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The methionine-rich domain of the 54 kd protein subunit of the signal recognition particle contains an RNA binding site and can be crosslinked to a signal sequence

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The 54 kd protein subunit of the signal recognition particle (SRP54) has been shown to bind signal sequences by UV crosslinking. Primary structure analysis and phylogenetic comparisons have suggested that SRP54 is composed of two domains: an amino-terminal domain that contains a putative GTP-binding site (G-domain) and a carboxy-terminal domain that contains a high abundance of methionine residues (M-domain). Partial proteolysis of SRP revealed that the two proposed domains of SRP54 indeed represent structurally discrete entities. Upon proteolysis the intact G-domain was released from SRP, whereas the M-domain remained attached to the core of the particle. Reconstitution experiments demonstrated that the isolated M-domain associates with 7SL RNA in the presence of SRP19. In addition, we observed a specific binding of the M-domain directly to 4.5S RNA of *Escherichia coli*, which contains a structural motif also present in 7SL RNA. This shows that the M-domain contains an RNA binding site, and suggests that SRP54 may be linked to the rest of SRP through this domain by a direct interaction with 7SL RNA. Using UV crosslinking, we found that in an *in vitro* translation system the preprolactin signal sequence contacts SRP through the M-domain of SRP54. These results imply that the M-domain contains the signal sequence binding site of SRP54, although we cannot exclude that the G-domain may also be in proximity to bound signal sequences. The results are consistent with our previous hypothesis that the M-domain contains a signal sequence binding pocket composed, in part, of a number of amphipathic α-helices with clusters of methionines exposed on one face.

Key words: GTP binding protein/protein-RNA interaction/proteolytic dissection/SRP/UV crosslinking

Introduction

The signal recognition particle (SRP) is an 11S cytoplasmic ribonucleoprotein which mediates targeting of secretory and membrane proteins to the membrane of the endoplasmic reticulum (reviewed in Walter and Lingappa, 1986). SRP is composed of six distinct protein subunits and one molecule of 7SL RNA (Walter and Blobel, 1982). Crosslinking experiments have shown that the 54 kd subunit of SRP (SRP54) interacts directly with signal sequences as they emerge from the ribosome (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986).

Recently the amino acid sequence of mammalian SRP54 was deduced from cDNA sequences (Bernstein *et al.*, 1989; Römisch *et al.*, 1989). The predicted protein is composed of an N-terminal segment that contains the consensus sequence for GTP binding (G-domain) and a C-terminal segment characterized by an unusually high content of methionine (M-domain). A closely homologous putative GTP binding segment was found in the α-subunit of the SRP receptor (SRα, docking protein), but in contrast to SRP54, SRα contains this region at its C terminus. Alignment of the SRP54 and SRα sequences therefore defined a sharp boundary between the two segments of SRP54, providing the rationale for their designation as 'domains'. GTP binding to SRP54 has recently been confirmed experimentally (J.Miller and P.Walter, unpublished). Other work has shown that SRα is also a GTP binding protein and that GTP is required for the release of SRP from the signal sequence and ribosome on targeting to the endoplasmic reticulum membrane (Connolly and Gilmore, 1989). The structural features of SRP54 are conserved in homologous proteins identified in *E.coli* and yeast (Bernstein *et al.*, 1989; Hann *et al.*, 1989; Römisch *et al.*, 1989; Amaya *et al.*, 1990).

The structure of SRP proteins has been analyzed by mild proteolytic digestion of SRP (Scoulia *et al.*, 1987). In the case of SRP54 it was shown that elastase generated a 45 kd proteolytic fragment which remained bound to the core particle. Increasing concentrations of elastase resulted in SRP54 fragments in the range of 27-35 kd which were released from the particle. The digestion products were not mapped with respect to the SRP54 primary structure, however.

In the experiments reported here we have continued to use partial proteolysis to examine whether there is a biochemical basis for the domain structure of SRP54 predicted from sequence analysis. Furthermore, this type of analysis has made it possible to map the attachment site of SRP54 to the core of SRP and to analyze the signal sequence binding site within SRP54. The latter question is of particular interest because a single SRP54 species appears to be able to recognize signal sequences that are very heterogeneous in primary sequence. This work is a first step towards discriminating experimentally between two apparently opposing models that have been proposed to explain the phenomenon of signal recognition in molecular terms. Based on secondary structure predictions and phylogenetic comparison we suggested that the M-domain mediates binding of signal sequences (Bernstein *et al.*, 1989). In contrast, based on a weak sequence homology of the G-domain to the heat shock protein hsp70, Römisch *et al.* (1989) suggested that the signal sequence binding site resides in the G-domain.

Results

To analyze the structure of SRP54 we generated antibodies that would recognize its extreme N- and C-termini. For this purpose, polyclonal antisera were raised against synthetic peptides comprising amino acids 2–10 and amino acids 485–493 of SRP54, respectively. SRP proteins were separated by SDS–PAGE (Figure 1A, lane 1), transferred onto nitrocellulose, and probed with the antiserum obtained against the N-terminal (Figure 1A, lane 4, 'αN/54') or C-terminal (Figure 1A, lane 6, 'αC/54') peptide. Note that both antisera recognized only SRP54. Antibody binding could be abolished by competition with the corresponding peptides (Figure 1A, lanes 3 and 5), thereby further corroborating the specificity of the sera. SRP54 was also recognized by a monoclonal antibody (Scoulia *et al.*, 1987) (Figure 1A, lane 2, 'mab54').

We next sought protease conditions that would allow us to dissect SRP54 into discrete proteolytic fragments. SRP was subjected to limited proteolysis using different proteases, and the digestion products derived from SRP54 were identified with the αN/54 and αC/54 sera by Western blot analysis. Figure 1B (lanes 2 and 3) shows that digestion with V8 protease generated a 22 kd fragment which reacted exclusively with αC/54 (Figure 1B, lane 3, 'C22'). A second proteolytic fragment of 33 kd was generated, which reacted exclusively with αN/54 (Figure 1B, lane 2, 'N33'), and contained the epitope recognized by mab 54 (Figure 1B, lane 1, 'mab54'). The sum of the molecular masses estimated for the N- and C-terminal fragments approximated 54 kd, suggesting that they were derived from a single proteolytic cleavage of SRP54. Most notably, the molecular masses of these fragments indicated that the cleavage occurred almost precisely at the boundary between the N-terminal G-domain and the C-terminal M-domain which was previously proposed on the basis of sequence analysis and phylogenetic comparison (Bernstein *et al.*, 1989; Hann *et al.*, 1989; Römisch *et al.*, 1989; Amaya *et al.*, 1990). From the primary structure the calculated molecular mass is 32.5 kd for the G-domain (corresponding to N33) and 23.3 kd for the M-domain (corresponding to C22).

These digestion conditions were used to determine which of the two domains contains the site of attachment of SRP54 to the core of SRP. After digestion with V8 protease, SRP was sedimented on sucrose gradients and the proteolytic fragments derived from SRP54 were detected by immunoblotting of each gradient fraction with both αN/54 and αC/54 sera (Figure 2A). Note that some SRP54, recognized by both sera, remained undigested and sedimented at ~11S, the position of intact SRP (Figure 2A, lane 5). The M-domain of SRP54 (C22) sedimented in the same fraction (Figure 2A, lane 5) indicating that it remained associated with SRP after digestion. In contrast, the G-domain (N33) was released from SRP and was recovered near the top of the gradient (Figure 2A, lane 2).

Digestion with a different protease, elastase, gave very similar results (Figure 2B). Elastase digestion resulted in two C-terminal fragments of 24.5 kd and 22.5 kd recognized by αC/54 which cosedimented with SRP (Figure 2B, lane 5, 'C24.5' and 'C22.5') and two N-terminal fragments of 33.5 kd and 32.5 kd recognized by αN/54 which were released from SRP (Figure 2B, lane 'N33.5' and 'N32.5'). These results indicate that the M- and G-domains of SRP54 are linked by a region that is susceptible to cleavage by

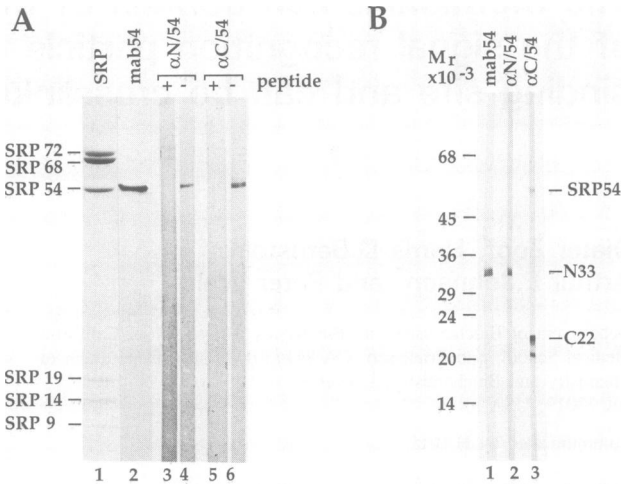

Fig. 1. Proteolytic dissection of SRP54. **Panel A:** SRP proteins (2 μg/lane) were fractionated by electrophoresis on a 10–15% SDS–polyacrylamide gradient gel, visualized by staining with Coomassie blue (lane 1) or transferred to nitrocellulose (lanes 2–5). The nitrocellulose strips were probed with a monoclonal antibody against SRP54 (lane 2; 'mab54'), or with polyclonal antisera against the N-terminal (lanes 3 and 4; 'αN/54') or the C-terminal peptide of SRP54 (lanes 5 and 6; 'αC/54'). Strips were probed in the absence (lanes 4 and 6, '–') or presence (lanes 3 and 5, '+') of 0.5 μg/ml of the corresponding peptide. The positions of the SRP proteins are indicated. **Panel B.** SRP (2 μg/lane) was digested with V8 protease and analyzed on Western blots using mab54 (lane 1), αN/54 (lane 2) and αC/54 (lane 3). Dilution of peptide antisera was 1:200. The proteolytic fragments of SRP54 ('C22' and 'N33') are indicated.

proteases with different substrate specificities, and hence may constitute a flexible hinge between the two domains. The relative protease resistance of the G- and M-domains suggests that they are indeed discrete compactly folded entities. Immunoblotting with a specific antiserum revealed that SRP19, which was previously shown to be required for stable binding of SRP54 to 7SL RNA, was not digested by the protease in these experiments (data not shown).

We next digested isolated SRP54 with protease in order to test whether the M-domain was capable of associating with 7SL RNA. SRP was dissociated into RNA and protein components under non-denaturing conditions as previously described (Siegel and Walter, 1985), and the proteins were fractionated from one another. Purified SRP54 was then digested with V8 protease (see Materials and methods) and the digestion products were fractionated by SDS–PAGE and visualized by silver staining (Figure 3, lane 1). Note that the pattern of proteolytic fragments generated was very similar to that observed after digestion of intact SRP (Figure 1B, lanes 2 and 3). N33 and C22 fragments were identified by immunoblotting (data not shown). An additional fragment of 27 kd ('N27') was observed after prolonged digestions. N27 was also immunoreactive with αN/54 (data not shown), and hence is likely to represent a further degradation product of N33. The two bands around 40 kd ('V8') were contributed by the V8 protease preparation.

After inhibition of the protease, the SRP54 digestion products (Figure 3, lane 1) were incubated with RNA under conditions that allow reconstitution of SRP (Siegel and Walter, 1985). We used 7SL RNA, *E.coli* 4.5S RNA [which was recently shown to bind specifically to SRP54 (K.Strub, D.Zopf, and P.Walter, unpublished) or tRNA, as a negative

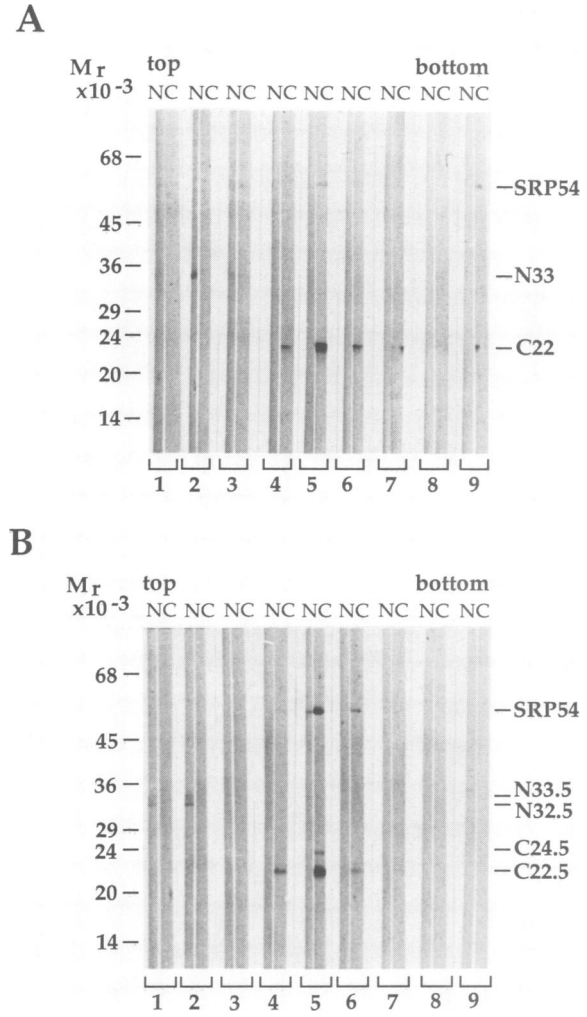

Fig. 2. Sedimentation of proteolytic fragments of SRP54 in sucrose gradients. Sucrose-gradient purified SRP (2 μg) was digested with *Staphylococcus aureas* V8 protease (**panel A**) or elastase (**panel B**) as described in Materials and methods and sedimented on a 5–20% (w/v) sucrose gradient in a SW60 rotor. The gradients were fractionated from the top into nine fractions. One-fourth of each fraction was analyzed by Western blot. After the fragments were transferred to nitrocellulose each lane was cut into two strips and reacted with either αN/54 ('N') or αC/54 ('C') antisera. Immunoreactive products are indicated on the left margin.

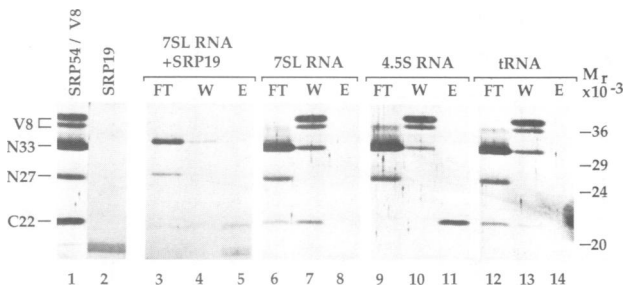

Fig. 3. The M-domain of SRP54 binds to 7SL RNA and *E.coli* 4.5S RNA. SRP54 was purified from disassembled SRP (Siegel and Walter, 1985) and digested with V8 protease (lane 1). Equal amounts of proteolytic products were added to reconstitution reactions containing 7SL RNA plus SRP19 (lanes 3–5), 7SL RNA alone (lanes 6–8), 4.5S RNA (lanes 9–11) or calf liver tRNA (lanes 12–14). The mixtures were loaded onto DEAE–Sepharose CL-6B columns in a buffer containing 250 mM KOAc. Equivalent amounts of the flow-through ('FT', lanes 3, 6, 9 and 12), wash ('W', lanes 4,7, 10 and 13; 350 mM KOAc, four column volumes) and eluate fractions ('E', lanes 5, 8, 11 and 14; 1 M KOAc, four column volumes) were analyzed by SDS–PAGE and silver staining. Bands contributed by V8 protease and proteolytic fragments of SRP54 are indicated. Due to a lack of uniformity in the silver staining procedure, the bands in lanes 2–5 are less pronounced than in the other lanes.

control (Figure 3)]. In addition, one reconstitution reaction containing 7SL RNA was also supplemented with SRP19. After reconstitution, the binding of RNA to protein was monitored by chromatography on DEAE–Sepharose columns (Lingelbach *et al.*, 1988; Strub and Walter, 1990). This assay is based on the observation that in the absence of an appropriate RNA, SRP proteins do not interact with the resin and are recovered in the flow-through and wash fractions (Figure 3, 'FT' and 'W'), whereas proteins that have assembled with the RNA are retained and elute at elevated salt (Figure 3, 'E'). Note that both proteolytic products which were derived from the G-domain of SRP54 (N33 and N27) were recovered exclusively in the flow-through or wash fractions (Figure 3, lanes 3, 4, 6, 7, 9, 10, 12 and 13), irrespective of the RNA species used for reconstitution. In contrast, the M-domain (C22) associated with 7SL RNA in the presence of SRP19 and was recovered mainly in the eluted fraction (Figure 3, lane 5). Some of the M-domain also appeared in the flow-through and wash fractions, probably due to limiting amounts of SRP19 present in the reconstitution reaction (Figure 3, lanes 3 and 4). No stable binding of the M-domain to 7SL RNA was observed under these conditions in the absence of SRP19 (Figure 3, lane 7). However, the M-domain bound very efficiently to *E.coli* 4.5S RNA (Figure 3, lane 11), even if SRP19 was not present.

The ability to dissect SRP54 into two defined domains with distinct properties allowed us to map the site to which signal sequences are crosslinked. For this purpose SRP was added to a wheat germ translation system in which a preprotein containing a photoreactive amino acid analog was synthesized. The translation reactions contained [^{35}S]methionine to label the nascent polypeptide chains and N^{ϵ}-(5-azido-2-nitrobenzoyl)-Lys-tRNA to render the nascent chains photoreactive through the incorporation of N^{ϵ}-(5-azido-2-nitrobenzoyl)-lysine at the N-terminal end of the preprolactin signal sequence (positions −27 and −22). Polypeptide elongation was arrested by SRP after the signal sequence became exposed outside the ribosome, and the signal sequence of the arrested preprolactin fragment (AF) was crosslinked to SRP54 by UV irradiation. The SRP*AF was then released from the ribosome by incubation with puromycin in high salt and isolated from the translation reactions by sucrose gradient centrifugation (Figure 4). The prominent 62 kd band sedimenting at 11S corresponds to the previously described SRP54*AF crosslink (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986). This was confirmed by immunoprecipitation with anti-prolactin and anti-SRP54 (not shown). Since AF has a molecular mass of ~8 kd, the molecular masses of AF and SRP54 are approximately additive in the crosslinked product.

Sucrose gradient fractions containing the crosslinked products (Figure 4, lane 11) were digested with V8 protease. One major digestion product of ~27 kd ('C22*AF') was detected (Figure 5, lane 1). Upon sedimentation of the digested material on the second sucrose gradient, C22*AF, as well as some undigested SRP54*AF, were found to

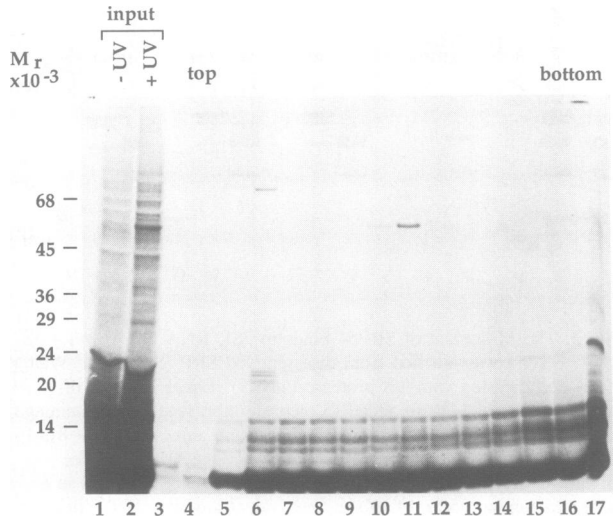

Fig. 4. Sucrose gradient purification of SRP*AF after release with puromycin. A 20 μl aliquot was removed from a 300 μl *in vitro* translation reaction before (lane 1, '−UV') or after UV irradiation (lane 2, '+UV'). The remainder was treated with 1 mM puromycin and 500 mM KCl (Blobel and Sabatini, 1971) to release nascent chains and SRP*AF from ribosomes and subsequently sedimented on a 5−20% (w/v) sucrose gradient in a SW40 rotor. One-tenth of each gradient fraction was analyzed. After precipitation with TCA, proteins were separated on 10−15% SDS−polyacrylamide gels. The AF and SRP*AF were visualized by autoradiography. As previously observed (Krieg *et al.*, 1986), the AF was distributed throughout the gradient, possibly due to self-aggregation. The less prominent crosslink product of 80 kd sedimenting at 11S (lane 11), previously noted but not identified (Krieg *et al.*, 1986), represents a crosslinked product between SRP72 and AF. We found that this product could be specifically immunoprecipitated with antibodies to SRP72 (Walter and Blobel, 1983c) and to prolactin (data not shown).

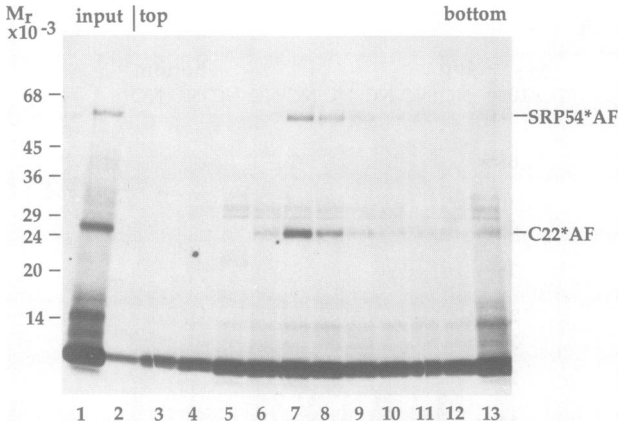

Fig. 5. Sedimentation of SRP*AF proteolytic products. SRP*AF was concentrated by DEAE−Sepharose chromatography and material derived from a 200 μl translation reaction was digested with V8 protease (see Materials and methods). One-fourth of the digestion reaction was immediately precipitated with 10% (w/v) TCA (lane 1 'input') and the remainder was sedimented on a 5−20% (w/v) sucrose gradient. Gradient fractions (lanes 2−12) and the pellet fraction recovered from the bottom of the tube (lane 13) were TCA precipitated, subjected to SDS−PAGE and analyzed by autoradiography.

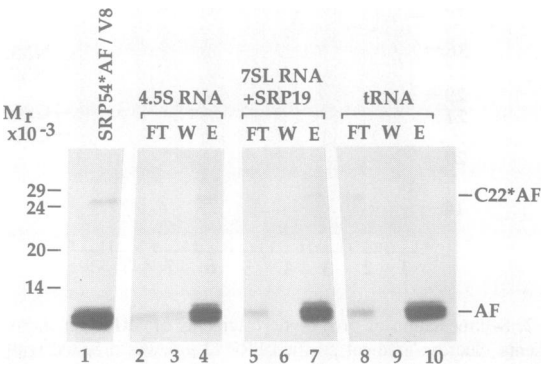

Fig. 6. Binding of the C22*AF proteolytic fragments to 7SL RNA and 4.5S RNA. Sucrose gradient purified SRP*AF derived from a 300 μl translation reaction (see Figure 4, lane 11) was disassembled and SRP54*AF was purified (see Materials and methods). SRP54*AF was digested with V8 protease and the reaction mixture was divided into four aliquots. One aliquot was analyzed directly by SDS−PAGE and autoradiography (lane 1) and the other aliquots were added to reconstitution reactions containing either 7SL RNA and SRP19 (lanes 5−7), 4.5S RNA (lanes 2−4) or calf liver tRNA (lanes 8−10). Analysis of reconstitution reactions was performed as described in Figure 3 except that the bands were visualized by autoradiography.

sediment at ~11S (Figure 5, lanes 7 and 8). This result suggested that AF was crosslinked to the M-domain of SRP54. In addition the apparent molecular mass of the crosslinked product approximates the sum of C22 and AF (22 kd + 8 kd ≈ 27 kd).

Unfortunately, we were unable to confirm these results with the anti-peptide antibodies. While these antibodies worked well on Western blots, under no condition was it possible to immunoprecipitate SRP54 or any of its fragments with αN/54 or αC/54. Therefore, to verify that C22*AF indeed contained the M-domain of SRP54, we tested whether isolated C22*AF could assemble with 7SL RNA and 4.5S RNA as shown above for C22. SRP*AF was purified on a sucrose gradient as shown in Figure 4, and then disassembled into protein and RNA components under conditions similar to those used to disassemble uncrosslinked SRP. Dissociated SRP54*AF was further enriched by chromatography on CM−Sepharose (see Materials and methods) and then digested with V8 protease, yielding C22*AF (Figure 6, lane 1). A considerable amount of non-covalently bound AF was also recovered in this fraction (Figure 6, lane 1). We reconstituted the digestion products with 7SL RNA in the presence of SRP19 (Figure 6, lanes 5−7), with 4.5S RNA (Figure 6, lanes 2−4) or with tRNA (Figure 6, lanes 8−10) and fractionated the mixture on DEAE−Sepharose as described in Figure 3. Note that C22*AF specifically associated with 7SL RNA in the presence of SRP19 and was recovered in the eluted fraction (Figure 6, lane 7). Specific binding was also observed to 4.5S RNA (Figure 6, lane 4), but not to tRNA (Figure 6, compare lanes 8 and 10). These data corroborate the results of the co-sedimentation experiment, and further suggest that C22*AF represents a crosslinked product of the M-domain and AF.

Discussion

We have defined proteolysis conditions which have permitted the dissection of SRP54, the signal sequence binding subunit of SRP, into two domains: the N-terminal G-domain and the C-terminal M-domain. The relative protease resistance of both domains suggests that they are indeed structurally separate, compact entities. In contrast, the region connecting the two domains is susceptible to cleavage by proteases with

completely different substrate specificities (Figure 2), and hence may provide a flexible hinge. These results confirm biochemically the domain structure of SRP54 that was predicted solely from the comparison of the primary structures of mammalian SRP54 and SRα and from the phylogenetic conservation of SRP54 homologs in yeast and *E.coli* (Bernstein *et al.*, 1989; Hann *et al.*, 1989; Römisch *et al.*, 1989; Amaya *et al.*, 1990).

The ability to separate physically the two domains of SRP54 has made it possible to investigate by UV crosslinking which domain contacts the signal sequence of preprolactin. A crosslink of the signal sequence to the M-domain of SRP54 was consistently observed. This result lends strong support to the notion that the M-domain is directly involved in signal sequence binding. In our approach, the crosslinking reagent was incorporated into the N-terminal region flanking the hydrophobic core of the signal sequence of a nascent protein and presented to SRP in the context of a translating ribosome. Signal sequences containing the crosslinking reagent were still fully competent to promote elongation arrest and membrane translocation. Hence the crosslinked products described here are likely to reflect a functionally meaningful interaction between the signal sequence and SRP.

The finding that the preprolactin signal sequence interacts with the M-domain is consistent with our previous proposal that the methionines in the M-domain play an important role in signal sequence recognition. We have proposed that the signal sequence binding pocket is composed, in part, of a number of amphipathic α-helices that bear clusters of methionines on one face (Bernstein *et al.*, 1989; Hann *et al.*, 1989). According to this model, the methionine side chains form a flexible hydrophobic groove with sufficient plasticity to accommodate different signal sequences despite their heterogeneity in primary structure. A precedent for the notion that methionine residues can be involved in the recognition of a diverse set of substrates comes from elegant studies on calmodulin, which binds to target proteins by recognizing amphipathic α-helices (reviewed in O'Neil and DeGrado, 1990). The X-ray structure of calmodulin reveals two hydrophobic surface patches that are rich in methionine residues (Babu *et al.*, 1988). Crosslinking studies of synthetic amphipathic helices to calmodulin have shown directly that, depending on the location of the crosslinking probe within the peptide, individual methionine residues are contacted (O'Neil *et al.*, 1989). Binding of the hydrophobic surface of amphipathic helices with vastly different sequences is thought to occur in a pocket that is flexible due to the contributions of the methionine side chains.

Although our crosslinking experiments show that the M-domain contacts the signal sequence, they have the limitation that the photoreaction probed only the environment of the two naturally occurring lysine residues at the far N-terminal end of preprolactin. Hence, these results cannot exclude the possibility that the G-domain might also be in proximity to some part of the bound signal sequence, but may not be as close to the photoreactive residues as the M-domain. To obtain a more detailed picture of the structural properties of the signal sequence binding pocket, it will be necessary in future experiments to move the photoreactive lysine(s) in a step-wise fashion to different locations in the signal sequence.

Römisch *et al.* (1989) proposed a model for signal recognition by SRP54 in which signal sequences interact with the G-domain through a region analogous to the effector region of other GTP-binding proteins. Signal sequence binding could then be controlled by a conformational switch of the G-domain between a GDP and GTP bound state. Allosteric changes, mediated by GDP or GTP binding, may allow the domains to move with respect to each other, possibly modulating the affinity for signal sequences. The view that both domains may function in concert in intact SRP is supported by the finding that alkylation of cysteine residues in SRP54 which are located exclusively in the G-domain abolished signal sequence recognition (Siegel and Walter, 1988b).

We have established that the M-domain is not only involved in signal sequence recognition, but also attaches SRP54 to SRP. Isolated M-domain reassociated with 7SL RNA only in the presence of SRP19 under the conditions we have used (Figure 3). However, several lines of evidence suggest that SRP54 may bind directly to 7SL RNA. For example, we have demonstrated that the M-domain (as well as intact SRP54) (K.Strub, D.Zopf and P.Walter, unpublished) bound directly to *E.coli* 4.5S RNA which shares a homologous domain with 7SL RNA (Figure 3). This interaction was specific because neither tRNA (Figure 3) nor yeast U4 RNA (Poritz *et al.*, 1990) bound either the isolated M-domain or SRP54. Moreover, under stringent high salt conditions, we estimated a K_d of $\sim 10^{-8}$ M for the binding of SRP54 to 4.5S RNA (L.Kahng and P.Walter, unpublished). These results show that SRP54 is indeed an RNA binding protein, and suggest that the putative binding site of SRP54 on 7SL RNA is most likely contained within the RNA domain that is conserved between 4.5S and 7SL RNA [domain IV (Poritz *et al.*, 1988; Struck *et al.*, 1988)]. This notion is further supported by the finding that intact SRP does not bind 4.5S RNA, possibly because the RNA binding site present on isolated SRP54 is already occupied (D.Zopf and P.Walter, unpublished).

Direct evidence for an SRP54−7SL interaction comes from recent observations that SRP54 formed a discrete complex with 7SL RNA in a gel shift assay in the absence of SRP19 (F.Janiak and A.E.Johnson, unpublished). Furthermore, SRP54 and 7SL RNA are necessary and sufficient to stimulate a GTPase activity in the presence of SRP receptor, indicating that both can interact in the absence of SRP19 (P.Walter, R.Gilmore and H.Wilhelm, unpublished).

These findings are unanticipated, since the M-domain (or intact SRP54) did not interact with its cognate mammalian RNA under high salt conditions that allowed for reconstitution of SRP (Walter and Blobel, 1983a). Binding to 7SL RNA was only observed when SRP19 (by itself an RNA binding protein) was also present [Figure 3 (Walter and Blobel, 1983a; Siegel and Walter, 1988a; Römisch *et al.*, 1989)]. This result could be explained, however, if other parts of 7SL RNA [such as, e.g., domain III which is absent in 4.5S RNA (Poritz *et al.*, 1988; Struck *et al.*, 1988)] would interfere with SRP54 binding in high salt. Interaction of 7SL RNA with SRP19 would then alleviate this interference. Thus, SRP19 may not be essential for SRP54 to interact with 7SL RNA under all conditions as previously thought, but rather may stabilize an interaction that can occur in its absence.

Given that, like many RNA binding proteins, the M-domain is highly basic (the pI is ~ 10), it is plausible that the abundance of positively charged amino acids may serve to neutralize negative charges contributed by the nucleic acid

backbone. In the SRP54 homologs of yeast and *E.coli*, which are also found in a particle that contains RNA (Poritz *et al.*, 1990; B.C.Hann and P.Walter, unpublished) the basic character of the M-domain is phylogenetically conserved (Bernstein *et al.*, 1989; Hann *et al.*, 1989; Amaya *et al.*, 1990). Unlike other classes of RNA binding proteins, however, SRP54 does not contain an RNP consensus sequence (Dreyfuss *et al.*, 1988).

We have noted that some protein components of other RNP complexes also contain regions with clustered methionine residues. This is most striking in two U1 snRNP associated proteins (A-protein and C-protein) which both contain regions that are extremely rich in methionine residues [C-protein: amino acids 60–159, 20% Met; A-protein: amino acids 143–196, 17% Met; (Sillekens *et al.*, 1987, 1988)], but is also observed in other proteins, e.g. the poly(A) binding protein (amino acids 432–475, 19% Met) (Adam *et al.*, 1986). These regions have not been demonstrated to be directly involved in protein–RNA recognition, however, and in the case of the poly(A) binding protein the region has been deleted with no deleterious effects on RNA binding *in vitro* or function of the protein *in vivo* (Sachs *et al.*, 1987). Hence although the function of the methionine residues is not clear, they are very likely not involved in the binding of RNA. More detailed structural and genetic analyses will be required to determine the elements important for RNA binding in the M-domain of SRP54.

Materials and methods

Materials

A monoclonal antibody against SRP54 ['mab54' (Scoulia *et al.*, 1987)] was a kind gift of B.Dobberstein (EMBL, Heidelberg). Phenylmethylsulfonyl fluoride (PMSF) and 3,4-dichloroisocoumarin (DCI) were obtained from Boehringer (Mannheim). Diisopropylfluorophosphate (DFP) was purchased from Aldrich (Milwaukee). A stock solution of 75 mM DFP in water was prepared freshly each time. A 20 mM stock solution of DCI was made in dimethylformamide.

Generation of antibodies against N- and C-terminal peptides of SRP54

The peptides VLADLGRKIC and CRQFQQGAAG corresponding to residues 2–10 and 485–493 of SRP54, respectively, were synthesized with C-terminal amide groups (Multiple Peptide Systems, San Diego) and used unpurified. The cysteine residues are not present in SRP54, but were added to allow coupling of the peptides to carrier proteins. Each peptide was reacted with keyhole limpet hemocyanine (KLH; Sigma) that had been treated with bifunctional crosslinking reagent *m*-maleimidobenzoyl *n*-hydroxysuccinimide ester (Sigma) as described (Lerner *et al.*, 1981). The unfractionated reaction mixture was used for injections. Rabbits were immunized subcutaneously with 1 mg of KLH–peptide conjugate in complete Freund's adjuvant and then boosted every 4 weeks with equal amounts of material in incomplete Freund's adjuvant. Rabbits were bled 7 and 14 days after each boost.

Immunoblotting was performed as described by Towbin *et al.* (1979). Proteins were transferred electrophoretically onto nitrocellulose. Anti-peptide antibodies were usually diluted from the crude serum 1:1000 (αN/54) and 1:2000 (αC/54). The monoclonal antibody mab54 (Scoulia *et al.*, 1987) was obtained as ascites fluid and used at a dilution of 1:5000. Alkaline phosphatase coupled to anti-rabbit IgG (BioRad) or coupled to anti-mouse IgG (Boehringer Mannheim) were used as secondary antibodies at a dilution of 1:3000.

Protease digestion of SRP and SRP*AF

SRP was prepared according to the procedure of Walter and Blobel (1983b). Sucrose gradient purified SRP (200 μg/ml) was digested for 30 min at 30°C with elastase (Sigma) at a concentration of 25 μg/ml in the presence of an equal concentration of aprotinin (Boehringer) or with *Staphylococcus aureus* V8 protease (25 μg/ml; Worthington) in SRP buffer [50 mM triethanolamine-HOAc, pH 7.5 (TEA), 500 mM KOAc, 5 mM $Mg(OAc)_2$, 0.01% (w/v) Nikkol detergent (octaethyleneglycol-*n*-dodecyl ether), 1 mM dithiothreitol (DTT)]. Digestions containing elastase were stopped by the addition of PMSF to a final concentration of 20 μM. V8 protease was inhibited by successively adding DCI to a final concentration of 20 μM (Harper *et al.*, 1985) and DFP to 5 mM. The digestion products were sedimented on a 5–20% (w/v) sucrose gradient in a SW60 rotor as previously described (Scoulia *et al.*, 1987). Gradient fractions were analyzed by Western blotting.

Sucrose gradient purified SRP*AF obtained from a 600 μl translation reaction was concentrated 10-fold on a 400 μl DEAE–Sepharose CL-6B column and eluted with SRP buffer containing 1 M KOAc (Siegel and Walter, 1985). Fractions containing SRP*AF were combined (520 μl) and one-third of the material was digested with V8 protease at a concentration of 100 μg/ml as described above. Digestion products were sedimented at 4°C on a 5–20% (w/v) sucrose gradient in SRP buffer containing 1 mM DFP and 20 μM DCI in a Beckman TLS 55 rotor at 55 000 r.p.m. for 5.5 h. Gradients were divided into 200 μl fractions.

Photocrosslinking

Crosslinking of the arrested fragment of preprolactin to SRP54 was carried out as described by Krieg *et al.* (1986). In brief, full length synthetic preprolactin mRNA was translated in a wheat germ system in the presence of [^{35}S]methionine (ICN; 1000 Ci/mmol), SRP (50 nM) and N^{ϵ}-(5-azido-2-nitrobenzoyl)-Lys-tRNA (0.23 μM), Polypeptide chain initiation proceeded for 10 min at 26°C and was then inhibited with 4 mM 7-methylguanosine-5′-monophosphate and 10 μM edeine. After the reactions were subjected to UV illumination, SRP was dissociated from ribosomes with 1 mM puromycin and 500 mM KCl (Blobel and Sabatini, 1971). Typically, 300 μl reactions were then layered on a 5–20% (w/v) linear sucrose gradient in SRP buffer and sedimented in a SW40 rotor at 40 000 r.p.m. for 20 h at 4°C. Gradients were fractionated from the top into 1 ml fractions. Trichloroacetic acid (TCA) was added to a concentration of 10% (w/v) to 100 μl of each fraction and the precipitated proteins were analyzed by SDS–PAGE.

Partial reconstitution of SRP

7SL RNA and SRP proteins were purified according to Siegel and Walter (1985). The CM–Sepharose CL-6B fractions that contained SRP54 were adjusted with 20 mM HEPES/KOH pH 7.5, 10 mM $Mg(OAc)_2$, 1 mM DTT and 0.01% Nikkol to obtain final concentrations of 250 mM KOAc, 5 mM $Mg(OAc)_2$ and 50 μg/ml protein. The purified protein was then digested for 20 min at 30°C with V8 protease (25 μg/ml). After the reaction was stopped by the addition of DCI, the digested protein was used directly and reconstituted with a 4-fold molar excess of either 7SL RNA, *E.coli* 4.5S RNA or calf liver tRNA (Sigma) as described (Siegel and Walter, 1985). Reconstitution experiments involving 7SL RNA were performed in the presence or absence of equimolar amounts of purified SRP19. *E.coli* 4.5S RNA was prepared as described by Poritz *et al.* (1990). Partially reconstituted SRP was fractionated on DEAE–Sepharose CL-6B minicolumns as described by Strub and Walter (1990). Proteins were subjected to SDS–PAGE and analyzed by silver staining (Wray *et al.*, 1981).

To purify SRP54*AF for reconstitution experiments, SRP containing the crosslinked product was disassembled on DE53 cellulose as described by Walter and Blobel (1983a). After CM–Sepharose CL-6B chromatography, fractions containing SRP54*AF were pooled (150 μl total volume) and the KOAc concentration was adjusted to 250 mM. SRP54*AF was then digested for 30 min at 30°C with V8 protease (100 μg/ml). After inhibition of V8 protease with DCI and DFP (see above) the reaction was divided into four aliquots (60 μl each). One aliquot was immediately precipitated with TCA and the other three aliquots were reconstituted with 7SL RNA and SRP19, 4.5S RNA or tRNA and analyzed as described above.

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Note added in proof

While this article was in press, Römisch *et al.* (1990) *J. Cell Biol.*, **11**, 1793–1802, also reported that the M-domain of SRP54 contains an RNA binding site.