At this level of digestion the 72-kDa protein is completely converted to the 68-kDa and 55-kDa forms and the 19-kDa protein to its 18.5-kDa form. By correlating this cleavage pattern to SRP function it can be concluded that the conversion of the 72-kDa protein to its 68-kDa forms and of the 19-kDa protein to its 18.5-kDa form might not affect SRP function negatively. SRP treated with 10 μg/ml elastase completely lost all of its functions. At this elastase concentration essentially all of the 72-kDa protein was converted to the free 55-kDa domain and the 54-kDa protein to the free 35-kDa domain. The 68-kDa protein was cleaved to its 54-kDa form. The 14-kDa and 9-kDa proteins appear to remain unaffected by this level of elastase. Most likely it is the release of the two large protein domains from the 72-kDa and 54-kDa proteins that is responsible for the loss in SRP functions.

The elongation-blocking and the translocation-promoting activities of SRP may reside in distinct portions of the SRP [15, 16]. Structurally SRP can be divided into two subparticles; a small one consisting of the two Alu-like segments at the 3′ and 5′ ends and the 14/9-kDa dimer, and a large one consisting of the central S fragment with the 72, 68, 54 and 19-kDa proteins. Siegel and Walter [16] showed that the S-fragment-containing particle still retained some translocation-promoting activity, but failed to block elongation of preprolactin. The elongation block activity might, therefore, require the part of SRP containing the Alu sequence. The loss of elongation block activity of SRP containing intact 7SL RNA and 14/9-kDa proteins would suggest that the proteins binding to the S fragment are also important for the elongation arrest function of SRP.

The identification of the SRP protein domains will enable studies on their functional interactions.

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