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The intrinsic ability of ribosomes to bind to endoplasmic reticulum membranes is regulated by signal recognition particle and nascent-polypeptide-associated complex

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ABSTRACT **Signal peptides direct the cotranslational targeting of nascent polypeptides to the endoplasmic reticulum (ER). It is currently believed that the signal recognition particle (SRP) mediates this targeting by first binding to signal peptides and then by directing the ribosome/nascent chain/SRP complex to the SRP receptor at the ER. We show that ribosomes can mediate targeting by directly binding to translocation sites. When purified away from cytosolic factors, including SRP and nascent-polypeptide-associated complex (NAC), *in vitro* assembled translation intermediates representing ribosome/nascent-chain complexes efficiently bound to microsomal membranes, and their nascent polypeptides could subsequently be efficiently translocated. Because removal of cytosolic factors from the ribosome/nascent-chain complexes also resulted in mistargeting of signalless nascent polypeptides, we previously investigated whether readdition of cytosolic factors, such as NAC and SRP, could restore fidelity to targeting. Without SRP, NAC prevented all nascent-chain-containing ribosomes from binding to the ER membrane. Furthermore, SRP prevented NAC from blocking ribosome–membrane association only when the nascent polypeptide contained a signal. Thus, NAC is a global ribosome-binding prevention factor regulated in activity by signal-peptide-directed SRP binding. A model presents ribosomes as the targeting vectors for delivering nascent polypeptides to translocation sites. In conjunction with signal peptides, SRP and NAC contribute to this specificity of ribosomal function by regulating exposure of a ribosomal membrane attachment site that binds to receptors in the ER membrane.**

Current models for the assembly of membrane-bound ribosomes maintain that targeting to the endoplasmic reticulum (ER) begins when signal recognition particle (SRP) binds to nascent signal peptides (1). The resulting translational elongation arrest (2, 3) both prevents nascent chains from folding into translocation incompetent states and retains them on the ribosomes until targeting has occurred. Because SRP is required for cotranslational translocation in the wheat germ lysate translation systems containing canine pancreas microsomes (4, 5) and because the SRP receptor (*i*) relieved the elongation arrest, (*ii*) was purified by affinity chromatography with immobilized SRP, and (*iii*) restored translocation activity to proteolyzed microsomes, targeting of ribosomes to the ER membrane is currently hypothesized to be mediated by the interaction of SRP with its receptor (5–8).

Nascent-polypeptide-associated complex (NAC) was discovered as being among the first nonribosomal factors that cotranslationally interact with newly synthesized polypeptides (9) and in the absence of NAC signalless nascent chains can be mistranslocated across the ER membrane *in vitro* (9). This mistranslocation can result from an SRP-independent targeting mechanism that functions by virtue of the intrinsic affinity of ribosomes for binding sites in the ER (10). NAC prevents this mistargeting of signalless polypeptides from occurring—most likely by blocking a ribosomal membrane attachment site. Although we have demonstrated that NAC prevents mistargeting, it was previously not possible to demonstrate a role for NAC in the SRP-dependent targeting of signal peptide containing nascent polypeptide chains because of the presence of endogenous reticulocyte lysate SRP (10).

In the present work we asked whether NAC plays a role in the SRP-dependent targeting of *bona fide* secretory proteins and show that in the absence of SRP and NAC ribosome binding alone can efficiently target secretory nascent chains to the ER such that efficient translocation can ensue. NAC prevents this targeting and translocation unless SRP binds to the signal peptide. We propose that SRP and NAC together provide for fidelity in protein targeting to the ER by coordinately regulating exposure of a ribosomal membrane attachment site.

MATERIALS AND METHODS

In vitro translation of truncated mRNAs was as described (11). Unless otherwise indicated, translations were for 20 min at 26°C. After translation, 9 vol of dilution buffer [DB: 40 mM Hepes/0.5 M KOAc/5 mM $Mg(OAc)_2$/2 mM dithiothreitol, pH 7.5] was added, and the ribosome/nascent-chain complexes were recovered by centrifugation (100 Krpm, 40 min, 4°C, TLA 100.4 rotor, Beckman) through a 1.5-ml high-salt-containing sucrose cushion [HSS: 0.5 M sucrose/DB supplemented with protease inhibitors (9) and RNasin at 0.8 unit/μl (Promega)]. The complexes were resuspended in translation blank buffer (TBB), as described (9), using about 0.5 vol of the original translation volume. Insoluble material was then removed by centrifugation at 14,000 × *g* for 10 min at 4°C. Recovery of the nascent-chains was typically 50–75%. These complexes were essentially free of NAC as assessed by immunoblotting (data not shown) or by a photocrosslinking approach (9).

Nascent-Chain Targeting Assay. This assay is as described (10). Basically, ribosome/nascent-chain complexes in buffer either lacking or supplemented with nucleotides and the energy-generating system, as indicated in the figures, were incubated with EDTA/KOAc stripped rough microsomes (EKRM) (12). Some reactions, as indicated in the figures, were preincubated with SRP and/or NAC before microsome addition. After incubation, 20-μl samples were mixed with 2.3 M sucrose in ribosome binding buffer [RBB: 50 mM Hepes/100

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Abbreviations: ER, endoplasmic reticulum; SRP, signal recognition particle; NAC, nascent-polypeptide-associated complex; ffLuc, firefly luciferase; pPL, preprolactin; EKRM, EDTA and KOAc stripped rough microsomes; CAT, chloramphenicol acetyltransferase.

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mM KOAc/5 mM $Mg(OAc)_2$/2 mM dithiothreitol/RNasin at 0.8 unit/μl/protease inhibitors (9)] to give a final sucrose concentration of 2.1 M. Samples were transferred to 750-μl tubes and overlaid with 360 μl of 1.9 M sucrose/RBB. Tubes were filled with RBB and then centrifuged (45,000 rpm, 1 hr, 4°C, SW 55 rotor, Beckman). Gradients were then frozen in liquid nitrogen and cut into three fractions with a sharp Rambo knife. The nascent chain content of each fraction was analyzed by SDS/PAGE and fluorography or by scintillation counting. Protease and endonuclease H treatments were as described (9).

RESULTS

Ribosome/nascent-chain complexes were produced by *in vitro* translation in a wheat germ lysate supplemented with [^{35}S]methionine of 3′-truncated mRNAs lacking stop codons (11) encoding either the first 86 aa of signal peptide-containing preprolactin (86aapPL-wt, where wt is wild type), a mutant (mut) in which the signal has been rendered nonfunctional (13) (86aapPL-mut), or the amino-terminal 77 aa of the peroxisomal firefly luciferase (77aaffLuc). After these complexes were stripped of NAC and purified by sedimentation through high-salt-containing sucrose cushions (9, 10), the complexes were incubated with SRP-depleted EKRM (12). Extent of ribosome/nascent-chain complex binding was monitored by flotation of membranes together with any bound ribosome/nascent-chain complexes in discontinuous sucrose gradients (10, 14, 15). After centrifugation, three fractions were collected and analyzed by SDS/PAGE and fluorography (Fig. 1). Top (T lanes) and middle (M lanes) fractions contain membranes with targeted nascent chains, whereas free ribosome/nascent-chain complexes remained in the bottom fraction (B lanes).

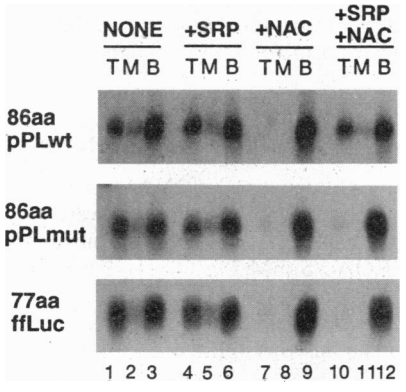

FIG. 1. NAC is a global ribosome-binding prevention factor that is specifically blocked by signal peptide-directed SRP binding. Salt-stripped ribosome/nascent-chain complexes (for details, see text and ref. 10) were resuspended in 0.8 vol of the original translation reaction volume in translation blank buffer (TBB) lacking nucleotides and the energy-generating system. Four microliters of 200 nM SRP (4) and 4 μl of 2 μM NAC (9) were mixed with 16 μl of 50 mM Hepes, pH 7.5/5 mM $Mg(OAc)_2$/2 mM dithiothreitol/protease inhibitors (9)/RNasin at 0.8 unit/μl. SRP or NAC blank buffers were used in control samples. Twenty-four microliters of this mixture was added to an 8-μl sample of stripped ribosome/nascent-chain complexes and incubated for 5 min at 26°C and on ice for 5 min. Next, samples containing SRP were adjusted to 1 mM GTP. Then, 8 equivalents of EKRM (stock is 1 equivalent/μl) was added to each sample, and mixtures were incubated as above. Twenty-microliter samples were analyzed for ribosome binding. Those ribosome/nascent-chain complexes that bound to microsomal membranes floated up from the loading zone at the bottom (lanes B) of the gradient to the top (lanes T) and middle (lanes M) fractions and were considered targeted. Salt-stripped ribosome/nascent-chain complexes, either containing (86aapPL-wt) or lacking (86aapPL-mut, 77aaffLuc) signal peptides, are targeted equally well in the absence of SRP and NAC (lanes 1–3). Addition of SRP alone is of no discernible consequence (lanes 4–6). NAC, when added before the membranes, prevented targeting of all nascent chains tested (lanes 7–9). Simultaneous addition of SRP and NAC before addition of microsomes restored fidelity in targeting like that seen in an unfractionated system (lanes 10–12).

In the absence of added factors, approximately equal proportions of the ribosome/nascent-chain complexes were targeted (Fig. 1, lanes 1–3). Binding occurred in the absence of SRP, and most likely resulted from the affinity of ribosomes for binding sites in the ER membrane (10, 14–20). Addition of SRP before addition of the membranes (lanes 4–6) did not increase the extent of targeting.

In contrast, addition of NAC before addition of microsomes prevented the targeting of all three polypeptides (lanes 7–9). Because in a previous study NAC did not prevent targeting of 86aapPL-wt ribosome/nascent-chain complexes that were assembled in reticulocyte lysate (10) in which endogenous SRP is bound to the signal peptide and is not salt extractable (21), it seemed likely that SRP interfered with the ability of NAC to prevent targeting. Indeed, Fig. 1 shows that when the wheat germ-assembled salt-stripped nascent-chain complexes [which lack functional SRP (22)] were simultaneously incubated with NAC and SRP before addition of EKRM, binding was restored only when the nascent chain contained a signal peptide (lanes 10–12), suggesting that signal peptide-directed SRP binding prevents NAC from blocking ribosome binding.

Fig. 2 shows saturation curves for the binding of salt-stripped 86aapPL-wt ribosome/nascent-chain complexes both with and without SRP and of salt-stripped 86aapPL-mut complexes. Neither signal peptide nor SRP altered the curves, suggesting that the extent of binding is determined primarily by the ribosome and that targeting occurs to the same sites whether SRP is present or not.

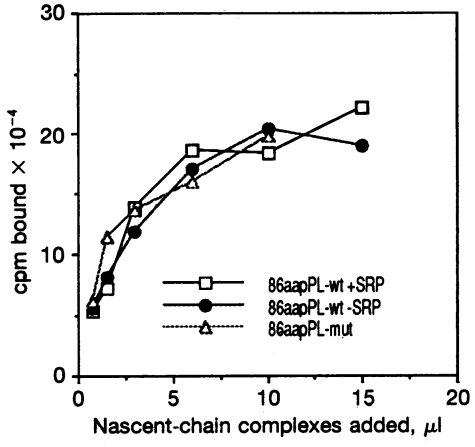

FIG. 2. Without NAC, ribosome-mediated targeting is not increased by SRP or a signal peptide. High-salt-stripped 86aapPL-wt and 86aapPL-mut ribosome/nascent-chain complexes were prepared as in Fig. 1 and resuspended in half the original translation mixture volume of buffer either lacking (−SRP samples) or containing SRP, as well as nucleotides and the energy-generating system (+SRP samples). The binding of 86aapPL-mut was analyzed without SRP and nucleotides. Thirty-five-microliter samples of stripped nascent-chain complexes were incubated with 3.5 μl of a 200 nM SRP stock or SRP blank buffer and incubated for 2 min at 26°C and then for 5 min on ice. Next, 0.75-, 1.5-, 3.0-, 6.0-, 10.0-, or 15.0-μl samples were added to separate assay tubes. The appropriate compensating buffer containing SRP or SRP buffer was added to all samples to adjust to a 15-μl vol. Samples were then incubated for 5 min at 26°C and 5 min on ice before adding 2 equivalents of EKRM in 5 μl of appropriate buffer (either with or without nucleotides, creatine phosphate, and creatine kinase). After 5 min at 26°C and 5 min on ice, samples were fractionated by using a flotation assay. Fractions were collected and subjected to scintillation counting. Nascent chains recovered in the top and middle fractions were considered targeted (see also Fig. 1).

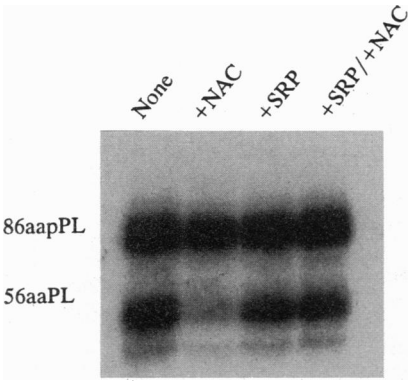

FIG. 3. 86aapPL chains can be efficiently translocated without SRP (compare lanes 1 and 3). Translocation was strongly inhibited by NAC (lane 2) but was rescued by simultaneous addition of SRP and NAC (lane 4). Samples were prepared exactly as in Fig. 1 but were analyzed for translocation rather than ribosome binding. After mixing of all assay components and incubation, samples were adjusted to 1 mM puromycin and incubated for 30 min at 37°C to induce translocation. Samples were analyzed by SDS/PAGE and fluorography after trichloroacetic acid precipitation.

Fig. 3 shows the analysis of reaction mixtures containing 86aapPL-wt ribosome/nascent-chain complexes prepared in the same way as those of Fig. 1 but analyzed for translocation by releasing the nascent chains from ribosomes with puromycin after targeting. Extent of translocation, as assessed by the appearance of the signal-cleaved form (56aaPL), was efficient without addition of SRP and NAC (lane 1). SRP did not increase the amount of translocation (lane 3), and NAC significantly blocked translocation (lane 2). SRP was able to "override" the NAC-mediated inhibition (lane 4). Because most salt-stripped nascent chains that bound to EKRM were subsequently translocated (compare extent of binding in Fig. 1 to extent of translocation in Fig. 3), ribosome binding alone can productively target nascent polypeptides to the translocon without NAC and SRP. Although in the absence of NAC and SRP, ribosome binding can efficiently target nascent chains, we cannot exclude the possibility that when NAC and SRP are present, SRP–SRP receptor interaction occurs before ribosome–membrane interaction. Currently, no data are published that indicate which interaction occurs first.

To exclude the possibility that immunologically undetectable levels of contaminating SRP that could mediate targeting remained in our system after salt extraction of the EKRM, samples lacking added SRP were also prepared without GTP. Because GTP omission has been shown to prevent SRP release from the signal peptide (21, 23), these conditions represent an SRP trap that blocks translocation. This trap also functions in our system using high-salt-extracted ribosome/nascent-chain complexes (10). Therefore, translocation that occurs without GTP (Fig. 3, lanes 1 and 2) occurs independently of SRP. This observation shows that no functional SRP was present unless added to the assay. Therefore, the targeting in Fig. 1, lanes 1–3, also occurred without functional SRP.

To measure the contribution of signal peptide to translocation with and without SRP, full-length pre-pro-α factor (encoding a signal peptide) or cytosolic chloramphenicol acetyltransferase (CAT, not encoding a signal peptide) mRNA was translated in wheat germ lysate for 3 min in the presence of [^{35}S]methionine to produce ribosome/nascent-chain complexes in which most polypeptides were shorter than full-length proteins; these were then immediately stripped of NAC and purified by sedimentation (Fig. 4, lanes 1 and 10). These stalled complexes were preincubated with NAC and then SRP, as indicated in Fig. 4, before addition of EKRM. After targeting had been given a chance to occur, fresh translation mixture lacking mRNA but containing endogenous NAC and excess

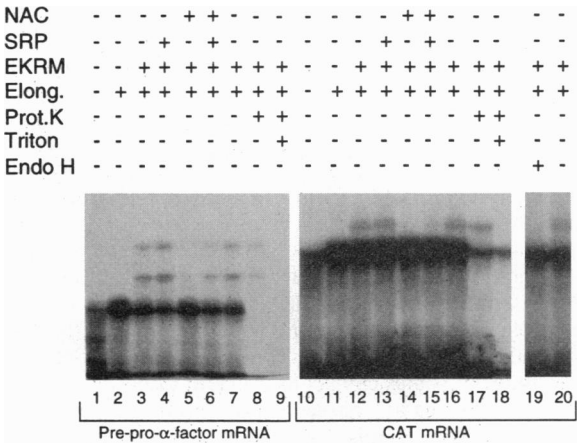

FIG. 4. NAC and SRP control translocation across the ER membrane. One hundred and fifty-microliter samples of wheat germ translation mixtures containing [^{35}S]methionine were preincubated for 7 min at 26°C and then programmed with full-length mRNA encoding pre-pro-α factor (lanes 1–9) or chloramphenicol acetyltransferase (lanes 10–20) for 3 min at 26°C to produce ribosome/nascent-chain complexes in which most of the nascent polypeptides are shorter than the full-length protein. Complexes were high-salt-stripped and isolated as usual. After resuspension in TBB, aliquots of the ribosome/nascent-chain complexes were preincubated with 200 nM NAC (lanes 5, 6, 14, and 15) or NAC buffer (lanes 1–4, 7–13, and 16–20), as indicated, for 3 min at 26°C and then 10 min on ice. Next, 20 nM SRP (lanes 4, 6, 13, and 15) or SRP buffer was added to the remaining samples and incubated in the same way as for NAC addition. EKRM (final concentration of 0.2 equivalent/μl) were added (samples 3–9 and 12–20), and targeting occurred for 3 min at 26°C and 10 min on ice. Except for samples 1 and 10, wheat germ translation mixture lacking mRNA but containing 2 mM methionine was added, and all samples were incubated for 15 min at 26°C. This treatment restored translational elongation capacity to the previously stalled ribosomes (compare lanes 1 and 10 with 2 and 11). Samples were analyzed by SDS/PAGE and fluorography after trichloroacetic acid precipitation. Cotranslational translocation, as assessed by N-glycosylation (lanes 3, 4, 6, 7, 8, 12, 13, 16, 17, and 20) and resistance to proteolysis in the absence of detergent (lanes 8 and 17) of both proteins into the ER occurred when microsomes were incubated with the ribosome/nascent-chain complexes before restoration of elongation. Lanes 7 and 16 represent mock-treated samples. Note that translocation was more efficient for the signal peptide-containing pre-pro-α factor than for chloramphenicol acetyltransferase. Translocation that was blocked by NAC (lanes 5 and 14) was rescued by SRP only when the nascent chain contained a signal peptide (compare lanes 6 and 15). The salt extraction procedure used to transiently remove NAC and halt elongation did not irreversibly damage the ribosomes. Elong., wheat germ translation system with 100 μm methionine; Prot. K, proteinase K; Endo H, endoglycosidase H.

unlabeled 100 μM methionine was returned to the samples. Upon warming to 30°C, the stalled ribosomes resumed elongation (e.g., lanes 2 and 11), and the short nascent chains that were previously targeted were further elongated, showing that the salt extraction neither irreversibly damaged the ribosomes nor impaired their ability to function in elongation and termination.

Both polypeptides were translocated, as assessed by N-glycosylation, when targeting occurred in the absence of NAC and SRP (Fig. 4, lanes 3 and 12), and preincubation of the ribosome/nascent-chain complexes with NAC prevented translocation of both polypeptides (lanes 5 and 14). Translocation of pre-pro-α factor did not require SRP unless NAC was also present (compare lanes 5 and 6). In the presence of NAC, the translocation of CAT could not be rescued by SRP (lane 15). Whereas targeting of ribosomes occurred equally well without regard to the presence of a signal peptide (see Figs. 1 and 2), the efficiency of the translocation step was increased when a signal peptide was present (Fig. 4, compare lanes 3 and

12). This experiment further shows that when a ribosome engaged in translation of a full-length mRNA binds to the ER, cotranslational translocation can proceed indistinguishably well with or without SRP.

DISCUSSION

Upon removing NAC from ribosome-associated nascent chains, the contribution of the ribosome itself to the targeting of nascent chains to the ER membrane became readily apparent. Although SRP is not required for targeting in the absence of NAC, fidelity in targeting is lost. With purified ribosome/nascent-chain complexes having nascent chains with or without signal peptides and being devoid of NAC and SRP, it was shown that ribosomes can act as efficient targeting vectors for properly delivering any nascent polypeptides to the translocation site. Although mistargeting is efficient (see Fig. 1), mistranslocation is not (see ref. 10 and Figs. 3 and 4). This observation is consistent with the idea that signal peptides serve a second function at the membrane—such as gating the translocon (24, 25). In contrast, ribosome-mediated targeting could support the efficient translocation of 86aapPL (Fig. 3). Interestingly, this SRP-independent translocation occurred without addition of ATP or GTP.

Rather than restoring specificity, addition of purified NAC to stripped ribosome/nascent-chain complexes blocked all ribosome binding. Earlier it was hypothesized that NAC, by binding near the ribosomal membrane attachment site, sterically blocks this site and thus prevents ribosome binding (10). When similar experiments were done using reticulocyte lysate-assembled 86aapPL ribosome/nascent-chain complexes that obligatorily contain the endogenous reticulocyte SRP that resists salt extraction (21), NAC could not block the targeting of these ribosome/nascent-chain complexes (10). This result suggested that SRP functions to prevent NAC from inhibiting the ribosome–membrane junction. As predicted, when SRP and NAC are simultaneously returned to stripped ribosome/nascent-chain complexes (Figs. 1 and 4), specificity, like that seen in an unfractionated *in vitro* translation lysate, is restored. Therefore, both NAC and SRP are needed to ensure fidelity in targeting. Also, SRP must bind to the signal peptide so that it prevents NAC from sterically blocking the M site. It seems unlikely that SRP itself blocks the M site because 86aapPL ribosome/nascent-chain complexes containing bound and unreleasable SRP bound indistinguishably from those lacking SRP (B.L., I. Möller, G.K., and M.W., unpublished data). On the other hand, NAC is a general ribosome-binding prevention factor whose activity is specifically blocked by signal peptide-directed SRP binding.

A model based on these findings is presented in Fig. 5, which postulates that ribosomes are targeting vectors for delivering nascent chains to translocation sites. SRP and NAC work in conjunction with the signal peptide to contribute to the specificity of ribosome binding by coordinately regulating the exposure of a putative membrane-attachment site on the ribosome that binds to receptor proteins in the ER membrane.

Two conditions had to be satisfied to completely circumvent the usual need for SRP in the efficient translocation (Figs. 3 and 4). (*i*) NAC had to be removed from the system. (*ii*) Translational elongation had to be stopped, at least temporarily. It follows that SRP binding to a signal peptide normally functions to keep the ribosomal M site free of NAC and that the use of truncated nascent chains masks the contributions of SRP to kinetics of targeting. It is likely that the elongation arrest effected by SRP (2, 3) increases the period in which the M site is free of NAC. Siegel and Walter (26) reported that SRP could bind to nascent polypeptides and mediate targeting only when nascent polypeptides were >70 but $<\approx 100$ amino acids. The size of this window seems to be determined by the distances at which SRP can prevent NAC from binding to the

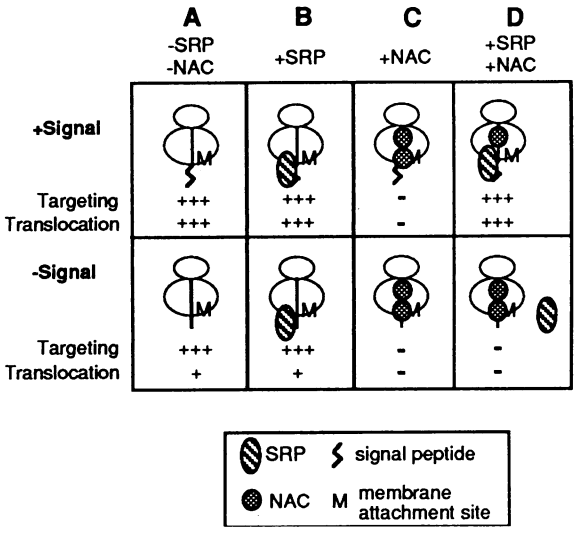

FIG. 5. Model for the roles of NAC and SRP in coordinately regulating the targeting of ribosomes synthesizing nascent polypeptides to the ER membrane. (*A*) The ribosome, because of its intrinsic affinity for receptors at the ER, can efficiently target nascent chains to the ER membrane. When translational elongation is stopped by the use of truncated mRNAs and in the absence of NAC, SRP is dispensable for targeting and translocation. Fidelity in targeting and translocation is lost. Although mistargeting is efficient, mistranslocation is not, presumably because the signal peptide gates the translocon (24, 25). (*B*) Without NAC, SRP does not increase targeting efficiency over that mediated by the ribosome. Although without NAC, SRP can be crosslinked to nonsignal peptides (9), the observed mistargeting can occur independently of SRP (10). (*C*) When bound to ribosome/nascent-chain complexes, NAC inhibits targeting of nascent chains with or without signal peptides by most likely blocking a putative ribosomal membrane attachment (M) site. (*D*) SRP provides specificity to targeting and therefore to translocation by binding to signal peptides so that it prevents NAC from blocking the M site. By slowing translational elongation (2, 3), SRP retains the signal peptide near the ribosome and may consequently increase targeting efficiency by extending the time span in which NAC cannot block the M site.

M site. In the absence of elongation arrest or when the signal peptide has emerged beyond the boundary of this window, nonsignal peptide regions of the emerging nascent chain would soon be located adjacent to the M site. NAC binding to these regions would then block ribosome binding. Possibly, cotranslational targeting could occur in the absence of SRP, but such targeting would be predicted to be much less efficient. Interestingly, Johnsson and Varshavsky (27) recently reported that SRP-deficient yeast are indeed capable of cotranslational translocation of indicator proteins, although with decreased efficiency. Furthermore, addition of low cycloheximide concentrations to slow down, but not completely halt, translation increased the efficiency of this SRP-independent cotranslational targeting.

Rather than a targeting factor, perhaps SRP, like many other GTPases, is best considered a regulatory protein. The ability to cotranslationally target nascent chains to the ER membrane seems to reside in ribosomal components. SRP and NAC regulate exposure of these ribosomal components—allowing exposure to their target molecules in the translocon only under appropriate conditions. This activity is dictated by the characteristics of the particular protein being synthesized on the ribosome in question, such as the presence of a signal peptide. This model is consistent with the signal hypothesis (28–30).

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