The Signal Sequence Interacts with the Methionine-rich Domain of the 54-kD Protein of Signal Recognition Particle

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Abstract. The signal sequence of nascent preprolactin interacts with the 54-kD protein of the signal recognition particle (SRP54). To identify the domain or site on SRP54 that interacts with the signal sequence we used a photocross-linking approach followed by limited proteolysis and immunoprecipitation using anti-peptide antibodies specific for defined regions of SRP54. We found that the previously identified methionine-rich RNA-binding domain of SRP54 (SRP54M domain) also interacts with the signal sequence. The smallest fragment that was found to be crosslinked to the signal sequence comprised the COOH-terminal 6-kD segment of the SRP54M domain. No cross-link to the putative GTP-binding domain of SRP54 (SRP54G domain) was found. Proteolytic cleavage between the SRP54M domain and SRP54G domain did not impair the subsequent interaction between the signal sequence and the SRP54M domain. Our results show that both the RNA binding and signal sequence binding functions of SRP54 are performed by the SRP54M domain.

SECRETORY and membrane proteins contain a signal sequence which mediates their targeting to and insertion into the ER membrane (Walter and Lingappa, 1986; Randall et al., 1987). These signal sequences are of widely differing primary structure with an overall hydrophobic nature being the only common feature (von Heijne, 1988). The way in which such widely diverse signals are recognized is poorly understood although it is central to the way in which such signals function. Eukaryotic signal sequences are known to be recognized cotranslationally by signal recognition particle (SRP),[1] a ribonucleoprotein particle consisting of a 7S RNA and six different proteins (Walter and Lingappa, 1986). The binding of SRP to the signal sequence of a protein causes a retardation or arrest of translation which is thought to increase the time during which the nascent chain remains competent for translocation (Walter and Blobel, 1981; Wolin and Walter, 1989). The subsequent interaction with the membrane-bound receptor for the nascent chain/ribosome/SRP complex, the docking protein (DP) (or SRP receptor), releases the arrest of translation and allows chain elongation to continue (Meyer et al., 1982; Gilmore et al., 1982). The protein is then either secreted or inserted into the membrane of the ER.

The 54-kD subunit of SRP (SRP54) has been identified as the polypeptide interacting with the signal sequence (Kurzchalia et al., 1986; Krieg et al., 1986). SRP54 contains a methionine-rich (M) domain at the COOH terminus and a putative GTP binding (G) domain (Bernstein et al., 1989; Römisch et al., 1989). It has been shown previously that the M domain of SRP54 (SRP54M domain) is a RNA binding domain and retains the protein in the SRP particle (Römisch et al., 1990). Both the M and G domains of SRP54 have been proposed as potential candidates for performing the signal sequence binding function of SRP54 (Bernstein et al., 1989; Römisch et al., 1989). The presence of a consensus GTP binding site within SRP54 led to the speculation that hydrolysis of GTP might regulate the binding of the signal sequence to SRP54 in the same way as GTP binding and hydrolysis plays a regulatory function in other GTP binding proteins such as Ras and EF-Tu (Bernstein et al., 1989; Römisch et al., 1989; Rothman, 1989). The DP has a functional GTP binding domain, homologous to that of SRP54, and it has been shown that the DP dependent displacement of SRP from a nascent polypeptide chain requires the presence of GTP (Connolly and Gilmore, 1989).

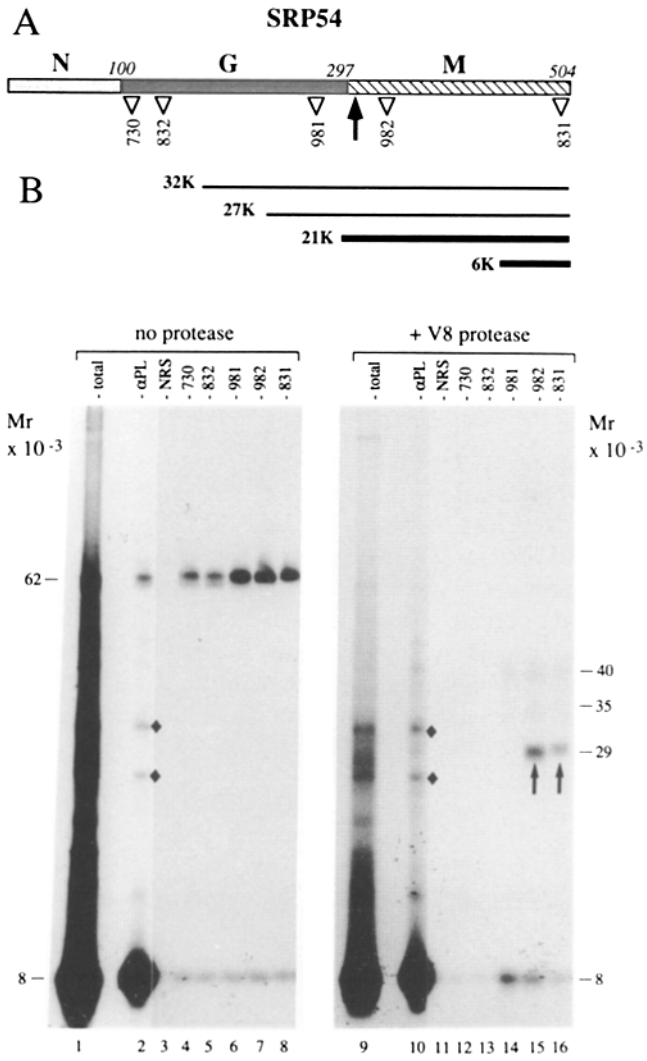
To identify the domain within SRP54 which binds to the signal sequence we have used a photocross-linking approach in combination with protease digestion and immunoprecipitation with peptide specific antibodies. We show here that the M domain of SRP54 is a major site of interaction between SRP54 and the signal sequence.

Materials and Methods

Materials

Restriction enzymes and SP6 RNA polymerase were from Boehringer Mannheim GmbH (Mannheim, FRG). Cycloheximide and 7-methyl-guanosine 5'-monophosphate were supplied by Sigma Chemical Co. (St. Louis,

1. *Abbreviations used in this paper*: DIFP, diisopropylfluorophosphate; DP, docking protein; PPL, preprolactin; PPL AF, signal recognition particle-arrested fragment of preprolactin; PPL_{86}, NH_2-terminal 86 amino acids of preprolactin; SRP, signal recognition particle.



Figures 1 and 2. (Fig. 1) (*A*) Location of peptides used to raise antibodies to SRP54. Triangles indicate the sites of the peptides used to raise antibodies against SRP54. The three previously defined domains of SRP54 (Bernstein et al., 1989; Römisch et al., 1989; Römisch et al., 1990) are indicated by: *N*, NH_2-terminal domain; *G*, putative GTP binding domain; and *M*, methionine-rich domain. The number of the amino acid residue at the putative boundaries between the different domains is indicated. The arrow indicates the V8 cleavage site in SRP54 from purified dog pancreas SRP (Römisch et al., 1990). (*B*) SRP54 fragments interacting with preprolactin signal sequence. The fragments of SRP54 found cross-linked to the PPL signal sequence are indicated by the black lines, their calculated molecular weights are given. (Fig. 2). PPL AF-SRP54 photocross-linking product before and after digestion with V8 protease. The SRP-arrested nascent chain of PPL (PPL AF) was cross-linked to interacting components. The total photocross-linked products before (lane *1*) and after (lane *9*) digestion with 10 $\mu g \cdot ml^{-1}$ V8 in the presence of 25 mM EDTA are shown. Cross-linked proteins were immunoprecipitated before (lanes *2–8*) or after V8 digestion (lanes *10–16*) with the following antibodies as indicated: anti-prolactin (*αPL*), lanes *2* and *10*; nonrelated serum (*NRS*), lanes *3* and *11*; and anti-SRP54 peptide antibodies 730, lanes *4* and *12*; 832, lanes *5* and *13*; 981, lanes *6* and *14*, 982; lanes *7* and *15*; 831, lanes *8* and *16*. Lanes *3–8* and *11–16* were autoradiographed for five times longer than lanes *1*, *2*, *9*, and *10*. The arrow indicates the 29-kD V8 cleavage product of the PPL AF-SRP54 cross-link. The diamond indicates two PPL derived species of 27 and 33 kD which do not represent photocross-links.

MO). [^{35}S]methionine was obtained from Amersham Buchler GmBH (Braunschweig, FRG) and 4-(3-trifluoro-methyldiazarino) benzoyloxysuccinimide was a gift from Dr. Josef Brunner, Swiss Federal Institute of Technology, Zürich, Switzerland.

Cell-free Translation

Wheat germ extract and SRP were prepared as described elsewhere (Erickson and Blobel, 1983; Walter and Blobel, 1983). The plasmid pSPBP4 (Siegel and Walter, 1988*a*) containing the cDNA coding region for preprolactin (PPL) was cut within the coding region using PvuII and transcribed in vitro. The resulting transcript encoding the NH_2-terminal 86 amino acids of PPL was translated in a wheat germ cell-free translation system containing 25 nM SRP and [^{35}S]methione essentially as described by Krieg et al. (1986) except that 7-methylguanosine 5′-monophosphate was added 10 min after the start of synthesis and chain elongation was inhibited after a further 5 min incubation by addition of cycloheximide to 2 mM. The translation contained ε-TDBA-Lys-tRNA prepared as described by Wiedmann et al. (1987). Samples in open polypropylene microfuge tubes were maintained at 0°C during UV irradiation which was done with a Spectroline model B-100/F black light lamp equipped with a 100-W mercury bulb and a 365-nm filter (Spectronics Corp., Westbury, NY). Irradiation was for 10 min with the samples at a distance of 15 cm from the light source.

Purification of Photocross-linked Product

After UV irradiation the nascent chain/ribosome/SRP complex was isolated by centrifugation through a 0.5 M sucrose cushion containing 0.5 M potassium acetate, 30 mM Hepes-KOH, pH 7.9, 3 mM magnesium acetate, and 1 mM cycloheximide. After centrifugation in a TLS-55 rotor (Beckman Instruments Inc., Palo Alto, CA) at 166,000 *g* at 4°C for 1 h the resulting pellet was resuspended in SB buffer (75 mM Hepes-KOH, pH 7.9, 100 mM potassium acetate, 3 mM magnesium acetate, and 1 mM cycloheximide) so that the final volume was equal to 0.75 times that loaded on to the cushion.

Proteolysis of Photocross-linking Products and Immunoprecipitation

20 μl of the resuspended, purified nascent chain/ribosome/SRP complex was digested for 1 h at 25°C with 10 $\mu g \cdot ml^{-1}$ V8 protease (Boehringer Mannheim) in the presence of 25 mM EDTA or with 500 $\mu g \cdot ml^{-1}$ V8 in the absence of EDTA as indicated in the figure legends. After proteolysis diisopropylfluorophosphate (DIFP) (Sigma Chemical Co.) was added to 1 $mg \cdot ml^{-1}$. SDS was added to 1% and samples were denatured by heating at 95°C for 5 min before immunoprecipitation as previously described (Römisch et al., 1990). The immunoprecipitates were analyzed by SDS-PAGE using 10–15% gradient gels and subjected to fluorography.

V8 Protease Digestion of SRP before Photocross-linking

250 nm SRP was incubated at 25°C for 1 h with 5 $\mu g \cdot ml^{-1}$ V8, with DIFP and V8 together or mock incubated. After this time DIFP was added to 1 $mg \cdot ml^{-1}$. The transcript from PvuII linearized pSPBP4 was translated in the absence of SRP. The resulting PPL_{86}/ribosome complex was purified by centrifugation through a high salt/sucrose cushion and resuspended in SB buffer as described above. The pretreated SRP samples were added to 20 μl aliquots of the purified PPL_{86}/ribosome complex and incubated at 25°C for 5 min. After UV irradiation on ice samples were denatured in SDS, immunoprecipitated, and analyzed by SDS-PAGE as described above.

Results

Photocross-linking of Nascent PPL to SRP54

A SRP-arrested nascent chain complex was formed by translating mRNA encoding the NH_2-terminal 86 amino acids of PPL (PPL_{86}) in a wheat germ cell-free translation system containing dog pancreas SRP, [^{35}S]methionine, and lys-tRNA modified with the UV-activatable photocross-linking

reagent 4-(3-trifluoromethyldiazarino) benzoic acid (TDBA) (Wiedmann et al., 1987). TDBA-modified lysine can be incorporated at amino acid positions 4 and 9 of the PPL signal sequence. The SRP-mediated arrest of translation occurs after the synthesis of about 70 amino acids (Kurzchalia et al., 1986; Krieg et al., 1986). SRP54 photocross-linked to the SRP-arrested fragment of PPL (PPL AF) has a reported apparent molecular mass of 62 kD (Kurzchalia et al., 1986; Krieg et al., 1986). A product of this molecular mass was seen when total cross-linked proteins were analyzed (Fig. 2, lane *1*). The 62-kD product was absent when samples were not UV irradiated or when ϵ-TDBA-Lys-tRNA was omitted from the cell-free translation mixture (data not shown). Since the 62-kD photocross-linking product was immunoprecipitated by a polyclonal antiserum against prolactin (Fig. 2, lane *2*), and by all the anti-SRP54 peptide antibodies tested (Fig. 2 *A*, lanes *4–8*), it clearly represented PPL AF cross-linked to SRP54.

V8 Digestion of Photocross-linking Products

To identify the domain of SRP54 that interacts with the PPL signal sequence we digested the cross-linked material with 10 $\mu g \cdot ml^{-1}$ V8 protease. This led to the loss of the 62-kD component and the appearance of several smaller species among which was a 29-kD V8 cleavage product (Fig. 2, lane *10*). Two PPL-derived products of 27 and 33 kD (Fig. 2, lane *2*, indicated by *diamonds*) were unaffected by treatment with V8 (Fig. 2, lane *10*). These products do not represent photocrosslinks since they were also seen in the absence of UV irradiation (data not shown). The 29-kD V8 cleavage product was the major species immunoprecipitated by the anti-SRP54 antipeptide antibodies 982 and 831 (Fig. 2, lanes *15* and *16*, indicated by an *arrow*). Since 831 is directed against the extreme COOH terminus of SRP54 (Fig. 1 *A*) while 982 is directed against the NH_2 terminus of the SRP54M domain (Fig. 1 *A*) the 29-kD cleavage product must comprise the entire M domain of SRP54.

The 29-kD V8 cleavage product is not recognized by any of the three antibodies: 730, 832, and 981, which are directed toward regions of the SRP54N+G domain (Fig. 1 *A* and Fig. 2, lanes *12–14*). These antibodies recognize the SRP54N+G domain produced by V8 cleavage of native SRP (Römisch et al., 1990). Thus the 29-kD fragment possesses the entire SRP54M domain but has no detectable regions of the SRP54N+G domain. When the 8-kD contribution of the PPL AF is subtracted from the size of the 29-kD V8 cleavage product of the SRP54-PPL AF cross-link, the major fragment of SRP54 which remains cross-linked to PPL AF corresponds to the COOH-terminal 21 kD (Fig. 1 *B*). This 21-kD fragment of SRP54 cross-linked to PPL AF is approximately the same size as the SRP54M domain resulting from the V8 cleavage of purified SRP (Römisch et al., 1990).

In addition to the major 29-kD V8 cleavage product of the SRP54-PPL AF cross-linked species two minor products of 35 and 40 kD (Fig. 2, lanes *14–16*) are seen. Like the 29-kD product these are immunoprecipitated by both antibodies 982 and 831 (Fig. 2, lanes *15* and *16*). In addition these two species are immunoprecipitated by antibody 981 (Fig. 1 *A* and Fig. 2, lane *14*). Since all three fragments are recognized by antibody 831 they must all have the same COOH terminus, and thus the larger species represent fragments with longer NH_2 termini. For the 35- and 40-kD fragments this must include the site recognized by antibody 981, and the estimated sizes of the corresponding SRP54 fragments remaining after V8 digestion are 27 and 32 kD, respectively (Fig. 1 *A*).

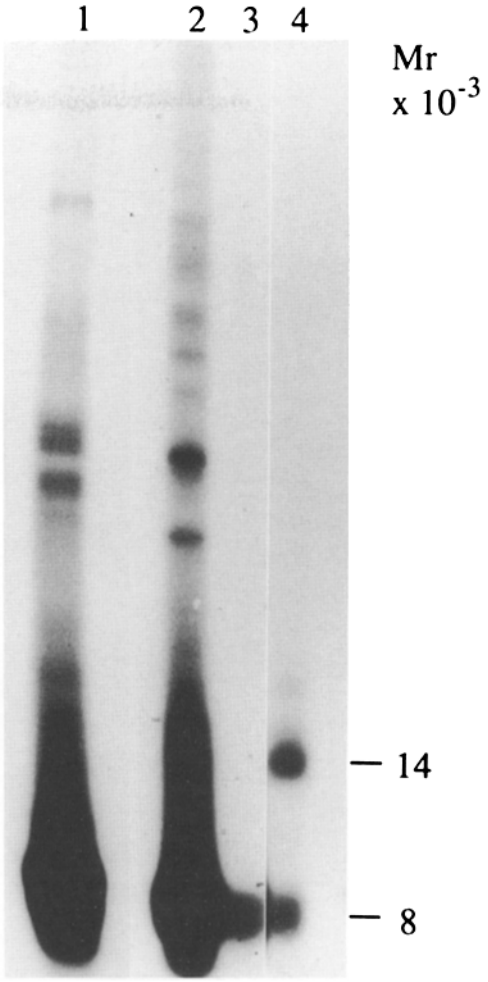


Figure 3. Photocross-linking products after digestion with high concentrations of V8. Photocross-linking products digested with 500 $\mu g \cdot ml^{-1}$ V8 in the absence of EDTA (lane *1*) were immunoprecipitated with anti-prolactin serum (lane *2*), nonimmune serum (lane *3*), and the anti-SRP54 peptide antibody 831 (lane *4*). The apparent molecular masses of PPL AF (8 kD) and PPL AF cross-linked to a SRP54 fragment (14 kD) are indicated. This identifies the COOH-terminal 6-kD segment of the SRP54M domain as one site of interaction with the PPL signal sequence.

Digestion with High Concentrations of V8

To more closely map the site of cross-linking between the PPL AF and the SRP54 we used high concentrations of V8 protease to generate smaller fragments of SRP54. Treatment with 500 $\mu g \cdot ml^{-1}$ V8 protease caused the loss of the 29-kD cross-linking product with a concomitant increase in smaller molecular mass components. Immunoprecipitation from this digested material with the SRP54 COOH-terminal antibody 831 (Fig. 1 *A*) specifically brought down a 14-kD radiolabeled fragment (Fig. 3, lane *4*). Treatment with 500

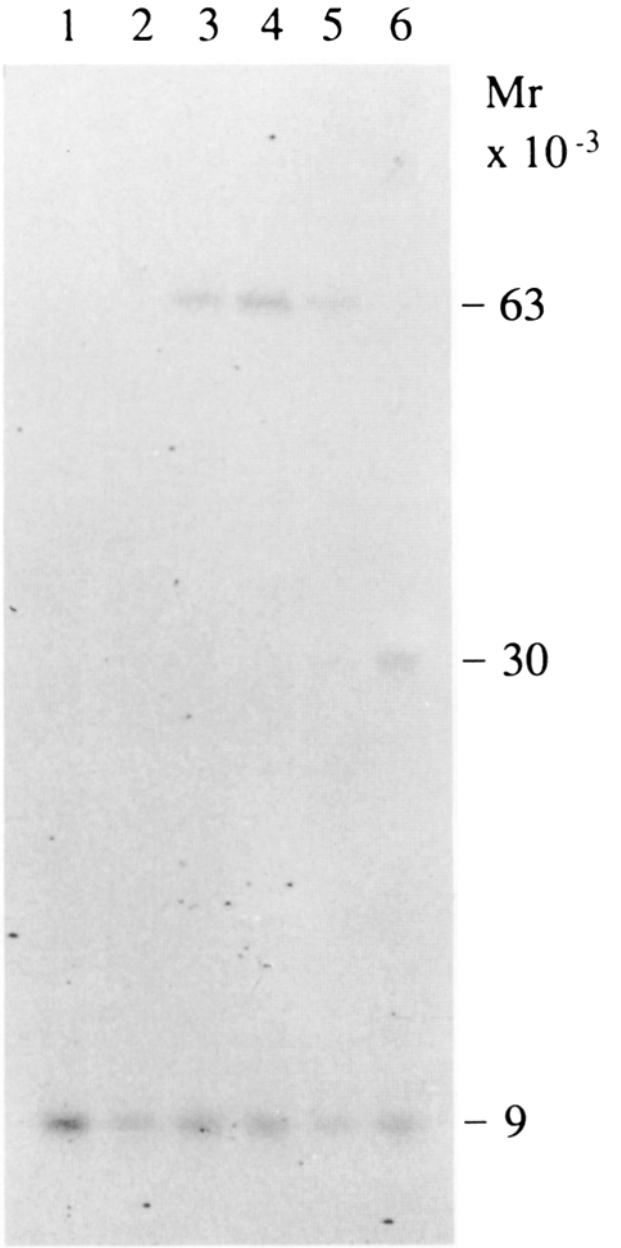


Figure 4. Interaction between the PPL signal sequence and the products of V8-cleaved SRP54. PPL nascent chains of 86 amino acids in length (PPL_{86}) were obtained by translating truncated mRNA in the absence of canine SRP. The resulting PPL_{86}/ribosome complex was isolated and incubated: in the presence of SRP and not subjected to photocross-linking (lane *2*); or photocross-linked either in the absence of SRP (lane *1*), or in the presence of: SRP (lane *3*); SRP that had been incubated with DIFP and V8 (lane *5*); or SRP incubated with V8 alone (lane *6*). As a further control SRP was also incubated in the absence of both V8 and DIFP and then the PPL_{86}/ribosome complex added (lane *4*). The samples were all immunoprecipitated using the anti-SRP54 antibody 831.

$\mu g \cdot ml^{-1}$ V8 did not cause any visible reduction in the size of the 8-kD PPL nascent chain. Thus, after the subtraction of the contribution from the nascent chain, the smallest SRP54 fragment which could be found cross-linked to PPL AF is the COOH-terminal 6 kD of SRP54.

V8 Digestion of SRP before Photocross-linking

It has been shown previously (Römisch et al., 1990) that the SRP54N+G domain is released from SRP upon cleavage with V8 protease while the SRP54M domain remains bound to the particle. To test whether cleavage of SRP with V8 affects the interaction with the PPL nascent chain we used a posttranslational SRP binding assay. In this assay a nascent chain/ribosome complex is formed by translation of truncated PPL mRNA coding for the NH_2-terminal 86 amino acid residues of PPL (PPL_{86}). The lack of a stop codon prevents release of the nascent PPL chains from the ribosome. It has previously been shown that SRP can bind to such nascent chains posttranslationally (Wiedmann et al., 1987; Siegel and Walter, 1988*b*). PPL_{86} has a molecular mass of 9 kD, slightly larger than that of PPL AF. When SRP was added to the isolated PPL_{86}/ribosome complex, photocross-linking to SRP54 was observed as judged by the appearance of a 63-kD radiolabeled product which was immunoprecipitated by the anti-SRP54 antibody 831 (Fig. 4, lane *3*). As expected, the product of PPL_{86} cross-linked to SRP54 was roughly 1 kD larger than that observed with PPL AF. When SRP was omitted (Fig. 4, lane *1*) or when the sample was not UV irradiated (Fig. 4, lane *2*) the 63-kD product was not observed. When SRP digested with 5 $\mu g \cdot ml^{-1}$ V8 protease was used in the posttranslational signal sequence binding assay, UV irradiation and subsequent immunoprecipitation resulted in a 30-kD cross-linking component (Fig. 4, lane *6*). The 63-kD cross-linking product was no longer visible confirming that the V8 cleavage of SRP54 had gone to completion. The 30-kD component was not seen when the SRP was incubated in the absence of V8 (Fig. 4, lane *4*) and was barely visible when the V8 protease was inhibited with DIFP before incubation (Fig. 4, lane *5*). When the 9-kD contribution of PPL_{86} is subtracted from the size of the 30-kD cross-linking component a 21-kD fragment results. This is the same size as the COOH-terminal domain of SRP54 generated by V8 cleavage of native SRP (Römisch et al., 1990 and Fig. 1 *A*). Since the antibody used for the immunoprecipitation, 831, recognizes the extreme COOH terminus of the SRP54M domain (Fig. 1 *A*) this furthermore confirms the identity of the fragment.

Discussion

We have used an established photocross-linking approach to identify the signal sequence binding domain of SRP54. A cross-linking reagent attached to lysine residues was incorporated into the PPL nascent chain and photoactivated in the presence of SRP. The highly reactive carbene radical generated upon photolysis of the diazarino group (see Wiedmann et al., 1987 and references therein) allows cross-linking to occur only to immediately adjacent regions of an interacting component. We have subjected the photocross-linking product of PPL AF and SRP54 to protease digestion with V8. V8 digestion of native SRP is known to cleave SRP54 between the G domain and the M domain (Römisch et al., 1990). The products were then analyzed using anti-SRP54 peptide antibodies which had been previously characterized (Römisch et al., 1990). The results showed that in each case the fragments generated could only be immunoprecipitated with

anti-SRP54 peptide antibodies directed against regions at the COOH terminus of the molecule. Further, in each case the fragments were always immunoprecipitated by antiserum 831 which is directed against the extreme COOH terminus of the SRP54M domain. The major 21-kD fragment generated upon digestion with 10 $\mu g \cdot ml^{-1}$ V8 proved to be equivalent to the M domain of SRP54. No fragments corresponding to the SRP54N+G domain, or portions thereof, were immunoprecipitated. It was furthermore found that the interaction between SRP and the PPL nascent chain does not require the intact SRP54 protein. The SRP54M domain of V8-digested SRP was also able to interact with the signal sequence of PPL when analyzed in a posttranslational binding assay.

Our results are consistent with the proposal that the signal sequence binding function of SRP54 is restricted to the M domain as predicted by Bernstein et al. (1989) and suggest that the SRP54N+G domain does not participate in the binding of SRP54 to the signal sequence. The observation that the SRP54N+G domain can be cleaved from the particle without preventing signal sequence binding suggests that it may not be essential for this function. Since the SRP54N+G domain is still present in solution after V8 digestion, we cannot rule out that it may noncovalently associate with the SRP subparticle containing the SRP54M domain and/or the PPL_{86}/ribosome complex during our assay. Even if the SRP54N+G domain is not essential for signal binding it is still possible that this region may play a regulatory role in the process (Bernstein et al., 1989; Römisch et al., 1989; Rothman, 1989). The interaction between SRP and a nascent presecretory protein has been shown to be of high affinity (Walter and Lingappa, 1986; Siegel and Walter, 1988*b*). It can even withstand extraction with 2 M urea, 0.5 M potassium acetate or 1% NP40 (High, S., unpublished data). It is therefore conceivable that the binding of GTP to the SRP54G domain may be required to allow the DP-mediated release of SRP from the signal sequence to occur. Future work on the role of the SRP54N+G domain should clarify this point and may also help to elucidate the significance of its homology to part of the docking protein (Römisch et al., 1989; Bernstein et al., 1989).

It has been previously shown that the SRP54M domain is a RNA binding domain and is responsible for the binding of SRP54 to the SRP (Römisch et al., 1990). The COOH-terminal 38 amino acids of SRP54 were found to be dispensable for particle binding (Römisch et al., 1990). In the work described here we demonstrate that the M domain of SRP54 can be cross-linked to the signal sequence of PPL. The COOH-terminal 6 kD of SRP54 is the smallest detectable fragment that is still cross-linked to the signal sequence. This may indicate that the SRP54M domain can be further subdivided into RNA binding and signal sequence binding regions. Secondary structure predictions have revealed segments in the SRP54M domain that are likely to form amphipathic helices (Bernstein et al., 1989). The hydrophobic faces of these helices have been proposed to function in signal sequence binding (Bernstein et al., 1989). This is an attractive hypothesis and if correct could imply that the polar faces of the helices are involved in RNA binding. A more detailed characterization is necessary to reveal the precise structural requirements for either function.

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Note Added in Proof: Zopf et al. (Zopf, D., H. D. Bernstein, A. E. Johnson, and P. Walter. 1990. *EMBO [Eur. Mol. Biol. Organ.] J.* 9:4511–4517) have reported that the M domain of SRP54 is responsible for signal sequence binding.

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