The EMBO Journal vol.11 no.4 pp.1543-1551, 1992

The methionine-rich domain of the 54 kDa subunit of signal recognition particle is sufficient for the interaction with signal sequences

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Communicated by B.Dobberstein

The signal recognition particle (SRP) binds to signal sequences when they emerge from a translating ribosome and targets the complex of ribosome, nascent chain and SRP to the membrane of the rough endoplasmic reticulum (rER) allowing the co-translational translocation of the nascent chain. By photo-crosslinking it has been shown that the signal sequence of preprolactin (PPL) only interacts with the methionine-rich (M) domain of the 54 kDa protein subunit (SRP54) of SRP. Here we show that (i) a signal-anchor sequence is likewise crosslinked only to the methionine-rich domain of SRP54, (ii) free SRP54 can interact with signal sequences independently of the other components of SRP, (iii) its M domain suffices to perform this function, and (iv) an essentially intact M domain is required for signal sequence recognition. Alkylation of the N+G domain in intact SRP54 with *N*-ethyl maleimide (NEM), but not after cleavage with V8 protease, prevents the binding of a signal sequence to the M domain. This suggests a proximity between the N+G and M domains of SRP54 and raises the possibility that the role of the N+G domain may be to regulate the binding and/or the release of signal sequences.

Key words: GTP binding protein/methionine-rich domain of SRP54/photo-crosslinking/signal sequence binding/signal recognition particle

Introduction

Three sequentially performed functions have been attributed to the signal recognition particle (SRP): (i) Recognition of and binding to signal sequences of proteins destined to enter the 'secretory pathway' upon their emergence from a translating ribosome (Walter and Lingappa, 1986), (ii) arrest or retardation of the elongation of the nascent polypeptide (Siegel and Walter, 1985; Wolin and Walter, 1989) until (iii) the interaction of the complex of ribosome, nascent chain and SRP with the docking protein (DPα) (or SRP receptor) on the membrane of the rough endoplasmic reticulum (rER) (Gilmore *et al.*, 1982; Meyer *et al.*, 1982). Upon binding to DPα the signal sequence is displaced from SRP in a GTP-dependent process (Connolly and Gilmore, 1989; High *et al.*, 1991a), the elongation arrest is released and translocation of the nascent polypeptide is initiated (Walter and Lingappa, 1986). SRP is subsequently released from the membrane of the rER in a step requiring GTP hydrolysis (Connolly *et al.*, 1991).

SRP consists of a 7S RNA and six different proteins: a 19 kDa (SRP19) and a 54 kDa (SRP54) protein both of which are RNA binding proteins (Lingelbach *et al.*, 1988; Ribes *et al.*, 1990; Römisch *et al.*, 1990; Zopf *et al.*, 1990) and two heterodimers of 9/14 kDa and 68/72 kDa respectively, which each by themselves also bind to 7S RNA (Walter and Blobel, 1983). The 9/14 kDa heterodimer is necessary for the elongation arrest function of SRP (Siegel and Walter, 1985) while the 68/72 kDa heterodimer has been implicated in the docking process (Siegel and Walter, 1988). By photo-crosslinking (Kurzchalia *et al.*, 1986; Krieg *et al.*, 1986; High *et al.*, 1991b) and by chemical modification (Siegel and Walter, 1988) SRP54 has been shown to be the protein responsible for the interaction of SRP with the signal sequences of nascent secretory and membrane proteins.

Signal sequences for rER targeting and insertion differ widely in primary structure and have as the only common feature an overall hydrophobic nature (von Heijne, 1988). How SRP54 recognizes and binds to such signal sequences is poorly understood. The investigation of this interaction may serve as a paradigm for other signal-mediated processes.

Based on amino acid sequence homologies SRP54 has been proposed to contain at least two domains, an N+G domain (SRP54N+G) which contains consensus motifs for binding GTP and a methionine-rich or M domain (SRP54M) (Bernstein *et al.*, 1989; Römisch *et al.*, 1989). The postulated domain structure is supported by the results of analyses using limited proteolysis of SRP and identification of the resulting fragments with peptide-specific antisera against SRP54 (Römisch *et al.*, 1990; Zopf *et al.*, 1990). These experiments located the RNA binding site of SRP54 to the M domain. Photo-crosslinking of the signal sequence of PPL to SRP, combined with limited proteolysis, fractionation and antibody mapping of the crosslinked products showed that SRP54M is also the domain which interacts with this signal sequence (Zopf *et al.*, 1990; High and Dobberstein, 1991). SRP54M contained in proteolytically cleaved SRP was found still to be capable of recognizing the signal sequence of PPL (High and Dobberstein, 1991). However, the experiment does not exclude that SRP54N+G was non-covalently associated with SRP after proteolysis and therefore required for signal sequence recognition.

Using a similar crosslinking approach to that used before (High and Dobberstein, 1991), we here report the mapping of the binding site on SRP54 for three signal sequences. Lysines modified with a photo-activatable crosslinking reagent were incorporated at different positions relative to the hydrophobic core of these signal sequences. Thus, the results were independent of the position of the photo-activatable crosslinker within the nascent chain. *In vitro* synthesized SRP54 was found to interact with signal

sequences with an efficiency similar to that of SRP54 present as part of the purified SRP complex. The effect of *N*-ethyl maleimide (NEM) modification and limited proteolysis upon the interaction between SRP54 and signal sequences was tested. Using mutant SRP54 proteins, deleted at the NH_2- or COOH-terminus, it was possible to define the minimal region of the protein necessary for signal sequence binding.

Results

The M domain of SRP54 interacts with different types of signal sequences

By a photo-crosslinking approach (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986) it has been shown previously (Zopf *et al.*, 1990; High and Dobberstein, 1991) that the cleavable signal sequence of nascent PPL interacts with the M domain of SRP54. A nascent chain–ribosome complex was formed by translation of a truncated PPL mRNA in a wheat germ cell-free translation system. [^{35}S]methionine was included as a label for the nascent chain. Lysyl-tRNA modified with the UV-activatable crosslinking reagent 4-(3-trifluoromethyl-diazarino)benzoic acid (TDBA) was incorporated into the nascent chain as a means to identify interacting components by photo-crosslinking. The site of crosslinking was then mapped by limited proteolysis and immunoprecipitation with peptide-specific antibodies.

As the arrested peptide of PPL contains lysine residues only at positions preceding the hydrophobic core of the PPL signal sequence (Figure 1) it remained unclear whether lysines in other positions in this or other signal sequences would interact with, and therefore be crosslinked to, other parts of SRP54 (High and Dobberstein, 1991). To test this we used the SRP-arrested translation product of the type I membrane protein IMC-CAT$^{\Delta 27}$ which contains an uncleaved signal–anchor (SA) sequence close to its NH_2-terminus (Figure 1, High *et al.*, 1991b). Crosslinking reagent is incorporated in this protein exclusively COOH-terminal of the hydrophobic core of the SA sequence (Figure 1). Truncated IMC-CAT$^{\Delta 27}$ mRNA codes for the NH_2-terminal 103 amino acids of IMC-CAT$^{\Delta 27}$ (IMC-CAT$_{103}^{\Delta 27}$). When the mRNA was translated in the presence of SRP one major polypeptide of the expected molecular mass of 11 kDa was synthesized, and upon UV irradiation a major specific crosslinked product of 65 kDa was seen (Figure 2, lane 1) which was absent when samples were not irradiated (not shown). As the 65 kDa crosslinked product could be immunoprecipitated with antisera specific for peptides derived from SRP54 (Figure 2, lanes 3–7) but not with a control serum (lane 2) it represents a crosslinked product between IMC-CAT$_{103}^{\Delta 27}$ and SRP54 (see Figure 1b for the locations of peptides used to raise the antibodies and Römisch *et al.*, 1990). To identify the portion of SRP54 interacting with the SA sequence of IMC-CAT$_{103}^{\Delta 27}$, we digested the purified crosslinked complexes of nascent chain, ribosome and SRP (Figure 2, lane 1) with V8 protease after dissociation of ribosomes with EDTA (lanes 8–14). These conditions had resulted in complete cleavage of SRP54 into its M and N+G domains when PPL_{86} (NH_2-terminal 86 amino acids of proprolactin) was the binding partner (High and Dobberstein, 1991). After digestion with V8 protease the major crosslinked portion of SRP54 had a relative molecular mass (M_r) of ~21 kDa. This was deduced by subtraction of the 8 kDa contribution of the cleaved IMC-CAT$_{103}^{\Delta 27}$ from the crosslinked product of 29 kDa. This corresponded to an essentially intact M domain as judged by its immunoprecipitation with the two anti-SRP54M (lanes 13 and 14) and the lack of precipitation with the three anti-SRP54N+G (lanes 10–12) antisera (cf. Figure 1b).

A minor crosslinked product migrating at ~43 kDa contained the entire M domain (precipitation by antisera 982 and 831) and contained a COOH-terminal portion of the G domain as it was also precipitated by antiserum 981 (lane 12). Note that upon cleavage by V8 protease IMC-CAT$_{103}^{\Delta 27}$ lost ~3 kDa of its NH_2-terminus (lane 8). This was deduced from the fact that the proteolysed nascent chain migrated at ~8 kDa and was no longer precipitated by an antibody raised against its NH_2-terminal 30 amino acid residues (not shown). Consistent with this cleavage is also the slightly increased mobility of the crosslinked product containing residual undigested SRP54 (Figure 2, lane 8).

Free SRP54 interacts with signal sequences

Since only SRP54 could be crosslinked to signal sequences it seemed possible that it was sufficient for binding of signal sequences. We therefore tested if SRP54 present as free

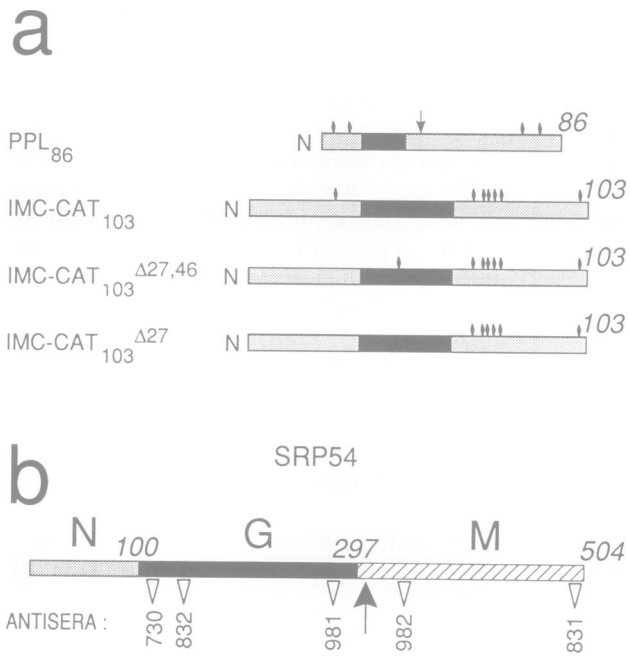

Fig. 1. (**a**) Outline of the nascent chains used in this study: the synthesis of the nascent chains of preprolactin (PPL) and IMC-CAT was arrested at amino acids 86 (PPL_{86}) and 103 (IMC-CAT$_{103}$), respectively. Black boxes indicate the hydrophobic cores of cleavable signal or SA sequences. The arrow on the PPL sequence points to the site of cleavage by signal peptidase. Lysine residues (♦) present in the nascent chains of COOH-terminally truncated PPL (PPL_{86}), IMC-CAT (IMC-CAT$_{103}$) and variants of IMC-CAT (IMC-CAT$_{103}^{\Delta 27}$ and IMC-CAT$_{103}^{\Delta 27,46}$) are found at positions 4, 9, 72 and 78 of the PPL_{86} and at positions 27, 69, 72, 73, 75, 77 and 101 of IMC-CAT$_{103}$. In the mutant IMC-CAT$^{\Delta 27}$ the lysine residue at position 27 is replaced by a glutamic acid residue. In IMC-CAT$^{\Delta 27,46}$ the lysine residue at position 27 is replaced by a glutamic acid residue and residue 46 is replaced by a lysine. (**b**) Antisera raised against peptides of SRP54 and used for all immunoprecipitations in this study: SRP54 and its N, G (SRP54N+G) and M (SRP54M) domains are delineated by the bordering amino acid positions (numbers in italics). The positions of peptides used to raise anti-peptide antisera 730, 832, 981, 982 and 831 (Römisch *et al.*, 1990) are indicated by arrowheads. The approximate position of cleavage by V8 protease is indicated by the arrow (Römisch *et al.*, 1990).

protein, rather than as a component of a ribonucleoprotein (RNP) particle, could be photo-crosslinked to PPL_{86} (Figure 3). SRP54 and, as a control, SRP19 were synthesized in the wheat germ system in the absence of [^{35}S] methionine. In a separate reaction PPL_{86} was synthesized in the presence of [^{35}S]methionine and N^{ϵ}-4-(3-trifluoromethyldiazarino)benzoyl-lysyl (ϵ-TDBA-lysyl) tRNA. The two translation mixtures were combined and incubated, whereupon crosslinking was induced by UV irradiation. In a control experiment PPL_{86} (M_r = 9 kDa) was crosslinked to the SRP54 subunit of added intact SRP (0.5 pmol) to yield the expected 63 kDa product (Figure 3, lane 1; High and Dobberstein, 1991). In the presence of *in vitro* synthesized SRP54 (Figure 3, lane 5) the major crosslinked product co-migrated with the 63 kDa of the control and was of similar intensity (Figure 3, compare lanes 1 and 5). However, when the translation mixture of SRP19 was incubated with PPL_{86} only a less intense 61 kDa crosslinked product was seen (Figure 3, lane 3, arrowhead). This was also observed when wheat germ lysate which had not been programmed with any mRNA was added (not shown). It corresponds to crosslinked product containing the SRP54 homologue ($SRP54^{WG}$) of the endogenous wheat germ SRP (Prehn *et al.*, 1987). The result suggested that free SRP54 could interact with a signal sequence.

To exclude that the *in vitro* synthesized SRP54 had bound to a partially assembled wheat germ SRP, we released the crosslinked complex from the ribosome by treatment with puromycin and separated free SRP54 from SRP by sucrose gradient centrifugation. We chose the centrifugation conditions such that SRP54 would remain on top of the gradient while SRP would move to the middle of the gradient. When PPL_{86} crosslinked to SRP was thus analysed the 63 kDa crosslinked complex was, as expected, found in an 11S particle in the middle of the gradient (Figure 4a). In contrast, when *in vitro* synthesized SRP54 was used in the crosslinking assay the 63 kDa complex was found on top of the gradient (Figure 4b). A small amount of the 61 kDa crosslinked complex containing the endogenous $SRP54^{WG}$ co-migrated with the 11S SRP (Figure 4b, arrow). From this experiment we conclude that *in vitro* synthesized SRP54 does not assemble into an RNP particle and is capable of recognizing the signal sequence of PPL_{86} as a free protein.

Free SRP54, like its SRP-bound form, interacts with signal sequences via its M domain

In order to test whether the interaction between the signal sequences of PPL or IMC-CAT and free SRP54 was the same as that with SRP-bound SRP54, we mapped the signal sequence binding domain of free SRP54 by photo-crosslinking and subsequent digestion with V8 protease. The crosslinked product of PPL_{86} and free SRP54 (Figure 5, lanes 1–8) was digested with V8 protease (lanes 9–16), and the signal sequence binding domain was identified by

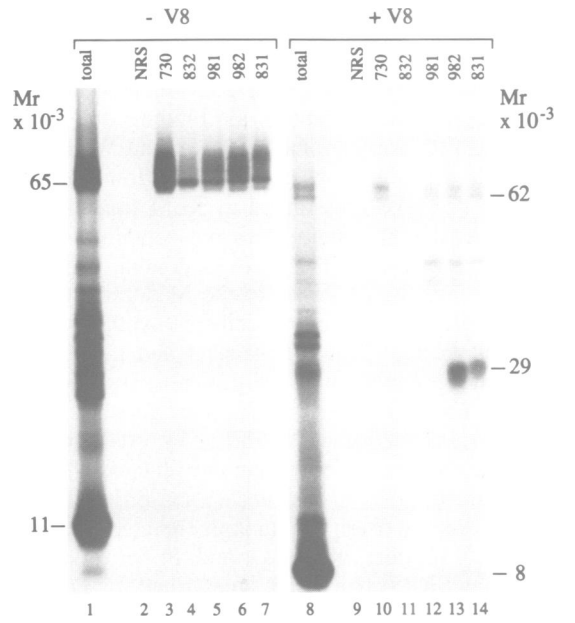

Fig. 2. The SA sequence of $IMC\text{-}CAT_{103}$ is exclusively crosslinked to the M domain of SRP54. The SRP- and truncation-arrested nascent chain of $IMC\text{-}CAT^{\Delta 27}$ ($IMC\text{-}CAT_{103}^{\Delta 27}$, 11 kDa band in lane 1) was photo-crosslinked to SRP54. The total crosslinked products are shown after purification by centrifugation through a sucrose cushion (lane 1). The purified material after proteolysis with 10 μg/ml V8 protease in the presence of 25 mM EDTA is shown in lane 8. Crosslinked products were immunoprecipitated before (lanes 2–7) or after proteolysis (lanes 9–14) with the following antisera as indicated: non-related serum (NRS), lanes 2 and 9; anti-SRP54 peptide antisera 730, lanes 3 and 10; 832, lanes 4 and 11; 981, lanes 5 and 12; 982, lanes 6 and 13; 831, lanes 7 and 14.

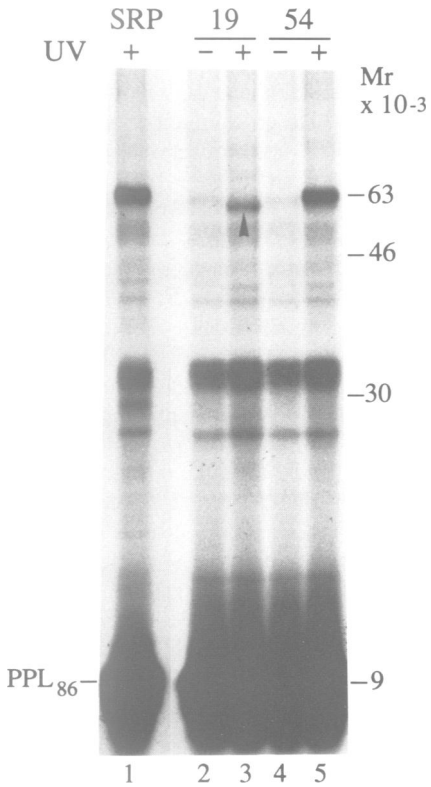

Fig. 3. Cell-free synthesized SRP54 interacts with the nascent chain of PPL_{86}. PPL_{86} was synthesized in the presence of ϵ-TDBA-lysyl tRNA and [^{35}S]methionine. SRP (lane 1) or unlabelled cell-free translation mixtures of mammalian SRP19 (lanes 2 and 3) or SRP54 (lanes 4 and 5) were added, and photo-crosslinking was induced by UV irradiation (lanes 1, 3 and 5) or not (lanes 2 and 4). The UV-dependent crosslinked products of PPL_{86} (9 kDa) to SRP54 contained in SRP (lane 1) or after translation *in vitro* (lane 5) are indicated (63 kDa). The 61 kDa crosslinked product of PPL_{86} to the endogenous wheat germ homologue of SRP54 is indicated in lane 3 (arrowhead).

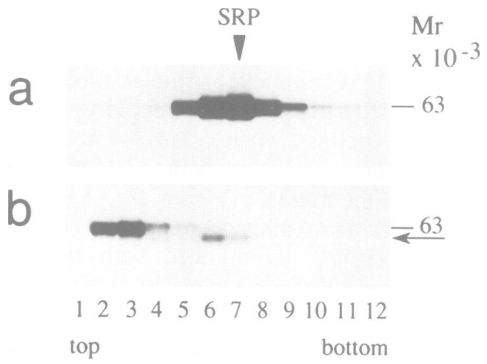

Fig. 4. Cell-free synthesized SRP54 is not associated with an RNP particle. Crosslinked complexes between PPL_{86} and SRP (**a**) or PPL_{86} and cell-free synthesized SRP54 (**b**) were released from the ribosome by puromycin/high salt and centrifuged through a 5–20% sucrose gradient. Equal fractions were collected from the top (fraction 1) to the bottom (fraction 12) of the gradient and analysed by SDS–PAGE and fluorography. Only the portions of the fluorograph containing the crosslinked complexes of PPL_{86} with free or SRP-contained SRP54 (63 kDa) or endogenous $SRP54^{WG}$ (arrow) are shown. Numbers below the lanes indicate fraction numbers.

immunoprecipitation using five SRP54 anti-peptide antisera (cf. Figure 1b).

As expected immunoprecipitation with all five antisera of the undigested crosslinked material yielded a product of 63 kDa (Figure 5, lanes 4–8) which was also precipitated by a prolactin-specific antiserum (lane 2). Digestion with V8 protease did not alter the apparent M_r of PPL_{86} (Figure 5, compare 9 kDa band in lane 10 with that in lane 2) although most of the 63 kDa crosslinked product was proteolysed (lane 10). As had been observed with SRP (High and Dobberstein, 1991; and Figure 2) the 63 kDa complex was digested to a major product of 30 kDa. This must be composed of the 9 kDa PPL_{86} peptide and the 21 kDa M domain of SRP54 as it was recognized only by antibodies specific for SRP54M (Figure 5, lanes 15 and 16) but not by those specific for SRP54N+G (Figure 5, lanes 11–14, cf. Figure 1b). A weaker crosslinked complex of 41 kDa comprises the entire M domain and the COOH-terminal part of the N+G domain as it was also immunoprecipitated by both M domain-specific antisera and by antiserum 981 which recognizes the COOH-terminal portion of the N+G domain (Figure 5, lanes 14–16; cf. Figure 1b). An experiment using $IMC\text{-}CAT_{103}^{\Delta 27}$ as the crosslinking partner, instead of PPL_{86}, yielded similar crosslinking products containing SRP54M (not shown). Thus the site of interaction of a signal sequence with free SRP54 or with SRP-bound SRP54 is the same.

The M domain of SRP54 alone is sufficient for interaction with signal sequences

So far all assays that tested the binding of SRP54 to signal sequences contained the N+G domain either as part of intact SRP54 or present in the reaction mixture after proteolytic cleavage (High and Dobberstein, 1991). Thus, a non-covalent requirement for SRP54N+G during the recognition of signal sequences could not be excluded. In order to test if signal sequences could be recognized by SRP54M alone we removed or inactivated SRP54N+G.

Proteolysis of SRP using V8 protease liberates SRP54N+G from SRP54M which remains bound to the RNP particle and can be quantitatively separated from SRP54N+G by absorption to DEAE–Sepharose (Römisch *et al.*, 1990; Zopf *et al.*, 1990). When we tested proteolysed SRP lacking SRP54N+G the M domain was efficiently crosslinked to PPL_{86} (not shown) as previously found for the unfractionated material (High and Dobberstein, 1991). This suggested that the N+G domain of SRP54 was dispensable for the interaction of SRP54M with signal sequences.

In order to determine if SRP54M alone, i.e. in the absence of 7S RNA and the other SRP proteins, was capable of signal recognition we synthesized unlabelled SRP54 *in vitro* as above (Figures 3 and 4) and digested it with V8 protease. After crosslinking to PPL_{86}, the interacting component was identified by immunoprecipitation using antibodies recognizing both mammalian and wheat germ SRP54N+G (antiserum 730) or specific for the COOH-terminus of mammalian SRP54M (antiserum 831). Figure 6, lanes 5 and 6 show that the 30 kDa proteolytic fragment of the crosslinked complex between SRP54 and PPL_{86} was recognized by antiserum 831 (Figure 6, lane 6), but not by antiserum 730 which only immunoprecipitated the intact endogenous $SRP54^{WG}$ (lane 5, arrow). Thus we conclude that SRP54M of V8 protease digested SRP54 can interact with the signal sequence of PPL_{86} while SRP54N+G cannot.

The N+G domain was still present in the mixture after proteolysis and could thus have assisted in signal sequence binding. In order to inactivate this domain we made use of the observation that intact SRP54 is inactivated by alkylation of cysteine residues with NEM (Siegel and Walter, 1988), and that the five cysteine residues present in mammalian SRP54 are exclusively located in the N+G domain (Römisch *et al.*, 1989; Bernstein *et al.*, 1989). Reconstitution of SRP with NEM treated SRP54 ($SRP54^{NEM}$) had rendered it inactive in assays for elongation arrest and protein translocation and prevented its binding to polysomes containing PPL nascent chains (Siegel and Walter, 1988). Consistent with these observations, we found that PPL_{86} could not be photo-crosslinked to NEM treated SRP (not shown). As the M domain does not contain any cysteine residues the NEM modified N+G domain was likely to impair the binding of PPL_{86} to the M domain.

To address this possibility, cell-free synthesized SRP54 was mock treated (Figure 6, lanes 1 and 2), treated with NEM (lanes 3 and 4), with V8 protease (lanes 5 and 6) or with both (lanes 7 and 8) and assayed for the capacity to bind the signal sequence of PPL_{86} by crosslinking. The resulting complexes were immunoprecipitated with antisera 730 (Figure 6, lanes 1, 3, 5 and 7) and 831 (lanes 2, 4, 6 and 8). Both antisera immunoprecipitated the 63 kDa crosslinked product of PPL_{86} and *in vitro* synthesized mammalian SRP54 (Figure 6, lanes 1 and 2). This product was not observed when the translation mixture of SRP54 was treated with NEM (Figure 6, lanes 3 and 4). The wheat germ homologue of SRP54 was present in all of the crosslinking assays in an unmodified form due to the addition of the PPL_{86} translation mixture. This gave rise to the 61 kDa crosslinked complex which was immunoprecipitated under all conditions by antiserum 730 (Figure 6, lanes 1, 3, 5 and 7, arrow). As already mentioned, digestion of SRP54 with V8 protease resulted in a 30 kDa crosslinked product of PPL_{86} and SRP54M which was immunoprecipitated by antiserum 831 (Figure 6, lane 6) but not by antiserum 730 (lane 5). NEM treatment of the V8 digested

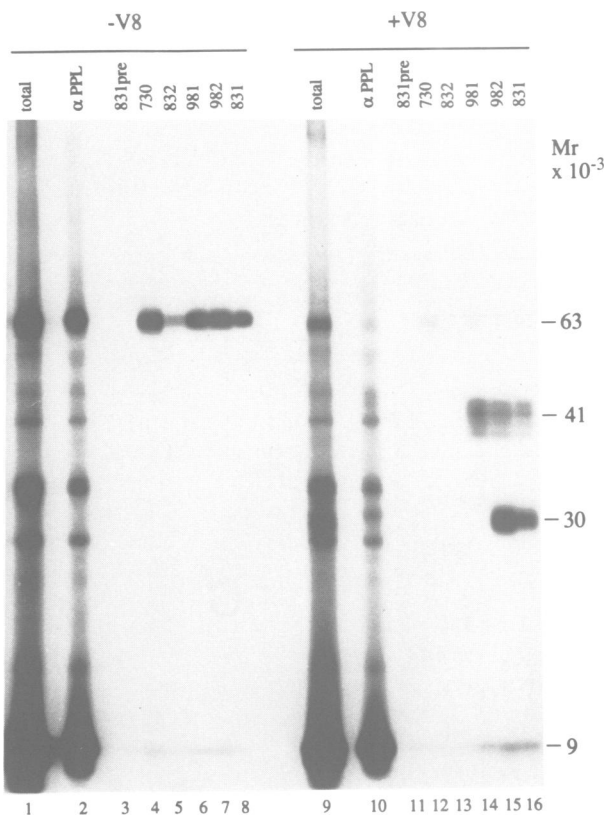

Fig. 5. The M domain of cell-free synthesized SRP54 interacts with the signal sequence of PPL_{86}. Crosslinked complexes between nascent PPL_{86} and cell-free synthesized SRP54 were made as described in the legend to Figure 3 and purified by centrifugation through a sucrose cushion. The total purified material (lane 1) was mock-digested (lanes 1–8) or digested with 10 μg/ml V8 protease in the presence of 25 mM EDTA (lanes 9–16) and immunoprecipitated with the following antisera: anti-prolactin (lanes 2 and 10); pre-immune serum 831 (lanes 3 and 11); the anti-SRP54 peptide antisera 730 (lanes 4 and 12); 832 (lanes 5 and 13); 981 (lanes 6 and 14); 982 (lanes 7 and 15); and 831 (lanes 8 and 16); no immunoprecipitation (lanes 1 and 9). See Figure 1b for the locations of the epitopes recognized by the antibodies. The total and immunoprecipitated material analysed by SDS–PAGE and fluorography is shown. The crosslinked products between PPL_{86} and intact SRP54 (63 kDa) or proteolytic fragments thereof (41 and 30 kDa) are indicated. Lanes 3–8 and 11–16 were fluorographed five times longer than lanes 1, 2, 9 and 10.

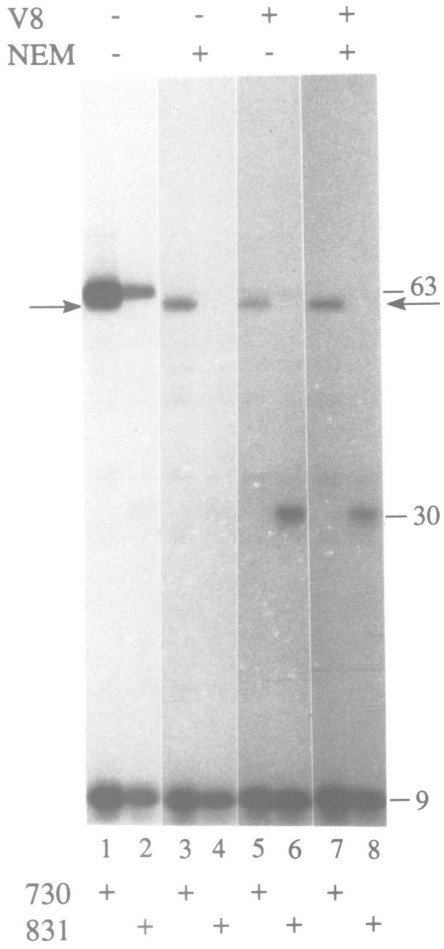

Fig. 6. NEM alkylation of SRP54 but not of V8 cleaved SRP54 prevents the binding of PPL_{86} to SRP54M. SRP54 synthesized in the wheat germ cell-free system was mock-digested or cleaved into its N+G and M domains by incubation with V8 protease after (lanes 1–4) or before (lanes 5–8) inactivation by diisopropyl fluorophosphate, respectively. Both, SRP54 and proteolysed SRP54 were treated with NEM after (lanes 1, 2, 5 and 6) or before (lanes 3, 4, 7 and 8) the addition of dithiothreitol. After photo-crosslinking to nascent PPL_{86} crosslinked products were analysed by immunoprecipitation with antisera specific for SRP54N+G (antiserum 730, lanes 1, 3, 5 and 7) or SRP54M (antiserum 831, lanes 2, 4, 6 and 8), SDS–PAGE and fluorography. Crosslinked products of PPL_{86} with mammalian SRP54 (63 kDa) or SRP54M (30 kDa) and endogenous $SRP54^{WG}$ (arrows) are indicated. Note that the wheat germ extract in which PPL_{86} had been synthesized was not treated with NEM.

mixture did not impair crosslinking of SRP54M (Figure 6, lane 8), whereas it completely inhibited any crosslinking to either intact SRP54 (lanes 3 and 4) or residual intact SRP54 present after V8 digestion (lanes 5 and 6) and visible in the non-alkylated samples after long exposure (not shown). Thus, the NEM modified N+G domain can only interfere with the binding of a signal sequence if it is part of SRP54, consistent with the N+G domain playing no direct role in signal sequence binding.

The regions of SRP54M required for binding to 7S RNA and signal sequences largely overlap

Besides being sufficient for interacting with signal sequences, SRP54M alone is also capable of binding to 7S RNA in the presence of SRP19 (Römisch *et al.*, 1990; Zopf *et al.*, 1990). The binding sites have been proposed to be formed by four amphipathic helices interacting via their hydrophobic faces with the highly variable signal sequences (Bernstein *et al.*, 1989) and via their hydrophilic ones with 7S RNA (High and Dobberstein, 1991). In order to map the binding sites for 7S RNA and signal sequences more closely we deleted various portions of the M domain of *in vitro* synthesized SRP54 and then tested the deleted proteins for these two binding activities. Figure 7a depicts the deletions of SRP54 and the positions of the four predicted amphipathic helices (Bernstein *et al.*, 1989). SRP54 truncated from its COOH-terminus by 19, 42, 65, 90 and 140 amino acids ('54-2' to '54-6') or a segment ('54-7') comprising the COOH-terminal 154 amino acids of SRP54M (amino acids 351–504) were used in both tests. In addition, we tested the *in vitro* synthesized M domain ('54-M', amino acids 297–504) which had been sufficient for binding to 7S RNA–SRP19 (Römisch *et al.*, 1990) for its capacity to interact with signal sequences.

Binding of SRP54, and truncations thereof, to 7S RNA was monitored by batch absorption of assembled RNP to DEAE–Sepharose as described previously (Lingelbach

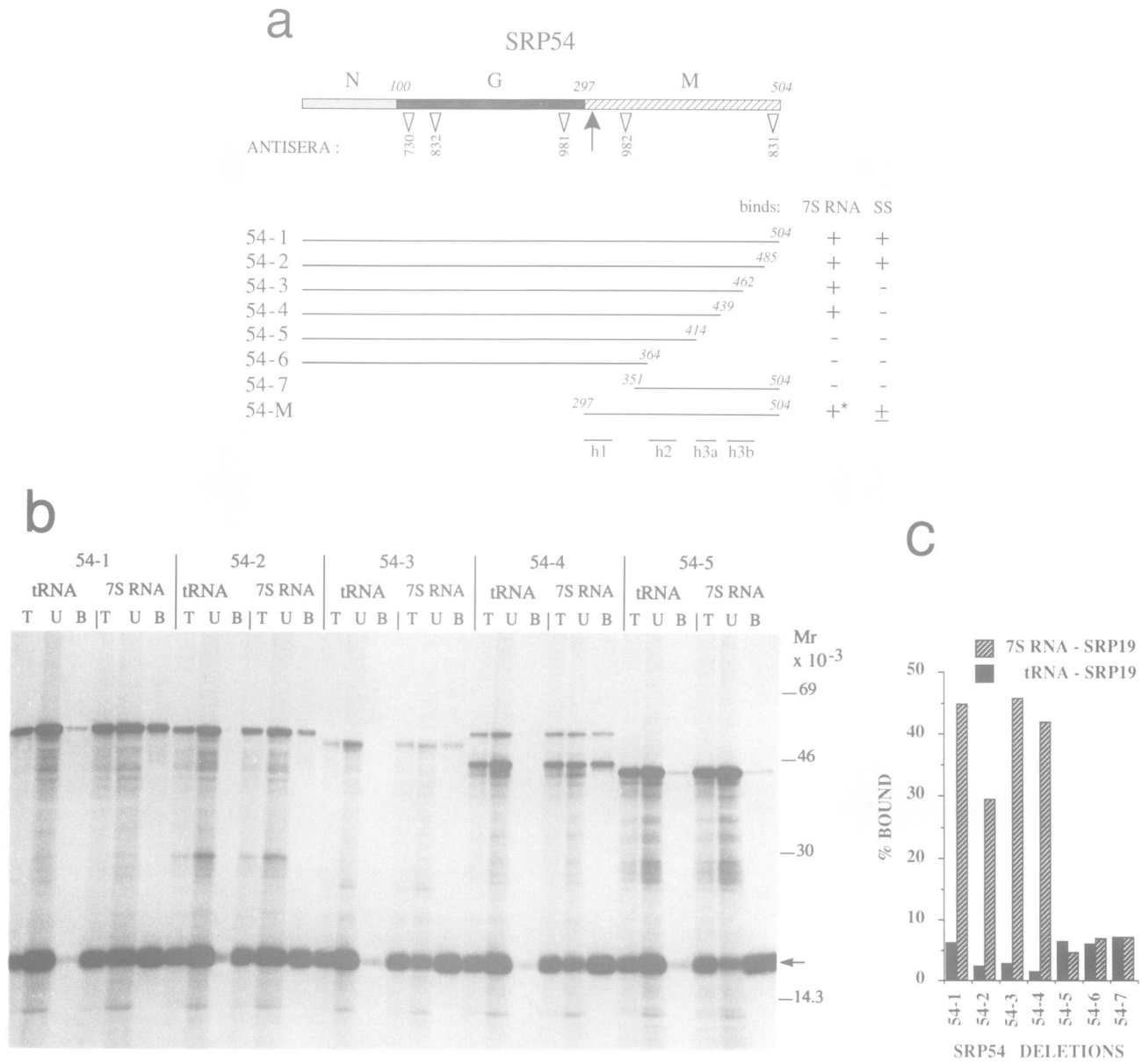

Fig. 7. Deletion mapping of SRP54M. (**a**) SRP54 and its domains are schematically represented as in Figure 1b. The approximate position of cleavage by V8 protease is indicated by an arrow (cf. Römisch *et al.*, 1990). The positions of peptides used to raise anti-peptide antisera 730, 832, 981, 982 and 831 are indicated by arrowheads. COOH-terminal (54-2 to 54-6) and NH_2-terminal (54-7 and 54-M) truncations of SRP54 (54-1) are represented by lines with numbers in italics indicating the positions of the last or first and last amino acids. Proposed amphipathic helices (Bernstein *et al.*, 1989) are depicted by short lines labelled h1 to h3b. The binding activities to 7S RNA and signal sequences (SS) of the truncated SRP54 proteins are indicated by + and −. The ± indicates inefficient binding. * Binding to 7S RNA of *in vitro* synthesized SRP54M ('54-M') had been demonstrated previously (Römisch *et al.*, 1990). (**b**) Binding of SRP54 ('54-1') and truncated SRP54 proteins ('54-2' to '54-5') in the presence of SRP19 (arrow) to 7S RNA or bovine liver tRNA was tested by batch absorption of RNP particles to DEAE−Sepharose. 20% of the total input ('T'), all of the DEAE-unbound material ('U') and all of the bound and eluted material ('B') were TCA precipitated and analysed by SDS−PAGE and fluorography. Numbers on the right indicate the relative molecular masses of marker proteins. Incomplete cleavage resulted in the residual full-length SRP54 observed with deletion 54-4 which however, did not compete with binding of 54-4. (**c**) Quantification of the relative amounts of DEAE-unbound (U) and -bound (B) material: the original fluorograph was scanned with an LKB Ultroscan XL laser densitometer, and binding to tRNA−SRP19 (black bars) or 7S RNA−SRP19 (hatched bars) was expressed as the percent of the combined bound and unbound fractions for each SRP54 deletion (% bound = 100 B $[U+B]^{-1}$, ordinate). Deletion mutants (54-2 to 54-7) of SRP54 (54-1) are indicated on the abscissa. The more efficient binding to 7S RNA of SRP19 (mean ± SD: 65.0% ± 2.5%) than of SRP54 (mean ± SD of 54-1 to 54-4: 40.5% ± 4.4%) has been consistently observed in repeated experiments independently of the amount of protein synthesized and has been discussed previously (Römisch *et al.*, 1990).

et al., 1988; Römisch *et al.*, 1990). Briefly, 7S RNA or tRNA (as a negative control) and SRP19 were mixed in an estimated 16-fold molar excess with SRP54 or its deletions and allowed to form RNPs. An aliquot of the 'total' (Figure 7b: T) and the DEAE-'bound' (B) and -'unbound' (U) material were analysed by SDS−PAGE and fluorography. The results of the RNA binding assay are displayed in Figure 7b, quantified by scanning of the fluorograph in Figure 7c and summarized in Figure 7a ('7S RNA'). Binding of SRP54 to 7S RNA−SRP19 still occurred when the COOH-terminal 65 amino acids were removed from its M domain (Figure 7a−c, '54-2' to '54-4') but was no longer possible when 90 or more amino acids were deleted (Figure 7a−c, '54-5' and '54-6'). While the entire

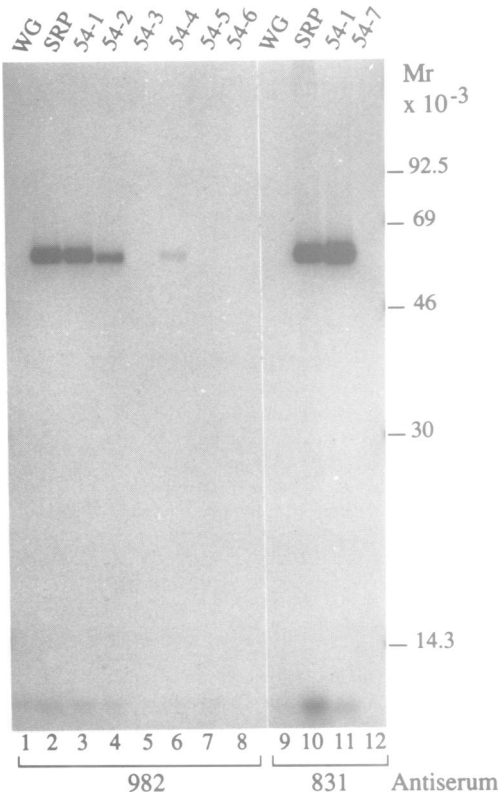

Fig. 8. Photo-crosslinking of signal sequences to SRP54 and its truncations: PPL_{86} was photo-crosslinked to unlabelled cell-free synthesized SRP54 (54-1) and its truncations (54-2 to 54-7, outlined in Figure 7a) (lanes 3–8, 11 and 12), to endogenous components of the wheat germ lysate (lanes 1 and 9) and to SRP (lanes 2 and 10). Crosslinked complexes were immunoprecipitated with anti-peptide antisera recognizing an epitope in the NH_2-terminal (982, lanes 1–8) or the COOH-terminal region (lanes 9–12) of SRP54M and specific for the mammalian SRP54. The products were analysed by SDS–PAGE and fluorography. Positions of molecular mass markers are indicated on the right.

M domain ('54-M') had been found to bind to 7S RNA–SRP19 (Römisch *et al.*, 1990), binding no longer was possible when the 53 NH_2-terminal amino acids of SRP54M were removed (Figure 7a and c, '54-7').

The same truncated SRP54 proteins were also tested in crosslinking assays (in the absence of 7S RNA and SRP19) for their abilities to interact with the signal sequences of PPL_{86} (Figure 8), $IMC\text{-}CAT_{103}$ and $IMC\text{-}CAT_{103}^{\Delta 27,46}$ (not shown). Crosslinked products were immunoprecipitated with the appropriate antisera, i.e. antiserum 982 for SRP54-1 to 54-6 (lanes 3–8) and antiserum 831 for SRP54-1 and SRP54-7 (lanes 11 and 12). Since neither antiserum precipitates the endogenous wheat germ SRP54 homologue no crosslinked complexes were observed when only wheat germ lysate (WG) was added (Figure 8, lanes 1 and 9). Both antisera precipitated SRP54 which had been added to the crosslinking reactions as a positive control and was either part of purified SRP ('SRP', Figure 8, lanes 2 and 10) or derived from translation *in vitro* ('54-1', Figure 8, lanes 3 and 11). Of the COOH-terminally truncated proteins, only SRP54-2 interacted with the signal sequences of PPL_{86} (Figure 8, lane 4), $IMC\text{-}CAT_{103}$ or $IMC\text{-}CAT_{103}^{\Delta 27,46}$ (not shown). Since in SRP54-2 only the COOH-terminal 19 amino acids had been removed from SRP54 only a small shift in M_r of ~2 kDa can be seen (Figure 8, cf. lanes 3 and 4; see also Figure 7b). All further COOH-terminal truncations (54-3 to 54-6) abolished the capability to interact with either of the three signal sequences tested. The minor crosslinked product seen in lane 6 of Figure 8 (63 kDa) is that of incompletely truncated full-length SRP54 (cf. Figure 7b '54-4').

The signal sequence binding capacity of *in vitro* synthesized SRP54M ('54-M', Römisch *et al.*, 1990) was found to be low (not shown) while the COOH-terminal 154 amino acids of SRP54 ('54-7') were incapable of binding any of the signal sequences (not shown and Figure 8, lane 12). Since the M domain derived from *in vitro* synthesized SRP54 by V8 proteolysis showed efficient signal sequence binding (Figure 6) this suggests that *in vitro* synthesized M domains may not be able to fold into the correct conformation for optimal signal sequence binding.

Discussion

Photo-crosslinking was combined with limited proteolysis and mapping with site-specific antibodies or deletion mutagenesis to characterize the interaction between signal sequences and SRP54. Previously, this interaction had only been investigated for the cleavable signal sequence of preprolactin, and the M domain of SRP54 was the only detectable site of interaction (Zopf *et al.*, 1990; High and Dobberstein, 1991). Since uncleaved SA sequences, which can mediate the membrane insertion of type I and type II membrane proteins, also interact with SRP54 (High *et al.*, 1991b) it was of interest to know if they also contacted the same domain of SRP54. For the type I SA sequence of IMC-CAT we show here that it also interacts with the M domain of SRP54. Thus, both a cleavable signal sequence and an uncleaved SA sequence are only in contact with SRP54M and not with SRP54N+G.

In PPL the lysine residues are found only NH_2-terminal of the hydrophobic core of the signal sequence. By contrast, in $IMC\text{-}CAT^{\Delta 27}$ the lysine residues from which crosslinking can occur are only found COOH-terminal of the hydrophobic core of the SA sequence. Thus, crosslinking from these molecules can occur from either side of the hydrophobic core of the signal sequence. As both proteins were crosslinked to the M domain but not to the N+G domain, it is likely that over their entire length signal sequences are only in contact with the M domain of SRP54.

Since the only component of SRP detected to interact with signal sequences was SRP54, it was conceivable that free SRP54 alone was sufficient to perform this function. This was indeed found to be the case since proteins bearing a cleavable signal sequence or a SA sequence were efficiently crosslinked to *in vitro* synthesized free SRP54. SRP54 synthesized in this cell-free system was not part of a RNP particle. This was clearly demonstrated by sucrose gradient analysis which showed that *in vitro* synthesized SRP54 did not migrate as an 11S RNP particle but as a free protein. The site of interaction of signal sequences with free SRP54 was found to be the same as that with SRP-bound SRP54 since again only SRP54M was found in contact with the signal sequences of PPL or IMC-CAT. The crosslinking of signal sequences to free SRP54 and SRP-bound SRP54 was of roughly similar efficiency, since comparable molar quantities of *in vitro* synthesized and particle-bound SRP54 (as tested by immunoblotting; not shown) resulted in crosslinked products of similar intensities.

SRP54M retained in the RNP particle and even in its free

form was found to interact with signal sequences. The presence of SRP54N+G was not required for this interaction, although alkylation of the N+G domain of intact SRP54 inhibited signal sequence binding. In a previous study, alkylation of SRP54 with NEM ($SRP54^{NEM}$) followed by its reconstitution into SRP rendered this particle unable to promote elongation arrest of PPL or to bind to a complex of ribosome and nascent chain (Siegel and Walter, 1988). This was interpreted as $SRP54^{NEM}$ being unable to recognize signal sequences. By photo-crosslinking we show that this is indeed the case, and that free $SRP54^{NEM}$ unlike its unmodified form is unable to interact with signal sequences. The physical separation of SRP54N+G from SRP54M by cleavage with V8 protease prior to alkylation allowed the subsequent cross-linking of SRP54M to PPL_{86}. In canine SRP54 only the N+G domain can be alkylated by NEM since all cysteine residues, the targets for modification by NEM, are found in this domain and none are present in the M domain.

What could be the mechanism by which the NEM modified N+G domain inhibits the binding of a signal sequence to SRP54M? At least two scenarios can explain this finding. (i) The N+G domain could be close to the signal sequence binding site of the M domain such that alkylation could then block the entry of a signal sequence into its binding site on SRP54M by steric hindrance. (ii) Alternatively, alkylation of any one cysteine residue could interfere with a GTP-related function of the N+G domain which contains consensus motifs typical for GTPases (Bernstein *et al.*, 1989; Römisch *et al.*, 1989). *In vivo* this domain may function to regulate the recognition and/or binding of signal sequences to the M domain by conformational changes induced by its GTP/GDP binding state. In either case, the removal of SRP54N+G by proteolysis would allow SRP54M to resume its function.

Fine mapping of the signal binding site on SRP54 using COOH-terminally deleted SRP54 proteins revealed that nearly the entire M domain was required for binding to three different signal sequences. Since the binding of a signal sequence was deduced from its ability to be crosslinked we used several nascent chains with lysine residues at various positions. This made it unlikely that a truncated M domain capable of signal sequence binding could not be crosslinked to the nascent chain and could therefore escape detection. For all signal sequences tested only the 19 most COOH-terminal amino acid residues of SRP54 could be deleted without destroying its signal sequence binding function. For binding to 7S RNA−SRP19 a slightly larger fragment comprising the 65 most COOH-terminal amino acids of SRP54 was found dispensable. SRP54M created by cleavage of SRP54 with V8 protease efficiently bound to both 7S RNA (Römisch *et al.*, 1990; Zopf *et al.*, 1990) and to signal sequences. Based on secondary structure predictions the primary amino acid sequence of SRP54M has been proposed to form four amphipathic helices the hydrophobic faces of which might constitute a binding pocket for signal sequences (Bernstein *et al.*, 1989). It was furthermore proposed that the hydrophilic faces of the helices could interact with 7S RNA (High and Dobberstein, 1991). This implies that the RNA and signal sequence binding functions of SRP54 would overlap on SRP54M. Alternatively, the RNA and signal sequence binding functions could be performed by separate sub-domains. Our results are consistent with the former view namely that largely overlapping regions of SRP54M are essential for both RNA and signal sequence binding.

Our finding that free, i.e. RNP-unbound SRP54 can interact with signal sequences raises the possibility that the function of SRP may require the sequential assembly and disassembly of SRP54 with the rest of the RNP complex. The possibility that a free form of SRP54 has a role in SRP-mediated targeting as proposed by Rapoport (1990) should now be considered.

Materials and methods

Materials

T7 RNA polymerase, RNase H, V8 protease, Klenow and restriction enzymes were from Boehringer Mannheim GmbH (Mannheim, Germany). Taq polymerase was from Perkin Elmer Cetus Co. (Emeryville, CA). [^{35}S]Methionine was from Amersham Buchler GmbH (Braunschweig, Germany). Cycloheximide, emetine, 7-methyl-guanosine 5′-monophosphate and puromycin were supplied by Sigma Chemical Co. (St Louis, MO). DEAE−Sepharose CL 6B was from Pharmacia. 4-(3-trifluoromethyl-diazarino) benzoic acid (TDBA) was a gift from Dr Josef Brunner, Swiss Federal Institute of Technology, Zürich, Switzerland.

SRP54 deletions

Deletion mutants 54-2 to 54-4 were made by annealing complementary oligonucleotides in large molar excess to the *in vitro* transcript of SRP54 at the position to be cleaved and incubating it in translation buffer with RNase H (0.5 U/25 μl) for 15 min at 37°C. As deduced from the positions of the complementary oligonucleotides the SRP54 proteins, 54-2, 54-3 and 54-4, were arrested at the COOH-termini of amino acids 484, 462 and 442, respectively. Deletions 54-5 and 54-6 were constructed on the cDNA level by substituting a fragment 3′ of the unique *Bgl*II site in pSRP54-1 (Römisch *et al.*, 1989) with corresponding PCR fragments coding for stop codons at amino acid positions 415 and 363, respectively. Deletion mutant 54-7 was constructed by removing the cDNA sequence coding for SRP54N+G up to the *Bgl*II site in pSRP54-1 (Römisch *et al.*, 1989). The first codon for methionine following the *Bgl*II site (amino acid position 351) was very efficiently used as the initiation site in the wheat germ translation system yielding a 17 kDa protein (not shown).

Transcription and translation

Transcription of pSRP54-1 and deletion constructs thereof with T7 polymerase was performed as recommended by the manufacturer. For the photo-crosslinking assays truncated mRNA coding for the NH_2-terminal regions of preprolactin (PPL_{86}) and the artificial type I membrane signal anchor protein IMC-CAT and its variants (Figure 1) was prepared as previously described (High *et al.*, 1991b). Translations in the wheat germ cell-free translation system were carried out as previously described (Stueber *et al.*, 1984).

Photo-crosslinking analysis

For photo-crosslinking translation was performed as described by Stueber *et al.* (1984) in the presence of 3.75 pmol ε-TDBA-Lys-tRNA per 25 μl of translation mixture. The ε-TDBA-Lys-tRNA was prepared as described by Wiedmann *et al.* (1987) except that the modified Lys-tRNA was not submitted to benzoyl DEAE−cellulose chromatography. Cell-free translations of PPL_{86} and $IMC\text{-}CAT_{103}$ were pulsed (Krieg *et al.*, 1986) by addition of 7-methylguanosine 5′-monophosphate to a final concentration of 2 mM after 10 min of incubation. Translations were continued for a further 5 min and then cycloheximide added to 2 mM to prevent further chain elongation. Translation of truncated mRNA yields nascent chains which due to the lack of a stop codon largely remain bound to the ribosomes.

When purified SRP was used it was present during the translation at, or added after the completion of translation to, a final concentration of 25 nM. When unlabelled SRP proteins made by cell-free translation were included they were added at an equal volume of translation mixture to that of the completed synthesis of PPL_{86} or $IMC\text{-}CAT_{103}$.

After the addition of SRP or cell-free translation products to the nascent chain−ribosome complex the mixture was incubated at 25°C for 5 min and then chilled on ice. Subsequent irradiation of the samples on ice was as previously described (High *et al.*, 1991b). After irradiation the samples were TCA precipitated for direct analysis or immunoprecipitated under denaturing conditions using peptide-specific antisera (Römisch *et al.*, 1990). When crosslinked products or ribosome−nascent chain complexes were

purified this was performed by centrifugation through a high salt/sucrose cushion as previously described (High *et al.*, 1991a).

Sucrose gradient analysis

Crosslinked complexes were formed between PPL_{86} and SRP or *in vitro* synthesized SRP54 as described above except that emetine was added to 2 mM instead of cycloheximide to inhibit further chain elongation. The crosslinked complexes were released from the ribosome by the addition of puromycin to 0.3 mM and the adjustment of KOAc to 0.4 M followed by an incubation at 25°C for 10 min. Samples were centrifuged for 20 h in an SW40 rotor at 39 000 r.p.m. and 4°C through a 5–20% sucrose gradient containing 0.5 M KOAc, 20 mM HEPES–KOH pH 7.5 and 5 mM $Mg(OAc)_2$. Fractions of 1 ml were collected from the top and TCA–acetone precipitated prior to analysis by SDS–PAGE and fluorography.

Proteolysis of SRP and in vitro synthesized SRP54 using V8 protease

SRP and cell-free synthesized SRP54 were digested with 25 μg/ml V8 protease (protease Glu-X from *Staphylococcus aureus*, strain V8) as described (High and Dobberstein, 1990) with inactivation of the protease using diisopropyl fluorophosphate before or after the incubation. After the treatment of cell-free synthesized SRP54 with V8 protease the pH was adjusted to 7.0 by the addition of 100 mM HEPES. *N*-ethyl maleimide was added to 10 mM and quenched by the addition of 15 mM dithiothreitol before or after an incubation for 30 min at 25° C.

Fractionation of proteolysed SRP using DEAE–Sepharose

After treatment of 12.5 pmol of SRP with V8 protease (Römisch *et al.*, 1990; High and Dobberstein, 1991) the products were allowed to bind 50 μl of DEAE–Sepharose slurry (25 μl beads suspended in 25 μl TMK buffer: 50 mM TEA pH 7.5, 5 mM $Mg(OAc)_2$ and 250 mM KOAc). After binding the beads were pelleted and the supernatant containing unbound material removed. The beads were washed three times in 1 ml of TMK buffer. The bound material was eluted in 100 μl of 50 mM TEA pH 7.5, 5 mM $Mg(OAc)_2$ and 600 mM KOAc for 20 min. The elution was repeated and the supernatants were combined. The eluted material was tested for signal sequence binding. Analysis of proteolysed and DEAE-fractionated products by Western blotting was as previously described (Römisch *et al.*, 1990).

RNA binding assay

Binding of SRP proteins to 7S RNA was monitored by batch absorption of assembled SRP sub-particles to DEAE as described previously (Römisch *et al.*, 1990; Lingelbach *et al.*, 1988).

Acknowledgements

We are grateful to Josef Brunner for the generous gift of TDBA. We thank Dr A.Helenius for critically reading the manuscript. This work was supported in part by a grant (Do 199/5–3) from Deutsche Forschungsgemeinschaft. H.Lütcke is the recipient of an EMBO long-term fellowship.

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Received on October 28, 1991; revised on December 2, 1991