The nascent polypeptide-associated complex modulates interactions between the signal recognition particle and the ribosome

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Background: The first step in the co-translational targeting of secretory proteins to the endoplasmic reticulum membrane involves the recognition of signal sequences by the 54 kDa subunit of the signal recognition particle (SRP) as they emerge from the ribosome. It has recently been proposed that the nascent polypeptide-associated complex (NAC) contributes to the fidelity of targeting by modulating interactions that occur between the ribosome–nascent chain complex, the SRP and the endoplasmic reticulum membrane. Precisely how NAC influences SRP function is presently unclear.

Results: We have used immunoblotting experiments to monitor interactions between the SRP and the ribosome–nascent chain complex, in the absence and presence of NAC. In the absence of NAC, SRP binds in a high-salt-resistant manner only to ribosomes that contain a signal sequence, confirming the specificity of SRP for signal sequences. Binding of SRP to signalless ribosome nascent chains is observed at lower salt concentrations; however, the amount of SRP bound to this complex is indistinguishable from that bound to ribosomes lacking nascent chains. Thus, this salt-sensitive binding is likely to be the result of interactions between SRP and the ribosome that occur independently of the nascent chain. A minimal particle consisting of SRP54 and SRP RNA is sufficient to confer salt-resistant binding to ribosomes that contain signal sequences, whereas all of the SRP subunits are required for salt-sensitive binding to ribosomes that lack nascent chains. This salt-sensitive binding by SRP is inhibited by the addition of purified NAC.

Conclusions: Based on our results, we define two distinct modes of interaction between SRP and the ribosome–nascent chain complex: salt-resistant interactions between SRP54 and signal sequences, and salt-sensitive interactions between additional components of SRP and the ribosome. We conclude that NAC does not directly influence signal sequence recognition by SRP but, rather, that it negatively modulates interactions that occur between SRP and the ribosome itself. These results are discussed in terms of a model wherein SRP and NAC regulate each others' activity during protein targeting.

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Background

In eucaryotic and procaryotic cells, signal sequences that direct proteins into the secretory pathway contain few structural features that distinguish them from other proteins. In general, clusters of as few as seven consecutive hydrophobic amino acids function as signal sequences. In yeast, about 20 % of random peptide sequences can function as signal sequences (although many function with low efficiency) [1], and in *E. coli*, some proteins lacking a recognizable signal sequence can still be exported in some mutant strains [2]. Given the paucity of information contained within signal sequences, it comes as no surprise that models describing the interaction of signal sequences with the protein export apparatus provoke questions of specificity and fidelity.

In higher eucaryotes, the first step of protein translocation across the endoplasmic reticulum (ER) membrane involves co-translational recognition of signal sequences by the 54 kDa subunit of the signal recognition particle (SRP) (reviewed in [3]). SRP is a ribonucleoprotein composed of 7SL RNA (also referred to as SRP RNA) and six proteins — two monomers, of 19 kDa (SRP19) and 54 kDa (SRP54), and two heterodimers, one composed of a 9 kDa and a 14 kDa polypeptide (SRP9/14), and the other composed of a 68 kDa and a 72 kDa polypeptide (SRP68/72).

Concomitant with signal sequence binding by SRP is an arrest or pause in translational elongation, consistent with the notion that SRP interacts directly with the ribosome as well as the nascent chain. The SRP–ribosome–nascent chain complex is then targeted to the ER by interactions between SRP and its receptor, located at the ER membrane [4,5]. Following this interaction, the ribosome–nascent chain complex is released by SRP and becomes associated with the translocation machinery located in the ER membrane, where a second signal sequence recognition event is postulated to occur [6].

Recently, Wiedmann *et al.* [7] have characterized a protein complex, termed nascent polypeptide-associated complex (NAC), that also interacts co-translationally with nascent polypeptides. NAC is composed of two polypeptides, αNAC and βNAC, with apparent molecular weights of 33 kDa and a 21 kDa, respectively [7]. The authors proposed that this complex performs a chaperone-like function, preventing otherwise promiscuous interactions between nascent chains and other proteins in the cytosol, including SRP. Specifically, they demonstrated that, in the absence of NAC, SRP can be crosslinked to nascent chains lacking a signal sequence, and that these proteins can be translocated across the ER, albeit inefficiently. These observations suggested that SRP may not be the major determinant of specificity in this system, and that it binds signal peptides at least partially by default because they are not substrates for NAC. More recently, it has been demonstrated that translocation of signalless nascent chains across the ER in the absence of NAC occurs in an SRP-independent manner, and that NAC prevents ribosomes bearing signalless nascent chains from interacting with the ER membrane [8,9]. Thus, NAC is also proposed to govern interactions between ribosomes and the ER membrane.

The notion that SRP lacks signal sequence specificity contradicts two recent reports, where *in vitro* analysis showed that functional synthetic signal peptides, but not mutant peptides, could inhibit the GTPase function of SRP54, as well as its *E. coli* homologue, Ffh [10,11]. These experiments were conducted with purified components in the absence of ribosomes and NAC. To address the question of the specificity of signal sequence recognition by SRP, and to gain additional insight into the function of NAC, we have examined directly the association of SRP with ribosome–nascent chain complexes in the presence and absence of NAC. Our results affirm the specificity of SRP for signal sequences and, moreover, suggest that NAC functions by modulating interactions between SRP and the ribosome.

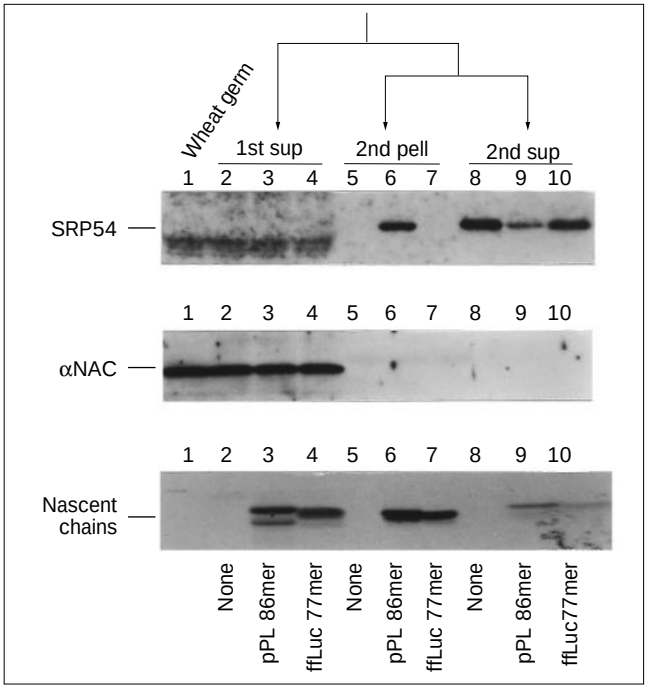
Results

Two different ribosomal binding modes for SRP

We wished to examine directly interactions between SRP and ribosomes bearing different nascent chains in the absence of NAC. To this end, we compared the ability of SRP to sediment through a sucrose cushion in a high-salt-resistant manner, either with vacant ribosomes, or with one of two ribosome-associated nascent chains: pL 86mer, which contained a signal sequence, or ffLuc 77mer, which lacked a signal sequence. These two ribosome–nascent chain complexes were produced by *in vitro* translation of truncated mRNAs, as described in Materials and methods. In each case, ribosomes and ribosome–nascent chains were salt-washed to remove NAC prior to addition of SRP [7]. We carried out immunoblotting experiments, using polyclonal antiserum raised against SRP54, to monitor the association between SRP and ribosomes.

We found that, in the presence of 500 mM potassium acetate (KAc), SRP associated only with ribosomes bearing pPL 86mer nascent chains (Fig. 1, upper panel, lane 6). In contrast, at these high salt concentrations, we

Figure 1



Specific association between SRP and signal-sequence-bearing ribosome–nascent chain complexes that have been stripped of NAC. SRP was incubated with salt-washed ribosomes lacking nascent chains ('none') and pPL 86mer or ffLuc 77mer ribosome–nascent chain complexes, as indicated, and analyzed by centrifugation through sucrose cushions containing 500 mM KAc, as described in Materials and methods. Western immunoblot analysis was carried out using the corresponding pellet (2nd pell, lanes 5–7) and supernatant (2nd sup, lanes 8–10) fractions from this centrifugation step, and the supernatant fractions (1st sup, lanes 2–4) after salt-washing of ribosomes following *in vitro* translation. Immunoblots were probed with polyclonal anti-SRP54 antiserum (upper panel) or polyclonal anti-αNAC antiserum (middle panel), and nascent chains were visualized by autoradiography (lower panel). An equivalent amount of wheat germ extract used for translation reactions was loaded for comparison (lane 1).

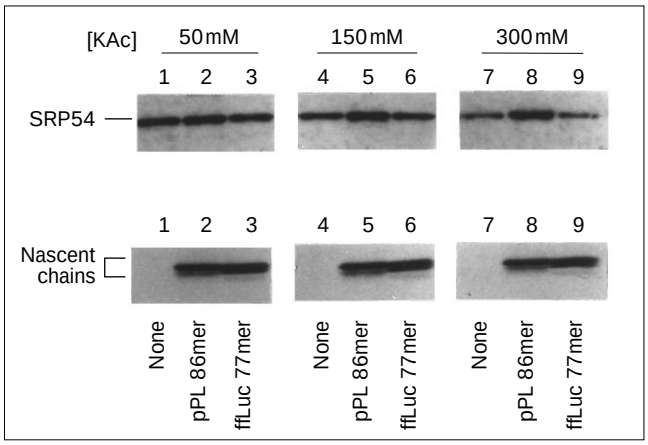
were unable to detect any binding of SRP to ribosomes bearing ffLuc 77mer nascent chains (Fig. 1, upper panel, lane 7), although all detectable NAC was removed during salt-washing of the ribosome–nascent chain complexes (Fig. 1, middle panel). Based on results from quantitative immunoblotting experiments, we estimated that greater than 95 % of NAC was removed from ribosomes by this procedure (data not shown). Quantitation of the radioactivity present in the nascent chains demonstrated that equimolar amounts of pPL 86mer and ffLuc 77mer nascent chains were associated with the ribosomal pellets (Fig. 1, lower panel, lanes 6 and 7). We conclude from these results that SRP, in the absence of NAC, remains selective for ribosomes bearing nascent chains that contain a signal sequence.

In the above experiment, ribosomes and ribosome–nascent chain complexes were washed with 500 mM KAc following translation to remove NAC. Identical results were obtained when ribosomes and ribosome–nascent chains were washed for a second time with 500 mM KAc, or twice with 1.0 M KAc before the addition of SRP (data not shown). Thus, we conclude that it is unlikely that any residual NAC could account for the selective binding of SRP to pPL 86mer nascent chains observed in Figure 1.

We next asked whether SRP could bind to ribosomes bearing ffLuc 77mer nascent chains under conditions that relaxed the stringency of the assay. To this end, we lowered the salt concentration used during the second centrifugation step (Fig. 2). A significant amount of SRP co-sedimented with the ffLuc 77mer ribosome–nascent chain complex when the salt concentration was reduced to 300 mM KAc (Fig. 2, lane 9), and nearly equal amounts of SRP bound to ffLuc 77mer and pPL 86mer ribosome–nascent chain complexes at 50 mM KAc (Fig. 2, compare lanes 2 and 3). However, at each salt concentration examined, the amount of SRP bound to ribosomes containing ffLuc 77mer nascent chains was indistinguishable from that bound to vacant ribosomes (for example, compare Fig. 2, lanes 4 and 6). Hence, this low-salt binding is likely to be the result of interactions between SRP and the ribosome that occur independently of the nascent chain. In a control experiment, we found that no SRP was present in the pellet fractions at low salt in the absence of ribosomes (data not shown), demonstrating that its appearance in the pellets resulted from its association with ribosomes, and was not simply because of aggregation at lower salt concentrations.

Taken together, these results suggest that there are two different ways in which SRP can interact with ribosomes: a high-salt-resistant interaction, which requires a signal sequence, and a high-salt-sensitive interaction, which occurs independently of the nascent chain.

Figure 2



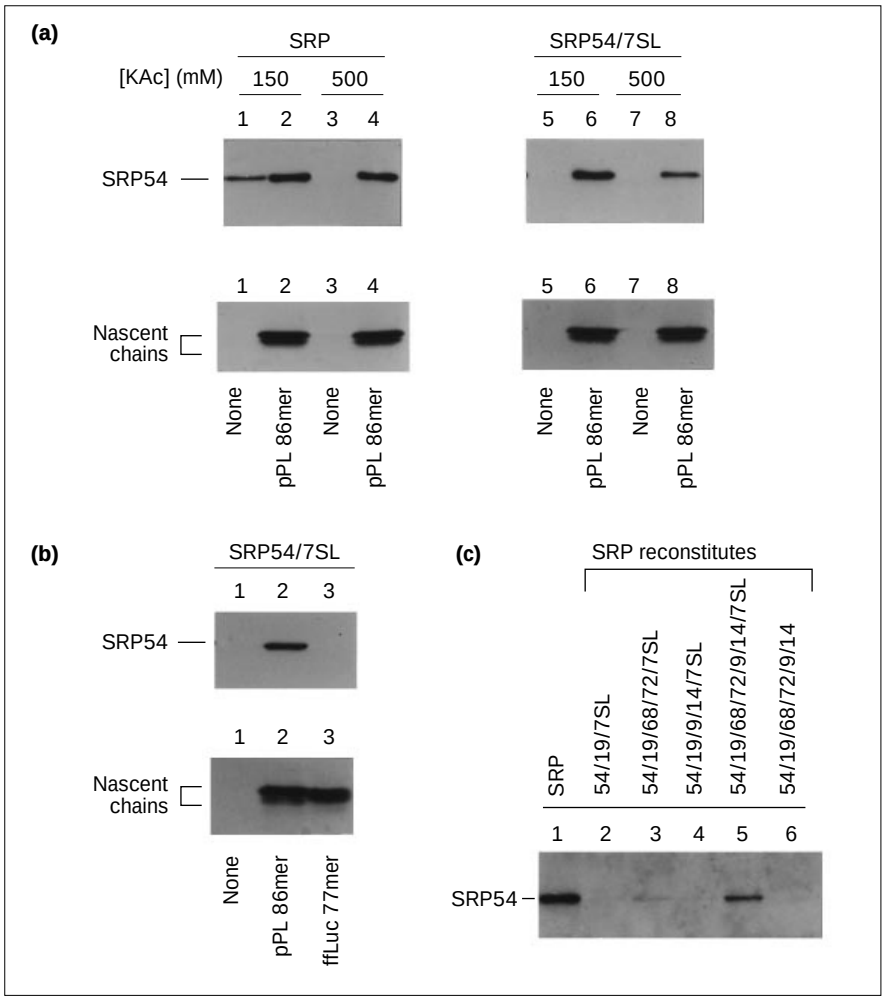
Testing the salt-sensitivity of SRP binding to ribosomes and ribosome–nascent chain complexes. The experiment was performed as described in Fig. 1, except that the concentration of KAc used during the second centrifugation step was varied as indicated. The final ribosomal pellets were used for western immunoblot analysis.

SRP components required for each ribosomal binding mode

To characterize the two different modes of SRP binding to ribosomes, we determined which subunits of SRP were required for each type of binding. We first asked which components were necessary for signal-sequence-dependent, high-salt-resistant binding. Previous studies have demonstrated that SRP54 interacts with signal sequences [10,12–16]. Accordingly, we tested the ability of an SRP subparticle consisting of SRP54 and 7SL RNA to interact with ribosome–nascent chains at two different salt concentrations, physiological (150 mM KAc) and high (500 mM KAc). We found that this minimal particle bound as well as intact SRP to signal sequence-bearing ribosomes at physiological salt (Fig. 3a, compare lanes 2 and 6). Significant binding of the minimal particle was also observed at high salt concentrations (Fig. 3a, lane 8). As earlier studies have demonstrated that SRP54 does not interact stably with 7SL RNA at high salt in the absence of SRP19 [17], this binding may reflect interactions with SRP54 alone (that is, in the absence of SRP RNA). Interaction of the minimal particle with ribosome–nascent chains required a signal sequence, as no binding to ffLuc 77mer ribosomes was detected at 150 mM KAc (Fig. 3b, lane 3). Taken together, these results indicate that interactions between SRP54 and the signal sequence are primarily responsible for high-salt-resistant binding of SRP to ribosomes. Resistance of this binding to high-salt conditions is consistent with predicted interactions between SRP54 and signal sequences which most likely are predominantly hydrophobic in nature [3].

In contrast to intact SRP, we were unable to detect binding of the SRP54/SRP RNA particle to vacant

Figure 3



Characterization of interactions between SRP and SRP subparticles with ribosomes and ribosome–nascent chain complexes. **(a)** Interaction between SRP (lanes 1–4) or a minimal SRP54/7SL particle (lanes 5–8) with vacant ribosomes or pPL 86mer ribosome–nascent chain complexes, in the presence of 150 mM or 500 mM KAc, as indicated. **(b)** Interaction of the SRP54/7SL particle with vacant ribosomes or pPL 86mer/ffLuc 77mer ribosome–nascent chain complexes at 150 mM KAc. **(c)** Binding of SRP or reconsituted SRP subparticles to vacant ribosomes at 150 mM KAc. In a–c, the final ribosomal pellets were used for western immunoblot analysis.

ribosomes (Fig. 3a, compare lanes 1 and 5), indicating that additional SRP components were required for nascent chain-independent binding. We therefore used a comprehensive set of reconstituted SRP subparticles to test which of the other subunits, in addition to SRP54, was required for binding to vacant ribosomes at 150 mM KAc. We found that each of the SRP protein subunits, as well as SRP RNA, was required for nascent chain-independent binding of SRP to the ribosome (Fig. 3c).

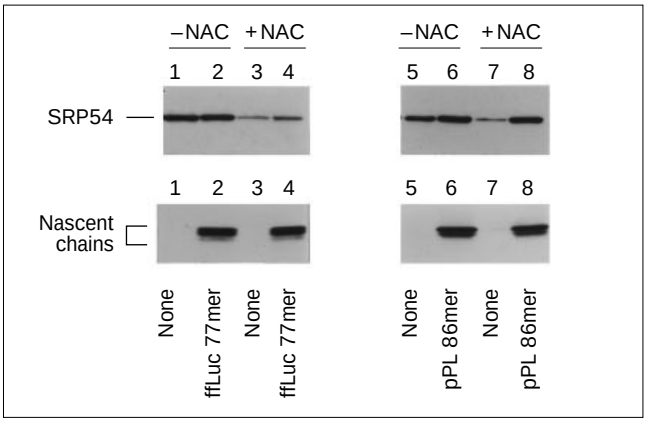
NAC prevents salt-sensitive interactions between SRP and the ribosome

It was previously suggested that NAC enhances the fidelity of protein targeting by preventing inappropriate interactions between SRP and signalless ribosome–nascent chain complexes [7]. Our results showing that SRP interacts similarly with ribosomes bearing signalless nascent chains and with vacant ribosomes (Fig. 2) suggested a possible mechanism for how NAC might regulate SRP, namely, by preventing salt-sensitive interactions between SRP and the ribosome. We tested this possibility directly by asking whether the addition of purified NAC interfered with binding of SRP to ribosomes at 150 mM KAc (Fig. 4). We found that NAC substantially reduced the binding of SRP to both vacant ribosomes as well as ffLuc 77mer ribosome–nascent chain complexes (Fig. 4, compare lanes 1 and 2 with lanes 3 and 4). In contrast, NAC had no effect upon binding of SRP to pPL 86mer ribosomes (Fig. 4, compare lanes 6 and 8). This result was specific because, in contrast to NAC, addition of other proteins, such as high concentrations of bovine serum albumin, had no effect on SRP binding to either signalless or vacant ribosomes (data not shown). We therefore conclude that NAC indeed interferes with salt-sensitive interactions between SRP and the ribosome.

Discussion

We have used immunoblotting experiments to examine the interaction between SRP and different ribosome–nascent chain complexes in the presence and absence of

Figure 4



NAC interferes with salt-sensitive binding of SRP to vacant or signalless ribosome–nascent chain complexes. SRP (30 nM) was incubated either alone or in the presence of NAC (300 nM) with salt-washed ribosomes and ribosome–nascent chains, and assayed by centrifugation in 150 mM KAc, as described in Materials and methods. Immunoblot analysis was carried out using the final ribosomal pellets.

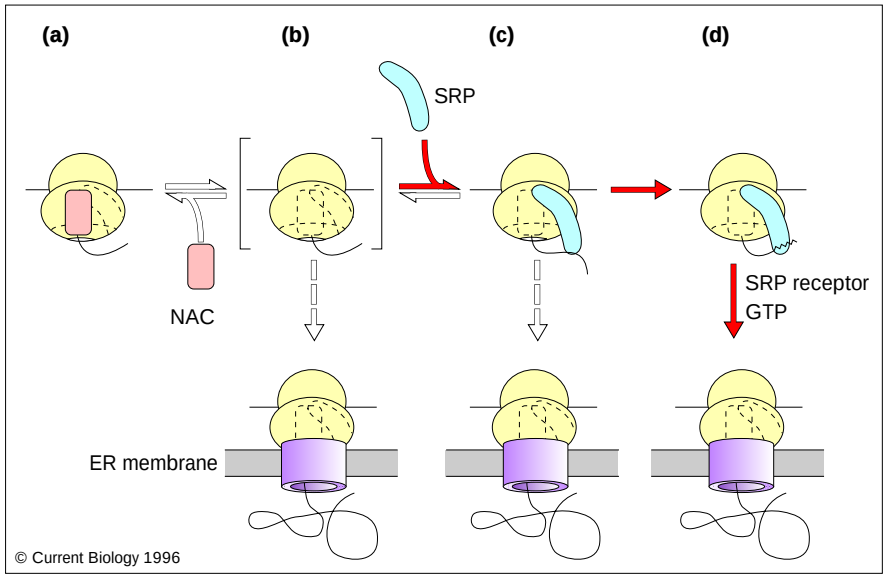
NAC. In the absence of NAC, we found that there are two distinct modes that SRP uses to interact with ribosome–nascent chain complexes, which are distinguishable by their salt sensitivity: salt-resistant interactions, which occur between SRP54 and the signal sequence, and salt-sensitive interactions, which take place between additional components of SRP and the ribosome. These latter interactions occur independently of the nascent chain, as SRP binds to signalless ribosome nascent chains and vacant ribosomes to the same extent. In addition, we found that this salt-sensitive binding by SRP is prevented by NAC. Thus, whereas SRP can interact exclusively with signal-sequence-containing ribosomes at high salt (500 mM KAc), NAC prevents binding of SRP to signalless ribosome nascent chains at physiological salt concentrations (150 mM KAc). We therefore conclude that both SRP and NAC are normally required for SRP to bind specifically to ribosome–nascent chain complexes that contain a signal sequence, in agreement with previous studies [7–9]. NAC, however, does not directly influence signal sequence recognition by SRP, as originally suggested [7], but, rather, it negatively modulates interactions that occur between SRP and the ribosome itself (Fig. 5).

Our finding that the SRP54/7SL minimal particle interacts with signal-sequence-containing but not signalless ribosomes (Fig. 3b) indicates that SRP54 is capable of signal sequence discrimination. Recently, Hauser *et al.* [18] have compared the ability of SRP54 to crosslink to salt-washed ribosomes containing pPL 86mer nascent chains bearing either wild-type or mutant signal sequences. These authors found that SRP54 could be crosslinked to a mutant signal sequence in the context of intact SRP, but not when it was part of a minimal particle together with SRP19 and 7SL RNA, in agreement with our direct binding results. Taken together, these studies offer compelling evidence that SRP54 possesses the intrinsic ability to discriminate functional from non-functional signal sequences. However, these results also indicate that interactions between additional components of SRP and the ribosome are sufficient to place SRP54 in the correct position to interact with all nascent chains. Thus, the crosslinks observed by Wiedmann *et al.* [7] between SRP54 and signalless nascent chains in the absence NAC can best be explained by their proximity to SRP54, rather than an intrinsic affinity by SRP54 for these signalless nascent chains. This explanation can also account for the fact that SRP promotes neither targeting nor translocation of these nascent chains [8,9].

Previous studies have examined the importance of individual subunits to the different functions carried out by SRP [18–21]. Both of its heterodimeric subunits, SRP9/14 and SRP68/72, have been shown to be important for SRP–ribosome interactions: in the absence of SRP9/14 the elongation-arrest function of SRP is impaired [19,20], and in the absence of SRP68/72 (or in the presence of alkylated forms of these proteins) its affinity for the ribosome is reduced [18,21]. These results correlate well with our findings that both SRP9/14 and SRP68/72, in addition to SRP RNA, are required for salt-sensitive association of SRP with ribosomes. It remains to be determined whether both of these protein components interact directly with the ribosome, or whether they assert their affects indirectly through structural changes in SRP.

How NAC prevents binding of SRP to signalless ribosome–nascent chain complexes is presently unknown, although our results seem to rule out the possibility that the two particles simply compete for interactions with the nascent chain, as NAC inhibited SRP from binding to vacant ribosomes (Fig. 4). Rather, we favor the idea that NAC directly affects interactions between ribosomes and SRP. This could occur if SRP and NAC possessed overlapping binding sites on the ribosome (Fig. 5). Results of crosslinking and protease protection experiments indicate that NAC protects an extensive length of nascent polypeptide on the ribosome, ranging from 17 to greater than 50 amino acids from the peptidyl transferase center [22]. This result suggests that multiple subunits of NAC can bind to the ribosome simultaneously, an idea supported by the fact that NAC is in stoichiometric excess compared to ribosomes (cited in [22]), or that at least more than a single ribosomal binding site exists for NAC. Thus, it is possible that at least one of these binding sites for NAC could overlap with the binding site for SRP. Formally, however, we cannot rule out more complicated scenarios where NAC and SRP inhibit each

Figure 5



Model for roles of SRP and NAC during co-translational targeting of nascent chains. (a) In the absence of a signal sequence, NAC prevents targeting of nascent chains by blocking the membrane binding site on the ribosome [8,9]. (b) In the absence of NAC, on the other hand, these nascent chains are targeted, albeit inefficiently, to the ER [7–9]. This condition is unlikely ever to occur in vivo, because of the presence of high concentrations of NAC [7,22]. (c) In the absence of NAC, SRP can bind to these signalless nascent chains by virtue of salt-sensitive interactions with the ribosome. This salt-sensitive association is likely to correspond to the low-affinity binding of SRP to vacant ribosomes observed previously [27]. SRP bound to ribosomes in this mode is not functionally engaged with the nascent chain, however, as targeting of these nascent chains occurs independently of SRP [9]. (d) In the presence of a signal sequence, SRP binds tightly to the ribosome, and effectively competes with NAC for binding. SRP bound in this mode efficiently targets these nascent chains to the ER, *via* interactions between SRP and the SRP receptor. Translocation across the ER is then initiated in a reaction requiring GTP [3,28,29].

others' binding indirectly, for example *via* conformational changes in the ribosome. Identification of the sites of interaction for both SRP and NAC on the ribosome will be required to distinguish between these different possibilities.

What role might competition between SRP and NAC for ribosome binding play during protein targeting? SRP is thought to examine translating ribosomes for the presence of a signal sequence; but because there are fewer SRPs than ribosomes in the cell, SRP must rapidly cycle on and off the ribosome in order to efficiently sample every nascent chain [3]. Recent experiments in yeast suggest that SRP accomplishes this by interacting with ribosomes only at a specific stage during translational elongation, at the step following peptide bond formation but preceding translocation of peptidyl tRNA [23]. If a signal sequence is present, SRP remains bound to the ribosome and elongation becomes slowed or arrested [3,24]. In the absence of a signal sequence, however, SRP dissociates from the ribosome and elongation continues. It is possible that NAC facilitates this sampling by SRP by increasing the rate at which SRP dissociates from signalless ribosome nascent chains. A prediction of this model is that depletion of NAC should increase the time window during which SRP can interact productively with a signal sequence. In principle, however, the cycling of ribosomes through the different states of elongation could suffice to displace SRP, by analogy to other factors that interact transiently with ribosomes. Intuitively, there is therefore no obvious need for NAC in this regard.

A more attractive possibility is suggested by recent results demonstrating that NAC prevents signalless ribosome nascent chains from interacting with the ER membrane [8,9], suggesting that in the absence of SRP, NAC blocks the site on the ribosome for binding to the ER. Thus, in the presence of SRP, NAC could be prevented from binding to the ribosome, allowing ribosomes bearing signal sequences to be efficiently targeted to the ER [8,9] (Fig. 5). The existence of overlapping binding sites for NAC and SRP on the ribosome would provide an obvious means for achieving this end. Of course, the two possibilities suggested here are not mutually exclusive. Indeed, the picture that emerges from these studies is that SRP and NAC work together, by modulating each others' binding to the ribosome, to target signal sequence-bearing nascent chains efficiently and accurately to the ER, as well as to prevent signalless nascent chains from gaining access to the protein export machinery in the ER membrane.

Materials and methods

Preparation of truncated mRNAs

Plasmid pSPBP4 [21], containing the coding sequence for preprolactin, was linearized with *Pvu*II to generate the template for a synthetic mRNA encoding the first 86 amino acids of preprolactin (pPL 86mer). Plasmid pGEM-luc (Promega), containing the coding sequence of firefly luciferase, was linearized with *Hin*fI to generate the template for a synthetic mRNA encoding the first 77 amino acids of firefly luciferase (ffLuc 77mer). Both mRNAs were prepared by transcription using a Megascript (Ambion) transcription system and SP6 polymerase. Transcripts were purified by phenol extraction, precipitated with isopropanol, and resuspended in water for use in *in vitro* translation reactions.

Purification and reconstitution of SRP and SRP subparticles

Purification of SRP, SRP protein subunits and 7SL RNA, and reconstitution of SRP and SRP subparticles was carried out as described previously [19]. Reconsitution reactions contained a final concentration of 200 nM SRP54, 300 nM of each of the other protein subunits and 300 nM 7SL RNA, as appropriate. Reactions were carried out in 10 μl of buffer, containing 50 mM triethanolamine acetate (TEA) (pH 7.5), 5 mM $Mg(Ac)_2$, 500 mM KAc, 0.01 % Nikkol and 1 mM DTT. Samples were mixed and incubated for 10 min on ice, followed by incubation for 10 min at 37 °C. The SRP reconstitutes were returned to ice for 10 min and then used immediately in ribosomal binding experiments.

In vitro *translations and ribosomal binding assays*

Wheat germ translations (10 μl), containing [^{35}S]methionine, were performed either in the absence of mRNA, or in the presence of truncated mRNAs to produce pPL 86mer or ffLuc 77mer ribosome–nascent chain complexes [7,25,26]. Each type of nascent chain contained four methionine residues [7]. Following addition of mRNA (~ 0.2 μg), reactions were allowed to proceed for 20 min at 25° C, after which cycloheximide (CH) was added to a final concentration of 0.25 mM. The salt concentration was then raised to 500 mM KAc and samples (in a final volume of 50 μl) were overlayed onto 150 μl sucrose cushions containing 25 mM TEA (pH 7.5), 0.5 M KAc, 2.5 mM $Mg(Ac)_2$, 1 mM DTT, 0.01 % Nikkol, 0.25 mM CH, and 0.5 M sucrose. Ribosomes were pelleted by centrifugation in a Beckman TLA 100 rotor at 60 000 rpm for 60 min at 4 °C. Following centrifugation, the ribosomal pellets were resuspended in 10 μl of resuspension buffer containing 50 mM TEA (pH 7.5), 50 mM KAc, 2.5 mM $Mg(Ac)_2$, 1 mM DTT, 0.01 % Nikkol and 0.25 mM CH. In addition, the supernatants from this centrifugation step were TCA-precipitated, and the resulting pellets were resuspended in SDS sample buffer (60 mM Tris–HCl (pH 6.8), 2 % SDS, 10 % glycerol, 100 mM DTT and 0.25 % bromophenol blue).

Next, 2 μl of a 200 nM solution of native SRP or the appropriate SRP reconstitute was added to the resuspended ribosomal pellet (SRP was present at a final concentration of ~ 30 nM, and KAc was present at 100 mM) and incubated for 10 min at 25 °C, followed by an additional incubation for 60 min on ice. The volume of the sample was then raised to 50 μl with resuspension buffer, containing an appropriate concentration of KAc so that the final concentration was 50–500 mM, as described in the figure legend for each experiment. Samples were then centrifuged in a TLA 100 rotor as described above, and the resulting ribosomal pellets were resuspended directly in SDS sample buffer. The corresponding supernatants were TCA precipitated and the resulting pellets resuspended in SDS sample buffer.

Ribosomal binding assays using both SRP and NAC were performed as described [9]. Salt-washed ribosomes or ribosome–nascent chain complexes were combined with 2 μl of 200 nM SRP and 1.5 μl of either 3 μM purified NAC (a generous gift from M. Wiedmann) or NAC buffer (20 mM Hepes–KOH (pH 7.5), 300 mM KCl). These amounts resulted in a final SRP to NAC ratio of 1:10, where NAC was approximately equimolar relative to ribosomes (estimated based on the absorbance at 260 nm of the salt-washed ribosomal pellet). Samples were then incubated for 5 min at 25 °C, followed by 20 min on ice, and overlayed directly without dilution onto a 150 μl sucrose cushion, containing 25 mM TEA (pH 7.5), 150 mM KAc, 2.5 mM $Mg(Ac)_2$, 1 mM DTT, 0.01 % Nikkol, 0.25 mM CH and 0.5 M sucrose. Ribosomes were then pelleted in a Beckman TLA 100 rotor at 100 000 rpm for 15 min at 4 °C and resuspended in SDS sample buffer.

SDS–PAGE analysis and immunoblotting

All samples were analyzed by SDS–PAGE on 10–15 % gradient gels. The bottom of the gels were cut off, dried, and exposed to X-ray film to detect the nascent chains, or quantified using a phosphoimager. The rest of the gels were used for western blot analysis and probed with polyclonal antisera raised against SRP54 or αNAC (a generous gift from M. Wiedmann). Detection of bound antibodies was carried out with the Renaissance chemiluminescence system (DuPont) according to the manufacturers' instructions. All exposures were within the linear range of the detection film, as determined by quantitative immunoblotting using known amounts of SRP54 and αNAC protein.

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