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Signal Recognition Particle-dependent Targeting of Ribosomes to the Rough Endoplasmic Reticulum in the Absence and Presence of the Nascent Polypeptide-associated Complex

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Proteins with RER-specific signal sequences are cotranslationally translocated across the rough endoplasmic reticulum through a proteinaceous channel composed of oligomers of the Sec61 complex. The Sec61 complex also binds ribosomes with high affinity. The dual function of the Sec61 complex necessitates a mechanism to prevent signal sequence-independent binding of ribosomes to the translocation channel. We have examined the hypothesis that the signal recognition particle (SRP) and the nascent polypeptide-associated complex (NAC), respectively, act as positive and negative regulatory factors to mediate the signal sequence-specific attachment of the ribosome-nascent chain complex (RNC) to the translocation channel. Here, SRP-independent translocation of a nascent secretory polypeptide was shown to occur in the presence of endogenous wheat germ or rabbit reticulocyte NAC. Furthermore, SRP markedly enhanced RNC binding to the translocation channel irrespective of the presence of NAC. Binding of RNCs, but not SRP-RNCs, to the Sec61 complex is competitively inhibited by 80S ribosomes. Thus, the SRP-dependent targeting pathway provides a mechanism for delivery of RNCs to the translocation channel that is not inhibited by the nonselective interaction between the ribosome and the Sec61 complex.

INTRODUCTION

The N-terminal hydrophobic signal sequence contains the sorting information that specifies transport of a protein across the rough endoplasmic reticulum (RER). Nascent polypeptides are cotranslationally translocated across the RER through an aqueous protein-conducting channel (Simon and Blobel, 1991; Crowley *et al.*, 1994) that is comprised of the heterotrimeric Sec61 complex (Görlich *et al.*, 1992; Görlich and Rapoport, 1993). Three to four Sec61 complexes oligomerize to form a quasi-pentagonal ring surrounding a 25Å diameter pore, that is believed to be the channel through which the nascent polypeptide traverses the membrane (Hanein *et al.*, 1996). The

Sec61 complex binds ribosomes with high affinity ($K_d = 5$ nM), hence the Sec61 complex serves a dual function acting as both the core of the translocation channel and the ribosome receptor (Görlich *et al.*, 1992; Kalies *et al.*, 1994). The molecular mechanism responsible for efficient, high fidelity targeting of the RNC to the Sec61 translocation channel has received considerable attention now that the dual function of the Sec61 complex has been appreciated. Ribosomes translating cytosolic proteins could conceivably interfere with protein translocation by nonselectively binding to the Sec61 complex. Furthermore, signal sequence-independent binding of an RNC to the Sec61 complex could result in the subsequent aberrant transport of a cytosolic protein into the lumen of the rough endoplasmic reticulum. Indeed, purified RNCs that lack signal sequences will bind to the Sec61 complex resulting in detectable, albeit inefficient, translocation of

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the nascent polypeptide (Wiedmann *et al.*, 1994; Jungnickel and Rapoport, 1995; Lauring *et al.*, 1995b). A specific interaction between the signal sequence and Sec61 may provide a fidelity checkpoint for the protein translocation reaction, thereby minimizing translocation of proteins that lack signal sequences (Jungnickel and Rapoport, 1995).

Until recently, the specificity of the translocation reaction was entirely attributed to the selective binding of the signal recognition particle (SRP) to the RNC once the signal sequence had emerged from the ribosome (Walter *et al.*, 1981; Walter and Blobel, 1981b; for a review see Walter and Johnson, 1994). According to this view, targeting of the SRP-RNC to the translocation channel is primarily dependent on the interaction between the SRP and the membrane-bound SRP receptor (SR) (Walter and Blobel, 1981a; Gilmore and Blobel, 1985). The GTP-dependent dissociation of the SRP from the signal sequence is coupled to the attachment of the RNC to the Sec61 complex (Connolly and Gilmore, 1989; Rapiejko and Gilmore, 1994). However, current models for the SRP-SR dependent targeting pathway do not describe a mechanism that prevents signal sequence-independent binding of RNCs or free ribosomes to the Sec61 complex.

The nascent polypeptide-associated complex (NAC) was initially proposed to enhance the fidelity of the sorting reaction by preventing the interaction between the SRP and nascent polypeptides that lack signal sequences (Wiedmann *et al.*, 1994). However, a subsequent report reaffirmed the earlier conclusion that the SRP has a low affinity for RNCs that lack signal sequences (Walter *et al.*, 1981), irrespective of the presence of NAC (Powers and Walter, 1996). Evidence was recently presented showing that NAC prevents the signal-sequence independent binding of mammalian (Lauring *et al.*, 1995b) and plant (Lauring *et al.*, 1995a) RNCs to the translocation channel. NAC and SRP are proposed to compete for binding to nascent polypeptides, with SRP having a higher affinity for signal sequences while NAC preferentially binds more hydrophilic sequences (Lauring *et al.*, 1995a; Wickner, 1995). According to this hypothesis, binding of NAC to the RNC prevents attachment of the ribosome to the translocation channel unless SRP suppresses the inhibitory action of NAC. Cooperation between NAC and SRP would provide an elegant solution to the dilemma raised by the dual function of the Sec61 complex. However, the intricate GTPase cycle of the SRP-SR complex (Miller *et al.*, 1993; Bacher *et al.*, 1996; Rapiejko and Gilmore, 1997) is difficult to accommodate in the context of a model wherein the postulated role of SRP is merely to suppress the inhibitory action of NAC on ribosome binding to the Sec61 complex.

Recent reports concerning the role of NAC in maintaining the fidelity of protein translocation have prompted a reevaluation of the role of SRP in early

events in the protein translocation reaction. Here we have explored the proposed role of NAC as an inhibitor of the interaction between the RNC and the Sec61 complex. On the basis of results obtained in these experiments, we examined the role of the SRP in stimulating the selective attachment of RNCs to microsomes in the presence of competing 80S ribosomes.

MATERIALS AND METHODS

Preparation of PK-RM

Rough microsomal membranes and SRP were isolated from canine pancreas as described (Walter *et al.*, 1981; Walter and Blobel, 1983). Puromycin-high salt extracted rough microsomes were prepared from rough microsomes essentially as described (Borgese *et al.*, 1974). Briefly, 10 ml of rough microsomes [1 eq/ μ l, eq as defined previously (Walter and Blobel, 1983)] were adjusted to 50 mM triethanolamine-acetate pH 7.5 (TEA-OAc), 600 mM KOAc, 12 mM Mg(OAc)₂, 0.2 mM GTP, 1 mM puromycin, 200 mM sucrose in a total volume of 15 ml. After a 30 min incubation at 4°C followed by a 10 min incubation at 25°C, the solution was adjusted to 1 mM CaCl₂ and 16 U/ml micrococcal nuclease and incubated for 10 min at 20°C. After the addition of 2 mM EGTA, the puromycin-high salt treated membranes were transferred to centrifuge tubes, underlaid with a cushion of 1.3 M sucrose, 50 mM TEA-OAc, 600 mM KOAc, 12 mM Mg(OAc)₂, 5 mM DTT, 1 mM EDTA and centrifuged for 2.5 h at 150,000 \times g in a Beckman Ti 50.2 rotor. The PK-RM were recovered at the cushion interface, diluted to 5 ml with 50 mM TEA-OAc, 600 mM KOAc, 12 mM Mg(OAc)₂, 1 mM DTT, 1 mM EDTA and centrifuged for 30 min at 150,000 \times g. After repeating the preceding centrifugation, the membranes were resuspended in 50 mM TEA-OAc, 250 mM sucrose, 1 mM DTT and stored at -80°C.

Cell-free Transcription and Translation

Truncated mRNAs encoding the N-terminal 86 residues of preprolactin (pPL86) (Connolly and Gilmore, 1986) and the 64 residues of the VSV G protein (pG64) (Connolly and Gilmore, 1989) were isolated from preparative-scale transcriptions as described previously (Rapiejko and Gilmore, 1994). The truncated mRNAs encoding the N-terminal 52 and 77 residues of firefly luciferase (ffLuc52 and ffLuc77) were transcribed using *Rsa*I and *Hin*fl linearized pGEMLuc (Promega, Madison, WI). The mRNA transcripts were translated at 25°C for 15 min in a wheat germ translation system that contained [³⁵S]methionine as described (Gilmore *et al.*, 1991). Rabbit reticulocyte lysate translations were conducted for 15 min at 30°C as described (Jackson and Hunt, 1983). Further protein synthesis was blocked by adding cycloheximide to a final concentration of 250 μ M. Peptidyl-tRNA was precipitated with cetyltrimethylammonium bromide (CTABr) as described previously (Gilmore and Blobel, 1985).

Isolation of NAC-depleted and Mock-depleted RNCs

NAC-depleted and mock-depleted RNCs were isolated as described previously (Lauring *et al.*, 1995b). Briefly, a 100- μ l translation was diluted with 10 volumes of either a high salt or a low salt dilution buffer. The high salt dilution buffer (40 mM HEPES [pH 7.5], 0.5 M KOAc, 5 mM Mg[OAc]₂, 2 mM DTT, 0.002% Nikkol) was used to extract NAC from the ribosomes, whereas the low salt buffer (40 mM HEPES (pH 7.5), 150 mM KOAc, 2 mM Mg(OAc)₂, 2 mM DTT, 0.002% Nikkol) was used for mock extraction of NAC. The diluted translation products were overlaid on a 1.5-ml cushion of 0.5 M sucrose in the appropriate dilution buffer. The samples were centrifuged for 40 min at 4°C in a TLA 100.4 rotor at 100,000 rpm. The RNCs were resuspended in 100 μ l of 40 mM HEPES (pH 7.5), 120

mM KOAc, 2.5 mM Mg(OAc)₂, 1 mM DTT, 0.002% Nikkol, 0.5 U/μl RNAsin, 1× PIC (protease inhibitor cocktail, as defined previously (Walter *et al.*, 1981). Resuspension was accomplished by multiple passes through a 25-gauge needle followed by a 10-min microcentrifugation to remove aggregates. NAC-depleted ribosomes were prepared from a wheat germ extract using a high salt extraction identical to that used to isolate the NAC-depleted RNCs. The concentration of wheat germ and reticulocyte lysate ribosomes was determined using a μM extinction coefficient of 61.4 at 260 nm. The extinction coefficient was calculated using 4.55×10^6 D as the molecular mass of the ribosome and an A₂₆₀ of 135 for a 1% solution of ribosomes (Tashiro and Siekevitz, 1965).

Translocation Assays

The RNCs or the SRP-RNCs (10 μl unless specified otherwise) were incubated with PK-RM (4 eq, unless specified otherwise) for 5 min at 25°C. The SRP-RNCs were supplemented with a ribonucleotide regenerating system (1 mM ATP, 0.1 mM GTP, 6 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase). Translocation of pPL86 was induced by releasing the peptidyl-tRNA from the ribosome by incubation with 25 mM EDTA for 10 min at 25°C (Connolly *et al.*, 1989). The assays were either prepared for electrophoresis as described previously (Connolly *et al.*, 1989) or were applied to 50 μl EDTA-sucrose cushions and centrifuged for 5 min at 20 psi in an airfuge (Connolly and Gilmore, 1986). The % translocation of pPL86 was calculated after quantification of PL56 and pPL86 using the following formula: % Translocation = $100 \times (1.33 \times (PL56_{S+P})) / ((1.33 \times (PL56_{S+P}) + pPL86_{S+P}))$, where S and P designate supernatant and pellet fractions obtained after the EDTA-sucrose gradient. The factor of 1.33 corrects for the loss of the N-terminal methionine residue from pPL86 upon signal sequence cleavage.

Binding of RNCs to PK-RM was assayed by centrifugal flotation on discontinuous sucrose gradients as described (Lauring *et al.*, 1995b) except that the gradients in Figure 1B were centrifuged for 3.5 h at 24,000 rpm in a SW50.1 rotor. The percentage RNC binding was calculated after quantification of pPL86 and PL56 in the top (T), middle (M) and bottom (B) fractions of the flotation gradient using the following formula: percentage RNC binding = $100 \times (pPL86_{T+M} + 1.33 \times PL56_T) / (pPL86_{T+M+B} + 1.33 \times PL56_T)$. Insertion of pPL86 into the Sec61 translocation channel was assayed by resistance to a 1 h digestion on ice with 400 μg/ml of proteinase K as described (Connolly *et al.*, 1989).

DSS Crosslinking

RNCs were incubated with disuccinimidyl suberate (DSS) to crosslink the radiolabeled nascent polypeptide to SRP54 or αNAC. The translation products were adjusted to 500 μM of DSS using a freshly prepared stock solution of DSS in dimethyl sulfoxide. After a 5-min incubation at 25°C, the crosslinking reactions were quenched by adjustment to 100 mM of glycine (pH 8.7) followed by a 5 min incubation at 25°C. The DSS-treated samples were prepared for PAGE in SDS as described (Kellaris *et al.*, 1991).

Protein Electrophoresis and Protein Immunoblots

Radiolabeled polypeptides were resolved by PAGE in SDS using Tris-Tricine buffered gels (Schägger and von Jagow, 1987). Dried gels were quantified using a Molecular Dynamics PhosphorImager and were exposed to X-ray film.

For protein immunoblots, cytosol and RNC preparations derived from reticulocyte and wheat germ lysates were resolved by gel electrophoresis on 10% SDS-polyacrylamide gels, and electrophoretically transferred onto 0.2-μm Bio-Rad Transblot membranes. The membranes were probed with rabbit antisera to αNAC that was provided by Dr. Martin Wiedmann (Wang *et al.*, 1995). Peroxidase-labeled secondary antibodies were detected by enhanced chemiluminescence using a kit from Kirkregard and Perry (Lumi-Glo).

RESULTS

Ribosome-bound NAC does not Inhibit SRP-independent Binding of Ribosomes to the Translocation Channel

Cotranslational protein translocation across the mammalian endoplasmic reticulum has been investigated using in vitro assay systems that are comprised of an in vitro protein translation system, an mRNA encoding a secretory protein and canine pancreas microsomes. The synthesis and transport phases of a cotranslational translocation assay can be separated by programming the translation with in vitro transcribed mRNAs that lack termination codons (Perara *et al.*, 1986). Because ribosomes remain bound to the 3' ends of truncated mRNA transcripts, stable RNCs can be prepared that are arrested at the point when the nascent polypeptide has just emerged from the ribosome (Gilmore *et al.*, 1991). Whereas high concentrations of microsomes inhibit in vitro translation systems, experimentally uncoupling the synthesis and transport phases of the translocation assay permits the addition of substantially more microsomes, hence more active translocation channels, per assay. The number of available translocation channels can be further increased by terminating the endogenous nascent polypeptides with puromycin, and subsequently extracting the membrane-bound ribosomes with high salt (Adelman *et al.*, 1973).

To determine whether wheat germ NAC inhibits ribosome attachment to the translocation channel, puromycin-high salt extracted rough microsomes (PK-RM) were tested for ribosome binding activity using RNCs or SRP-RNCs bearing an 86-residue preprolactin chain (pPL86) as a substrate. NAC was extracted from the RNCs and the SRP-RNCs by centrifugation through a high-salt sucrose cushion that has been shown to quantitatively remove NAC from RNCs assembled in the wheat germ system (Lauring *et al.*, 1995a; Wang *et al.*, 1995; Powers and Walter, 1996). RNCs and SRP-RNCs that contain ribosome-bound NAC were isolated by centrifugation through a low-salt sucrose cushion, as a previous report indicates that reticulocyte RNCs isolated by this procedure do not bind to microsomes in a signal sequence-independent manner (Lauring *et al.*, 1995b). The inhibitory effect of NAC on RNC targeting to the translocation channel was analyzed by separating unbound RNCs from membrane-bound RNCs by flotation on discontinuous sucrose gradients (Lauring *et al.*, 1995b). Microsomes, together with membrane-bound RNCs, float into the low-density top (T) and middle (M) fractions of the sucrose step-gradient, whereas unbound RNCs remain in the bottom (B) high-density load fraction. In addition to pPL86, which was predominantly recovered in the top and bottom fractions, low amounts of processed PL56 were recovered with

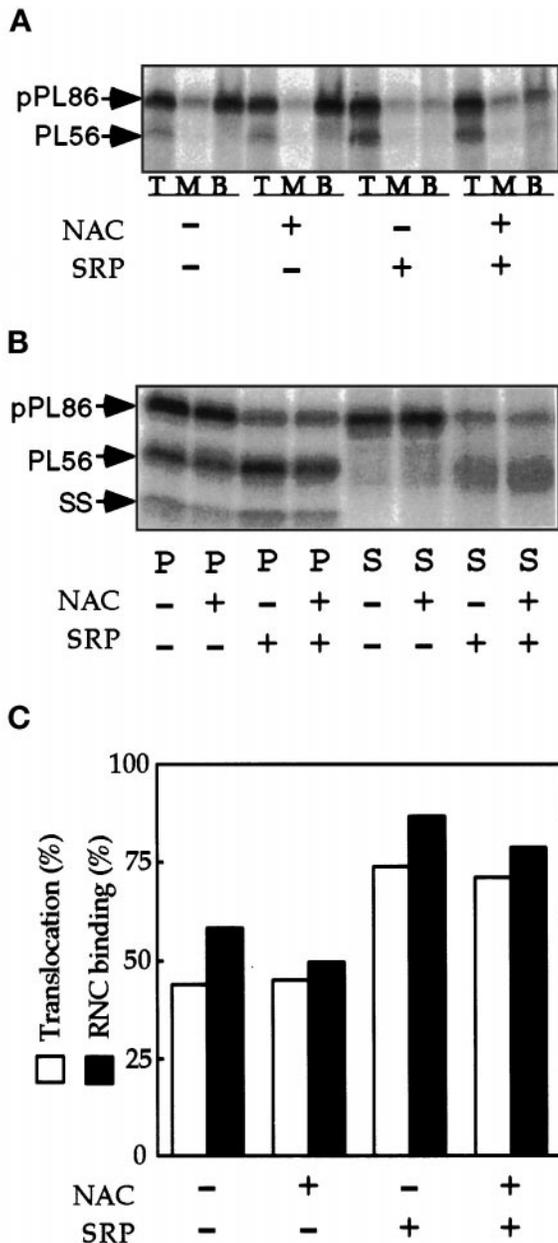


Figure 1. SRP-dependent and SRP-independent binding of ribosomes to PK-RM. The pPL86 mRNA was translated in the presence or absence of SRP. RNCs or SRP-RNCs were isolated by centrifugation through a high-salt (500 mM KOAc) sucrose cushion to deplete NAC (-) or through a low-salt (150 mM KOAc) sucrose cushion to mock-deplete NAC (+). Aliquots (10 μ l) of the SRP-RNCs (+SRP) and the RNCs (-SRP) were incubated for 5 min with 4 eq of PK-RM. GTP was only added to assays that contained SRP. Aliquots were removed for ribosome binding assays (panel A) and translocation assays (panel B). (A) To monitor binding of RNCs to PK-RM, the samples were adjusted to 2.1 M sucrose and applied as the bottom layer of a three-step discontinuous sucrose gradient (see MATERIALS AND METHODS). After centrifugation, membrane-bound pPL86 and PL56 were recovered in the top (T) and middle (M) fractions, while unbound pPL86 remained in the bottom (B) fraction. (B) To assay translocation, the samples were adjusted to 25

the microsomes in the top fraction (Figure 1A). The distribution of pPL86 and PL56 between the membrane-bound and unbound fractions was quantified (Figure 1C, solid columns). More than 80% of the SRP-RNC complexes bound to the microsomes both in the presence and absence of NAC. In the absence of SRP, binding of RNCs to the microsomes was reduced, but not eliminated (Figure 1A and 1C). SRP-independent binding of RNCs to the microsomes cannot be explained by SRP contamination of the PK-RM as GTP was only added to the assays that contained purified SRP. Previous studies have demonstrated that the SRP-dependent targeting pathway is exquisitely sensitive to deletion of GTP (Connolly and Gilmore, 1986; Connolly *et al.*, 1991; Rapiejko and Gilmore, 1992; Rapiejko and Gilmore, 1997).

To determine whether the membrane associated RNCs had engaged the translocation channel, the pPL86 nascent chains were released from the ribosome with EDTA (Sabatini *et al.*, 1966). Cetyltrimethylammonium bromide (CTABr) precipitation experiments show that the ester linkage between the nascent polypeptide and the tRNA is hydrolyzed upon addition of 25 mM EDTA (our unpublished results), hence pPL86 is freed for subsequent transport into the ER lumen as observed previously (Connolly *et al.*, 1989; Nicchitta *et al.*, 1995). Centrifugation of the assays to obtain membrane pellet (P) and supernatant (S) fractions confirmed that the processed prolactin nascent chain (PL56) and the signal sequence (SS) were associated with the microsomal vesicles (Figure 1B). Incubation of the assay products with proteases demonstrated that the processed products (i.e. PL56 and the SS), but not the precursor (pPL86), were inaccessible to proteases (our unpublished results), hence were genuine translocation products. After quantification of PL56 and pPL86 in both the supernatant and pellet fractions, translocation was calculated as the % conversion of pPL86 into PL56 (Figure 1C, open columns). Clearly, the majority of the RNCs and the SRP-RNCs were bound to the microsomes in a translocation-competent manner (Figure 1C, compare open and closed columns). When puromycin was used as an alternative method to release nascent pPL86 from membrane-bound RNCs, similar percentages of translocated PL56 chains were obtained (our unpublished results). The RNC binding and the translocation assays both demonstrate that SRP stimulates productive

Figure 1 (cont). mM EDTA and incubated for 10 min at 25°C before centrifugation to separate PK-RM in the pellet (P) fraction from soluble components in the supernatant (S) fraction. Radioactive bands corresponding to pPL86, PL56 and the signal sequence (SS) are labeled. (C) The % RNC binding and the % translocation were calculated (see MATERIALS AND METHODS) after quantification of pPL86 and PL56 in the gels shown in panels A and B.

targeting of RNC complexes to the Sec61 complex. However, these results challenge the hypothesis that ribosome-bound NAC inhibits SRP-independent binding of wheat germ RNCs to the translocation channel.

Conceivably, the inhibitory action of NAC on RNC binding to the microsomes may have been obscured in the preceding experiment by the heterologous assay system consisting of mammalian microsomes and plant NAC and RNCs. Although the majority of the evidence showing that NAC inhibits binding of mammalian RNCs to canine microsomes has been obtained using purified bovine NAC, ribosome-bound reticulocyte NAC was shown to inhibit targeting of ribosome-pL86 complexes to microsomes (Lauring *et al.*, 1995b). We next asked whether RNC-bound reticulocyte NAC inhibits SRP-independent binding of ribosome-pL86 complexes to the canine Sec61 complex. As the reticulocyte lysate contains functional SRP (Meyer *et al.*, 1982), a mixed population of SRP-RNCs and RNCs is produced when the pL86 mRNA is translated in the reticulocyte lysate system. After high or low salt extraction to prepare RNCs, the amount of pL86 chains bound to the ribosome via tRNA was determined by precipitation of the peptidyl-tRNA with CTABr (Figure 2A). The RNCs were incubated with microsomes in the presence or absence of GTP. As the SRP-dependent targeting pathway is completely blocked by deletion of GTP (Connolly and Gilmore, 1989; Rapiejko and Gilmore, 1997), SRP-independent binding of RNCs to the microsomes can be evaluated using samples lacking GTP. A protease digestion assay was used to differentiate between free RNCs and membrane-bound RNCs (Figure 2A). In the absence of microsomes, proteolysis of pL86 yields a ribosome-protected fragment of approximately 30 residues (30 mer) as observed previously (Connolly *et al.*, 1989). In contrast, pL86 is resistant to proteinase K digestion when the RNC is attached to the ribosome receptor and the nascent polypeptide is inserted into the translocation channel (Connolly *et al.*, 1989; Jungnickel and Rapoport, 1995). A greater fraction of the pL86 was protease resistant when the SRP pathway was activated by addition of GTP. As quantified in Figure 2B, ribosome-bound mammalian NAC did not inhibit SRP-independent binding of the RNCs to the microsomes.

SRP Stimulates RNC Binding to the Translocation Channel

SRP and NAC have been proposed to act as positive and negative regulators of ribosome binding to the translocation channel. According to this model, SRP merely suppresses the inhibitory action of NAC, thereby allowing ribosome attachment to the Sec61 translocation channel. In contrast, the preceding ex-

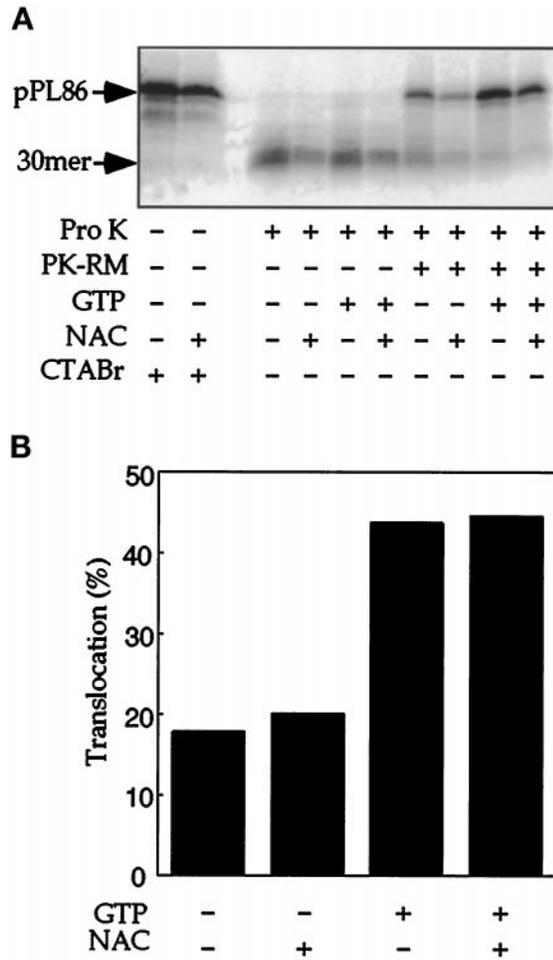


Figure 2. SRP-independent translocation in the reticulocyte lysate system. Ribosome-pL86 complexes were assembled in a reticulocyte lysate system. The RNCs were resuspended in the absence of the protease inhibitor cocktail. Peptidyl-tRNA was precipitated from an aliquot of the NAC-depleted and mock-depleted ribosome-pL86 complexes with CTABr. Additional aliquots of the NAC-depleted and mock-depleted ribosome-pL86 complexes were incubated in the presence or absence of 3 eq of PK-RM. The assays were chilled on ice before incubation with proteinase K. (A) Proteinase K resistant pL86 was resolved from the ribosome-protected fragment (30 mer) by PAGE in SDS. (B) The percent translocation was calculated as $(100 \times \text{protease resistant pL86}) / (\text{CTABr precipitated pL86})$.

periments (Figures 1 and 2) show that SRP stimulates binding of plant or mammalian RNCs to microsomes in the absence of NAC. Unlike the experiments reported here, wherein SRP was included during translation of pL86, Lauring *et al.* (1995a) added purified SRP to the plant RNCs after centrifugation to remove NAC. Conceivably, this difference in experimental design could be responsible for the apparent inability of SRP to stimulate translocation after extraction of NAC. The cotranslational presence of SRP stimulated pL86 translocation both in the presence and absence of

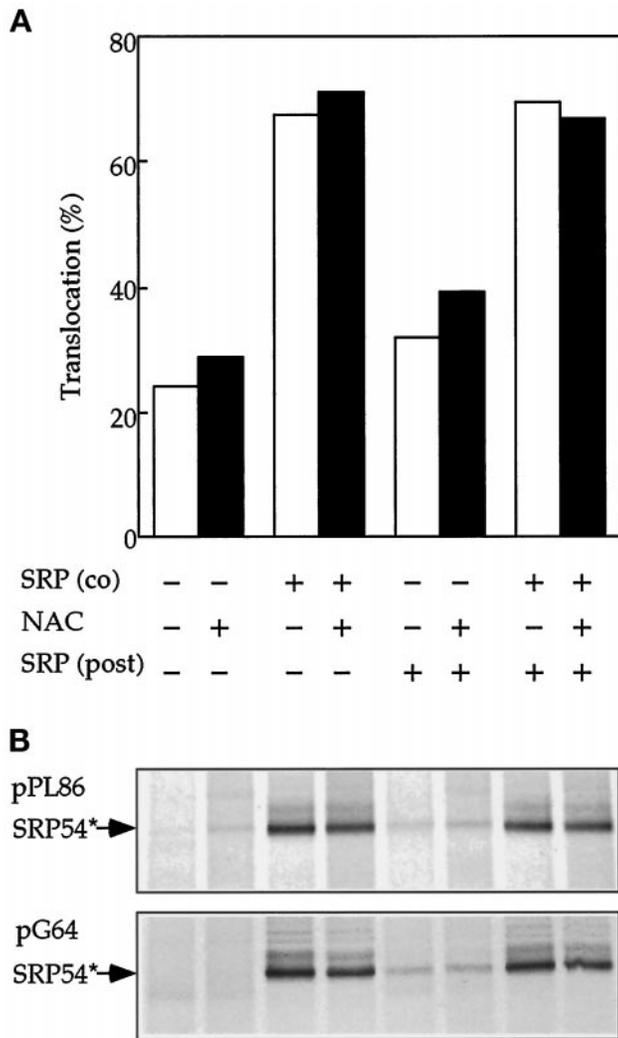


Figure 3. SRP stimulates translocation in the presence or absence of NAC. pPL86 mRNA or pG64 mRNA was translated in a wheat germ system in the absence or presence of SRP (SRP(co)). NAC-depleted or mock-depleted ribosome-pPL86 complexes were isolated as in Figure 1, and half the aliquots were supplemented with 50 nM SRP (SRP (post)). (A) Aliquots of the ribosome-pPL86 complexes were incubated with PK-RM, and translocation was assayed as in Figure 1B. (B) Aliquots of the ribosome-pPL86 complexes and the ribosome-pG64 complexes were incubated with DSS in the absence of PK-RM to crosslink the signal sequence of the nascent polypeptide to SRP54. The crosslinked product (SRP54*) was resolved from pG64 or pPL86 by PAGE in SDS. The chart in panel A also corresponds to the lanes in panel B.

NAC (Figure 3A). In contrast, the posttranslational addition of SRP to the NAC-depleted RNCs resulted in a barely detectable increase in pPL86 translocation relative to that observed without SRP. Thus, when we add SRP after isolation of the RNCs we were able to recapitulate the observation that SRP appears not to stimulate translocation in the absence of NAC. However, the posttranslational addition of SRP to the

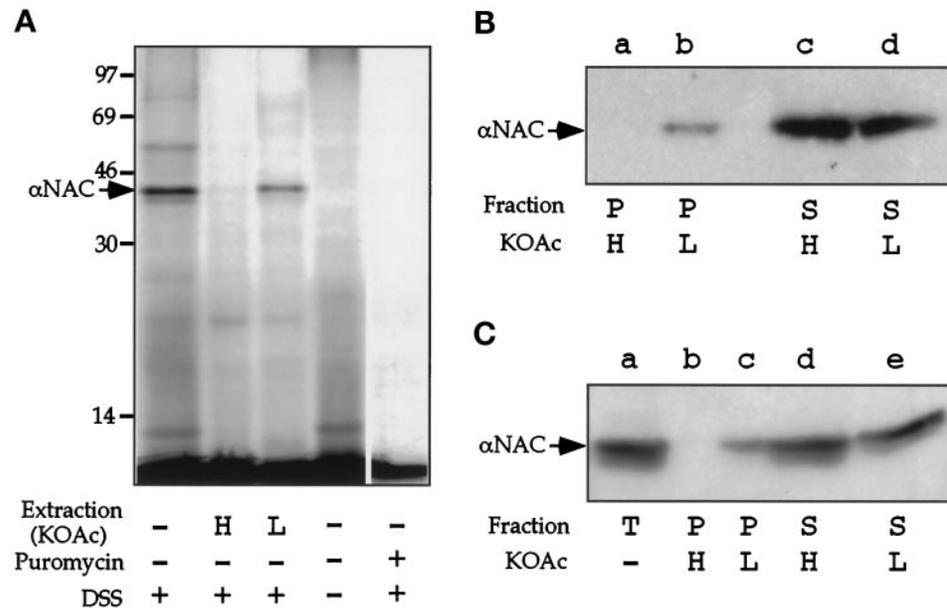
mock-depleted RNCs was also ineffective (Figure 3A) suggesting that SRP recognition of pPL86 may be impaired by the manipulations involved in the isolation of the RNCs.

Recognition of the signal sequence by SRP is mediated by the 54 kDa subunit (SRP54) (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986). To test the possibility that posttranslational binding of SRP to the RNCs was impaired, the homo-bifunctional lysine-directed crosslinker disuccinimidylsuberate (DSS) was used to crosslink SRP54 to pPL86 (Figure 3B, upper panel) or to pG64, a 64-residue nascent polypeptide derived from the membrane glycoprotein (G protein) of vesicular stomatitis virus (Figure 3B, lower panel). Crosslinking of the radiolabeled nascent polypeptide to SRP54 yields the product designated SRP54*. As expected, formation of SRP54* was dependent on the addition of both DSS (Kellaris *et al.*, 1991) and purified SRP. Production of SRP54* was far more efficient when SRP was present during translation than when SRP was added after isolation of the RNCs. Thus, the apparent inability of SRP to stimulate translocation in the absence of NAC is readily explained by the inefficient binding of SRP to the purified RNCs.

Distribution of NAC between Ribosome-bound and Free Pools

As the preceding experiments did not disclose an inhibitory effect of ribosome-bound NAC on RNC binding to microsomes, it was essential to establish that the high-salt and low-salt extracted RNCs differed with respect to NAC content. First, we determined whether contact between NAC and the nascent polypeptide persisted after isolation of the high salt extracted RNCs. NAC contacts nascent polypeptides shortly after they emerge from the peptidyltransferase site of the ribosome (Wang *et al.*, 1995). Consequently, the α subunit of NAC can be crosslinked to nascent polypeptides that contain photoreactive lysine analogues located between 10 and 30 residues from the tRNA moiety (Wiedmann *et al.*, 1994; Wang *et al.*, 1995). A truncated firefly luciferase nascent chain (ffLuc52) is an optimal target for crosslinking to NAC (Wiedmann *et al.*, 1994) because ffLuc52 contains several lysine residues in the region that has been shown to contact NAC. Here, crosslinking with DSS was used to determine whether NAC was efficiently separated from the RNCs by the high-salt extraction procedure (Figure 4A). The ribosome-bound ffLuc52 was crosslinked to a protein with an apparent molecular weight of 33–35 kDa when DSS was added directly to total translation products. In contrast, the crosslinked product was not observed after RNCs were extracted with high salt to remove NAC, or were incubated with puromycin to release the nascent

Figure 4. Depletion and mock-depletion of NAC from RNCs and 80S ribosomes. (A) Ribosome-ffLuc52 complexes assembled in the wheat germ system were incubated with DSS before (-) or after centrifugation through a high-salt (H) or a low-salt (L) sucrose cushion. As controls, DSS was not added, or puromycin was added before the addition of DSS. The crosslinked product between ffLuc52 and α NAC is indicated by the arrow. (B) Ribosome-ffLuc77 complexes assembled in a reticulocyte lysate translation system were centrifuged through a high-salt (H) or low-salt (L) sucrose cushions to obtain RNC pellet (P) and supernatant (S) fractions. Aliquots of the high-salt and low-salt extracted RNCs (a and b) and the high-salt and low-salt supernatants (c and d) were resolved by PAGE in SDS and probed with an antibody to α NAC. The P and S aliquots were derived from identical volumes of total reticulocyte translation products. (C) Wheat germ translation extract (T) was centrifuged as described in panel B obtain ribosome pellet (P) and supernatant (S) fractions. Aliquots derived from equal volumes of the wheat germ translation extract (a), high-salt and low-salt extracted ribosomes (b and c) and the high-salt and low-salt supernatants (d and e) were resolved by PAGE in SDS and probed with an antibody to α NAC.



polypeptide from the ribosome. When RNCs were isolated by centrifugation through the low-salt sucrose cushion, the 33-kDa protein could be crosslinked to nascent ffLuc52, albeit with reduced efficiency (Figure 4A). On the basis of the electrophoretic mobility of the crosslinked product and the two conditions that abolish crosslink formation, the 33-kDa protein was tentatively identified as α NAC, as these properties are characteristic of authentic NAC (Wiedmann *et al.*, 1994).

To confirm the results of the crosslinking experiments, an antibody to α NAC was used to probe immunoblots of the RNC preparations. The immunoblots showed that high-salt extracted rabbit reticulocyte RNCs (Figure 4B), or wheat germ RNCs (not shown) lack detectable α NAC. These results confirm previous reports that NAC does not interact with ribosome-bound nascent chains with sufficient affinity to withstand centrifugation through a high-salt sucrose cushion (Wang *et al.*, 1995; Powers and Walter,

1996). Thus, the results in Figures 1–3 cannot be ascribed to incomplete extraction of NAC from the RNCs.

Interestingly, the majority of reticulocyte NAC (Figure 4B) and wheat germ NAC (not shown) was recovered in the supernatant fraction after centrifugation of the RNCs through the low-salt sucrose cushion. The antibody to α NAC was also used to evaluate the distribution of NAC between ribosome-bound and unbound pools after centrifugation of a wheat germ translation extract (Figure 4C). Most of the NAC in a wheat germ extract did not cosediment with the 80S ribosomes through a low-salt sucrose cushion, but instead remained in the supernatant fraction, as observed for the RNCs. After a high-salt extraction, α NAC was not detected in the 80S ribosome preparation. These results strongly suggest that the majority of α NAC in the translation extracts is not stably bound to ribosomes or RNCs at physiological ionic strength.

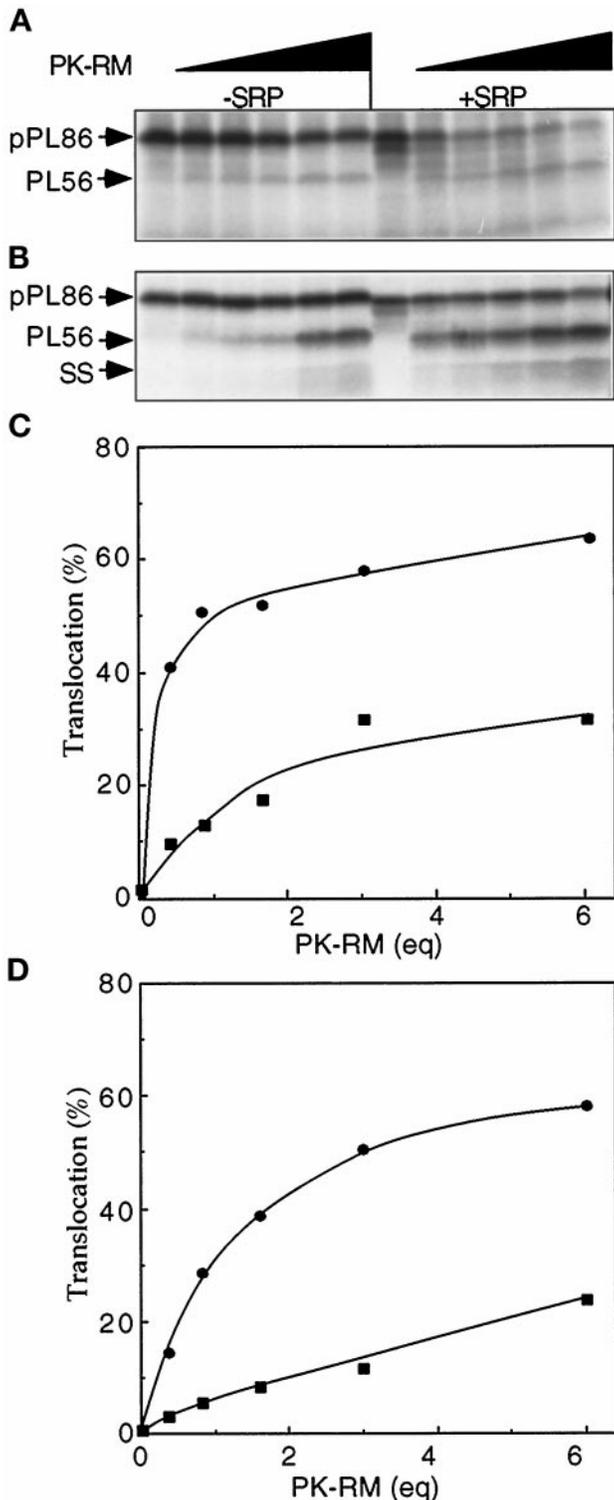


Figure 5. SRP-dependent and SRP-independent translocation in the presence of endogenous wheat germ NAC. Ribosome-pPL86 complexes were assembled in a wheat germ system in the absence or presence of 50 nM SRP. (A-C) Aliquots (15 μ L) of the translation products were incubated with 0–6 eq of PK-RM. Translocation of

SRP-dependent and SRP-independent Translocation in the Presence of Endogenous Wheat Germ NAC

Semiquantitative protein immunoblots using recombinant human α NAC as a standard indicate that our wheat germ and reticulocyte lysate translation systems contain between 1 and 2 μ M α NAC (our unpublished results). Previous studies (Lauring *et al.*, 1995a), using a final NAC concentration of 200 nM, have shown that purified NAC inhibits SRP-independent targeting of wheat germ ribosomes to the translocation channel. Having observed that the majority of the NAC in wheat germ cytosol does not copurify with the mock-depleted RNCs, the following experiment was designed to determine whether wheat germ cytosol, due to a substantially higher NAC content, would inhibit SRP-independent binding of RNCs to microsomes. After translating pPL86 in the absence or presence of SRP, the total translation products were incubated with either low (0–0.8 eq) or high (1.6–6 eq) amounts of PK-RM, and translocation of pPL86 was assayed as in Figure 1B. SRP-dependent translocation of preprolactin nascent chains was very efficient when low amounts (< 1 eq) of microsomes were present (Figure 5C, circles). When higher amounts of PK-RM were added, SRP-independent translocation was readily detectable (Figure 5C, squares). Whereas the SRP-independent translocation of pPL86 in the presence of micromolar concentrations of endogenous wheat germ NAC was surprisingly robust, we next tested whether SRP-independent translocation of pPL86 was stimulated by removal of NAC (Figure 5D). Although SRP-independent translocation of pPL86 was observed after NAC depletion (Figure 5D), the translocation efficiency was remarkably similar to that observed in the presence of endogenous wheat germ NAC (Figure 5C). Thus, depletion of endogenous wheat germ NAC did not stimulate SRP-independent translocation of pPL86. The biphasic titration curve observed for the assays containing SRP is a consequence of precursor heterogeneity; the translation products contain a mixture of SRP-RNCs and RNCs that lack bound SRP.

Figure 5 (cont). pPL86 was assayed as in Figure 1B. The supernatants (A) and pellets (B) were resolved by PAGE in SDS. The assays in panels A and B contained 0, 0.4, 0.8, 1.6, 3, and 6 eq of PK-RM for reactions that lacked or contained SRP. Radioactive bands corresponding to pPL86, PL56, and the signal sequence (SS) are labeled. (C) Translocation of pPL86 was calculated as described in Figure 1C. Translocation (%) is shown for assays that contained (circles) or lacked (squares) SRP. (D) NAC was removed from the RNC complexes. Aliquots (15 μ L) of the NAC-depleted ribosome-pPL86 complexes were incubated with PK-RM as described above. GTP (50 μ M) was not added to assays that lacked SRP during translation. Translocation of pPL86 was quantified as described above for assays that contained (circles) or lacked (squares) SRP.

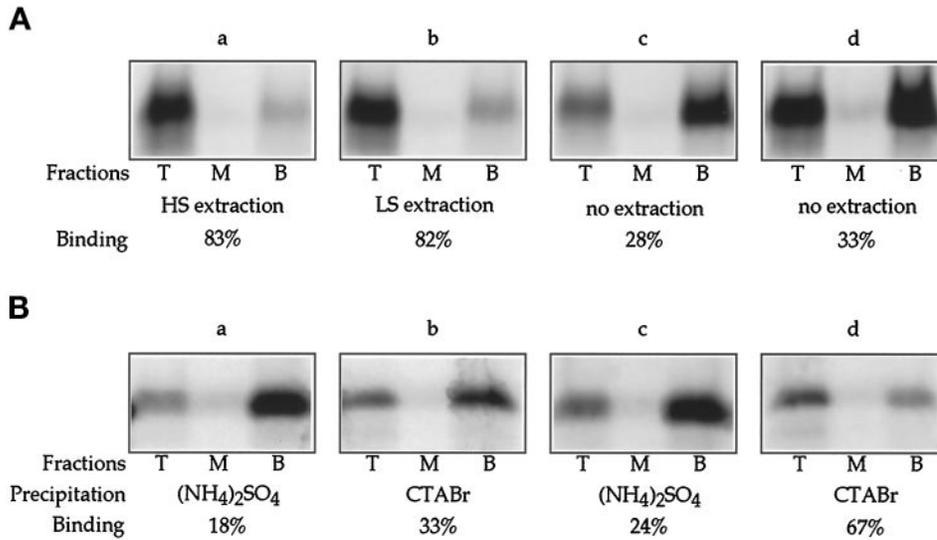


Figure 6. Signal sequence-independent binding of RNCs to PK-RM. Ribosome-ffLuc77 complexes were assembled in a reticulocyte lysate system. (A) Aliquots of (a) high-salt extracted RNCs (-NAC), (b) low-salt extracted RNCs (+NAC) and (c, d) total translation products (c, 2.5 μ l; d, 5 μ l) were incubated with 8 eq of PK-RM. The RNCs (a and b) were derived from 10 μ l of total translation products. Membrane-bound ffLuc77 was separated from unbound ffLuc77 by flotation on discontinuous sucrose gradients as in Figure 1A. (B) Total ffLuc77 translation products were incubated with 2 eq of PK-RM (a and b) or 4 eq of PK-RM (c and d) before flotation on discontinuous sucrose gradients. Aliquots of the three fractions from the gradients were precipitated with either 67% saturated ammonium sulfate (a and c) or with CTABr (b and d) before PAGE in SDS. The percent of ffLuc77 that was recovered with the microsomes was calculated as described in MATERIALS AND METHODS.

Signal Sequence-independent Binding of RNCs to Microsomes in the Presence of Mammalian NAC

Ribosomes bearing a 77-residue firefly luciferase nascent chain (ffLuc77) were used to investigate signal sequence-independent binding of mammalian RNCs to microsomes. Mock-depleted and NAC-depleted RNC complexes were incubated with 8 eq of PK-RM to determine whether ribosome-bound NAC would inhibit targeting of the RNCs to the microsomes. More than 80% of the RNCs and the NAC-depleted RNCs were recovered with the microsomes after flotation on a sucrose step gradient (Figure 6A, samples a and b). In agreement with a previous report (Lauring *et al.*, 1995b), the majority of the membrane-bound RNC complexes were insensitive to extraction with 500 mM KOAc (not shown) suggesting that the RNCs were bound to the Sec61 complex. The ribosome-bound NAC in the mock-depleted RNCs did not detectably inhibit signal sequence-independent attachment of the RNCs to the microsomes. To determine whether RNC binding to the microsomes was prevented when the NAC concentration was higher (i.e. \sim 1 μ M), total ffLuc77 translation products were incubated directly with the PK-RM. Approximately 25% of the in vitro synthesized ffLuc77 was typically recovered in the RNC preparations after centrifugation to remove aggregates. The majority of the ffLuc77 translation product (\sim 50%) did not precipitate with CTABr, hence did not cosediment with the ribosomes as a peptidyl-

tRNA. For these reasons, the volume of total translation products that were incubated with the microsomes (samples c and d) corresponded to 25 and 50% of the volume used to isolate the RNCs (samples a and b). Roughly 30% of the ffLuc77 was targeted to the microsomes when total translation products were incubated with the PK-RM (Figure 6A, samples c and d). The amount of membrane-bound ffLuc77 was proportional to the volume of added translation product, and could exceed that obtained using the isolated RNCs (e.g. Figure 6A, sample d). We postulated that prematurely released ffLuc77 was responsible for the apparent reduction in binding efficiency in samples c and d. To test this explanation, 10 μ l of the total translation products were incubated with either 2 eq (Figure 6B, a and b) or 4 eq (Figure 6B, c and d) of PK-RM before flotation on sucrose step gradients. The fractions from the flotation gradients were divided in half and precipitated with either ammonium sulfate or CTABr to differentiate between total ffLuc77 and ribosome-bound ffLuc77. Essentially identical amounts of ffLuc77 were precipitated by ammonium sulfate and CTABr when the top fractions from the flotation gradients were analyzed, demonstrating that the membrane-bound ffLuc77 was a ribosome-bound peptidyl-tRNA. In contrast, a substantial fraction of the ffLuc77 that remained in the bottom fraction of the gradient could be precipitated with ammonium sulfate but not with CTABr, indicating that the unbound fraction con-

tains both ribosome-bound and unbound fLuc77. Thus, mammalian RNCs lacking an RER signal sequence can bind to the microsomes in the presence of reticulocyte cytosol, which contains micromolar concentrations of NAC.

Competition between 80S Ribosomes and RNCs for Binding to the Translocation Channel

SRP-independent translocation of pPL86 was proportional to the quantity of added microsomes (Figure 5D), indicating that binding of NAC-depleted RNCs to microsomes was dependent on a saturable component that was limiting at all concentrations of PK-RM that were tested. The Sec61 complex is the most obvious candidate for the limiting component for SRP-independent translocation of pPL86. Canine microsomes contain approximately 1 pmol of Sec61 complex per eq (Kalies *et al.*, 1994). However, equilibrium binding studies reveal the presence of ~270 fmol of ribosome binding sites/eq of PK-RM (Kalies *et al.*, 1994) consistent with the recent discovery that the Sec61 complex forms oligomeric rings comprised of 3–4 Sec61 heterotrimers (Hanein *et al.*, 1996). The quantity of ribosome-pPL86 complexes added to each sample in Figure 5 was calculated to be between 150–300 fmol. Hence, Sec61 oligomers were present in excess relative to the quantity of added RNCs in all assays in Figure 5D that contained more than 1 eq of PK-RM. The preceding considerations suggested that the purified NAC-deficient RNCs contain an inhibitory component. A good candidate for an internal competitor was inactive 80S ribosomes, which have been shown to bind to the Sec61 complex with high affinity (Kalies *et al.*, 1994). Indeed, the signal sequence-independent binding of ribosome-fLuc77 complexes to the Sec61 complex is inhibited by an excess of 80S ribosomes (Lauring *et al.*, 1995b). Each assay shown in Figure 5 contained approximately 4.5 pmol of 80S ribosomes, a value that exceeded the total quantity of Sec61 oligomers. High-salt extracted wheat germ ribosomes, that lack detectable α NAC (see Figure 4C), were tested for the ability to inhibit SRP-dependent and SRP-independent translocation reactions (Figure 7). Excess 80S ribosomes are an effective competitive inhibitor of SRP-independent binding of ribosome-pPL86 complexes to PK-RM, whether or not NAC is present (Figure 7, open symbols). In contrast, the 80S ribosomes did not effectively compete with SRP-RNCs for attachment to Sec61 translocation channel (Figure 7, closed symbols). When a 10-fold excess of competing ribosomes were added, the inactive ribosomes were approximately 30-fold more abundant than Sec61 oligomers, and roughly 200-fold more abundant than the SRP-RNCs. The presence (Figure 7, filled circles) or absence (Figure 7, filled squares) of NAC did not reproducibly

influence the ability of 80S ribosomes to compete with SRP-RNCs for binding to the translocation channel.

DISCUSSION

The objective of this study was to determine how SRP and NAC mediate the efficient and accurate delivery of an RNC to the translocation channel. Several observations were made that impact on our understanding of the protein translocation reaction. First, RNCs can functionally engage the mammalian protein translocation channel in an SRP-independent reaction. Second, the ability of SRP to promote the efficient targeting of ribosomes to the endoplasmic reticulum was neither positively nor negatively influenced by ribosome-bound NAC. Third, the SRP-dependent binding of RNCs to the translocation channel was remarkably insensitive to the presence of a vast excess of a high affinity ligand for the Sec61 translocation channel. Each of these observations will now be considered in more detail.

Artificial Conditions Promote SRP-independent Translocation

The cotranslational binding of SRP to the signal sequence is considered to be an obligatory event during protein translocation across the mammalian endoplasmic reticulum (Walter and Johnson, 1994). The few noteworthy exceptions include several low molecular weight proteins that escape SRP recognition before chain termination and are targeted to the membrane by a ribonucleoprotein-independent pathway (Muller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987). The current study confirmed several recent reports describing SRP-independent targeting of RNCs to microsomes (Wiedmann *et al.*, 1994; Jungnickel and Rapoport, 1995). How can we explain the apparent contradiction between SRP-independent targeting of RNCs to the translocation channel, and the essential role for SRP in the cotranslational transport of full-length polypeptides? There are several important experimental differences that are likely responsible for the relaxed requirement for SRP in the experiments reported here. The majority of the Sec61 oligomers in rough microsomes are occluded by endogenous membrane-bound ribosomes (Görlich and Rapoport, 1993; Kalies *et al.*, 1994). Thus, if we calculate the concentration of ribosomes and Sec61 complexes that are present in a typical cotranslational translocation assay (10 μ l of wheat germ translation products, 1 eq RM), competing 80S ribosomes derived from the microsomes and the wheat germ lysate are present in roughly 15-fold M excess relative to Sec61 oligomers. To observe significant SRP-independent targeting of RNCs, the total amount of ribosomes had to be similar to the content of unoccupied Sec61 oli-

gomers. In our experiments, this was achieved by detaching endogenous membrane-bound ribosomes (e.g. PK-RM), and by increasing the quantity of microsomes relative to the amount of ribosomes derived from the translation system. Secondly, by using a truncated mRNA to prepare the RNCs, elongation of the protein is halted at a stage where the nascent polypeptide is an optimal length for insertion into the Sec61 complex, thereby drastically extending the time period within which the ribosome can productively interact with the translocation channel. Here, the pPL86-ribosome complexes were incubated with microsomes for 5 min before further analysis. Although this incubation period is somewhat shorter than the 8–10 min required to synthesize the 230-residue preprolactin chain *in vitro*, it exceeds the time period that elongating preprolactin chains are competent for cotranslational translocation (Siegel and Walter, 1985). Synchronized translation experiments using elongation arrest-deficient SRP particles have revealed that continued elongation of the polypeptide after SRP recognition interferes with the subsequent productive binding of the ribosome to the membrane (Siegel and Walter, 1985).

Reconsideration of the Role of NAC in the Protein Translocation Reaction

NAC was proposed to act as an antiassociation factor to prevent the SRP-independent binding of ribosomes to the translocation channel (Lauring *et al.*, 1995a). Unexpectedly, the ribosome-bound NAC that copurifies with wheat germ or rabbit reticulocyte RNCs did not interfere with signal sequence-dependent or signal sequence-independent binding of ribosomes to the Sec61 complex. Several explanations were considered for the discrepancy between our results and the current view that NAC performs a pivotal role in regulating ribosome attachment to the RER. First, an examination of the distribution of α NAC between the ribosome-bound and unbound fractions revealed that the majority of the NAC in wheat germ and reticulocyte translation extracts does not copurify with 80S ribosomes or RNCs. Our wheat germ and reticulocyte lysate translations contain 450 nM and 75–100 nM ribosomes, respectively. Semi-quantitative protein immunoblots using the antibody to α NAC indicate that the concentration of NAC in the two translation systems is between 1 and 2 μ M. Consequently, complete sedimentation of NAC with 80S ribosomes would be dependent on multiple NAC binding sites per ribosome. Nonetheless, additional experiments were performed to determine whether the unfractionated translation extract, by virtue of a higher NAC concentration, would prevent SRP-independent binding of RNCs to the microsomes. Our results do not support the hypothesis that NAC pre-

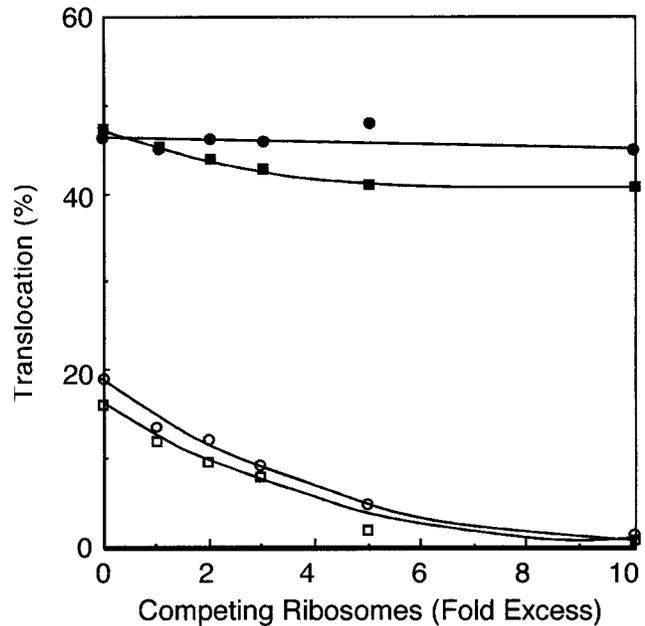


Figure 7. Competition between 80S ribosomes and RNCs for binding to the translocation channel. SRP-RNCs and RNCs assembled by translation of pPL86 mRNA in the wheat germ system were NAC depleted or mock-depleted (open squares, -SRP/-NAC; open circles, -SRP/+NAC; filled squares, +SRP/-NAC; filled circles, +SRP/+NAC). These four RNC preparations (4.5 pmol of 80S ribosomes, ~250 fmol of pPL86) were mixed with 45–45 pmol of purified NAC-depleted 80S ribosomes (see Figure 4C) and PK-RM. Assays of SRP-RNCs and RNCs contained 2 and 4 eq of PK-RM, respectively. Translocation of pPL86 was assayed as in Figure 1B.

vents signal-independent or SRP-independent attachment of RNCs to the ribosome-stripped microsomes. Second, as a source of ribosome-stripped microsomes, the previous studies characterizing NAC used EDTA-high salt extracted microsomes (EK-RM) instead of PK-RM (Lauring *et al.*, 1995a; Lauring *et al.*, 1995b). We believe that PK-RM are preferable for these experiments, as PK-RM are essentially ribosome-free, unlike EK-RM, which retain nonnative 60S ribosomal subunits (Sabatini *et al.*, 1966). However, EK-RM were assayed as in Figure 5, and we observed that endogenous wheat germ NAC did not prevent SRP-independent translocation of pPL86 (our unpublished results). A third possibility for the observed differences may be the use of purified NAC in conjunction with the high-salt extracted RNCs. Conceivably, the interactions between purified bovine NAC, the EK-RM and high-salt extracted ribosomes may differ significantly from that which occurs when total cytosol is present. Although we have not conducted experiments using purified NAC to test this possibility, the evidence that we have obtained using total translation extracts suggests that endogenous NAC, even when present in excess relative to ribosomes, does not perform a piv-

otal role as a negative regulator of RNC targeting to the endoplasmic reticulum. Additional research will be required to define the physiological role of NAC, an abundant cytosolic protein that is ubiquitously expressed in fungal, plant, and vertebrate organisms.

One objective of this study was to test the hypothesis that SRP promotes RNC binding to the translocation channel by suppressing the antiassociation activity of NAC. Several lines of evidence reported in this study are also in conflict with this proposal. First, SRP-dependent translocation was more efficient than SRP-independent translocation both in the presence and absence of NAC. If endogenous NAC acted as an antiassociation factor, SRP-independent and SRP-dependent translocation activity should have been equal when NAC was not present. Instead, our evidence confirms the previous conclusion that SRP actively stimulates ribosome delivery to the translocation channel (Walter and Blobel, 1981a). Second, the translocation-promoting activity of SRP was greatest when the SRP was present during synthesis of the nascent polypeptide, rather than when SRP was added after isolation of the RNCs. Crosslinking with DSS demonstrated that SRP54 binds to the signal sequence with reduced efficiency after isolation of the RNCs. Notably, poor binding of SRP54 to the RNC complexes was not nascent chain size or sequence specific, as essentially identical results were obtained with pPL86 and pG64. Although an explanation for the inefficient binding of SRP to the isolated RNCs is not readily apparent, this phenomenon provides an explanation for the reported failure of SRP to stimulate translocation in the absence of NAC (Lauring *et al.*, 1995a).

Competition between Ribosomes and RNCs for Binding to the Sec61 Complex

Clearly, the SRP-independent attachment of an RNC to the translocation channel is mediated by the inherent affinity between the ribosome and the Sec61 complex. We had anticipated that the affinity between the Sec61 complex and an RNC would be similar or identical to the affinity between an inactive ribosome and the Sec61 complex. However, when the total concentration of competing ribosomes was doubled we observed less than a twofold decrease in SRP-independent translocation of pPL86. This observation suggests that an inactive ribosome and the ribosome-pPL86 complex do not bind to the Sec61 complex with an identical affinity. Presumably, the interaction between the signal sequence and Sec61 α further stabilizes the interaction between the ribosome and the Sec61 complex. Although the ribosome-Sec61 interaction is sensitive to high salt (Görlich *et al.*, 1992; Kalies *et al.*, 1994), membrane-bound RNCs are not extracted by high salt unless the nascent polypeptide is released

from the ribosome with puromycin (Gilmore and Blobel, 1985).

The most pronounced stimulation by SRP of translocation occurred when low amounts of microsomes, hence low amounts of the Sec61 complex, were present in a translocation assay (Figure 1). This observation considered together with the experimentally determined concentrations of the crucial assay components (ribosomes, RNCs and Sec61 complexes) suggested that SRP circumvents the competition between inactive ribosomes and RNCs for binding to the translocation channel. This hypothesis was confirmed by the failure of inactive ribosomes to compete with SRP-RNCs for targeting to vacant Sec61 complexes even when the total amount of ribosomes was in considerable excess relative to the SRP-RNC complexes and the Sec61 oligomers. A large excess of reticulocyte 80S ribosomes has also been shown not to compete with SRP-RNC complexes for targeting to the Sec61 complex (Murphy *et al.*, 1997). We conclude that one role of the SRP-SR targeting pathway is to confer an overwhelming advantage during the targeting of a RNC complex to the translocation channel. The high in vivo efficiency of protein translocation demands a mechanism to insure that protein translocation sites are not occluded by ribosomes that are not actively engaged in the synthesis of proteins that contain RER signal sequences. The molecular mechanism responsible for preventing the futile attachment of mistargeted ribosomes to the Sec61 complex remains unresolved, yet these results suggest that SRP is intimately involved, and that further analysis of the targeting pathway will disclose the mechanism.

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