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Dual recognition of the ribosome and the signal recognition particle by the SRP receptor during protein targeting to the endoplasmic reticulum

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We have analyzed the interactions between the signal recognition particle (SRP), the SRP receptor (SR), and the ribosome using GTPase assays, biosensor experiments, and ribosome binding assays. Possible mechanisms that could contribute to an enhanced affinity between the SR and the SRP–ribosome nascent chain complex to promote protein translocation under physiological ionic strength conditions have been explored. Ribosomes or 60S large ribosomal subunits activate the GTPase cycle of SRP54 and SRα by providing a platform for assembly of the SRP–SR complex. Biosensor experiments revealed high-affinity, saturable binding of ribosomes or large ribosomal subunits to the SR. Remarkably, the SR has a 100-fold higher affinity for the ribosome than for SRP. Proteoliposomes that contain the SR bind nontranslating ribosomes with an affinity comparable to that shown by the Sec61 complex. An NH2-terminal 319-residue segment of SRα is necessary and sufficient for binding of SR to the ribosome. We propose that the ribosome–SR interaction accelerates targeting of the ribosome nascent chain complex to the RER, while the SRP–SR interaction is crucial for maintaining the fidelity of the targeting reaction.

Introduction

Ribosomes synthesizing proteins with RER-specific signal sequences are selectively attached to protein translocation channels on the cytoplasmic surface of the RER by the combined action of the signal recognition particle (SRP) and the SRP receptor (SR) (for review see Walter and Johnson, 1994). High-affinity binding of the SRP54 subunit of SRP to the signal sequence as it emerges from the polypeptide exit site on the large ribosomal subunit is the initial sorting step that ultimately partitions ribosome nascent chain complexes (RNCs) between RER-bound and cytosolic polysome populations. Contact between the SRP–RNC complex and the SR initiates a cooperative GTPase cycle that is catalyzed by SRP54 and the SRα subunit of the SR (Connolly and Gilmore, 1993; Miller et al., 1993; Rapiejko and Gilmore, 1997). Prior to complex formation, the GTP binding sites in SRP54 and SRα exist in an “empty site” conformation that is characterized by a low nucleotide affinity with rapid and reversible binding of GTP, GDP, or Gpp(NH)p (Rapiejko and Gilmore, 1994, 1997). Cooperative binding of GTP to SRP54 and SRα is followed by the transfer of the RNC to an unoccupied protein translocation channel (Song et al., 2000). Eukaryotic translocation channels are oligomeric assemblies of Sec61 heterotrimers (Görlich and Rapoport, 1993; Beckmann et al., 2001) that incorporate additional accessory proteins (Menetret et al., 2000). The specific binding interactions that are responsible for rapid, yet selective, targeting of an SRP–RNC to the SR are not fully understood. The binding affinity between SRP and the SR is surprisingly low (Kd ≈ 125 nM) in a physiological ionic strength buffer (Connolly and Gilmore, 1993), yet targeting of the SRP–RNC complex to the SR is efficient. It has been proposed that the ribosome acts as a classical guanine nucleotide exchange factor (GEF) for SRP54 (Bacher et al., 1996), resulting in an enhanced affinity between the SR and a GTP-bound form of the SRP–RNC complex. However, SRP–RNCs target to SR proteoliposomes in the absence of GTP (Song et al., 2000), indicating that the translocon, as well as GTP, is dispensable for the targeting reaction. Here we have analyzed the interactions between SRP, the SR, and the ribosome to determine how the RNC promotes...
the interaction between SRP and the SR. Our results suggest a novel model for the targeting reaction that involves simultaneous recognition of the ribosome and the SRP by the SR. We propose that the ribosome–SR interaction accelerates the rate of RNC targeting, while the SRP–SR interaction is essential for the fidelity of RNC targeting to the RER.

Results

Selective binding of an RNC to the translocon is dependent upon the interaction between two ribonucleoproteins (SRP and the ribosome) and two RNP receptors (SR and the Sec61 complex). The interaction between SRP and the SR is remarkably sensitive to a physiological concentration of monovalent cations (Connolly and Gilmore, 1993). Consequently, one might predict that the targeting phase of the protein translocation reaction would be inhibited by modest increases in ionic strength. To test this prediction, SRP–RNC complexes bearing a nascent opsin polypeptide (op156) were assembled by in vitro translation of a truncated opsin mRNA transcript lacking a termination codon. The SRP–RNC complexes were incubated with microsomes in buffers containing 50 mM stepwise increases in KOAc concentration (Fig. 1 A). Integration of op156 into the membranes was detected by the decreased gel mobility that accompanies transfer of N-linked glycans onto one or both of the glycosylation sites that precede the first TM span of opsin to yield glycosylated op156 (g-op156; Fig. 1 A, top). In the absence of GTP (not depicted, but see Rapiejko and Gilmore, 1992), or when membranes lack intact SRx (C,PK-RM), we observe greatly reduced quantities of membrane-integrated op156 and no detectable g-op156 (Fig. 1 A, bottom). SRP-dependent integration of g-op156 is surprisingly insensitive to increased ionic strength, with ~50% inhibition observed at 300 mM KOAc (Fig. 1 B).

Ribosomes stimulate the GTPase activity of the SRP–SR complex

We used a previously described GTPase assay (Bacher et al., 1996, 1999) to test whether RNCs promote the interaction between SRP and the SR. The GTPase activity of the SRP–SR complex is readily detected when equimolar amounts of purified SRP, SR proteoliposomes, and in vitro–assembled RNCs are combined in an assay buffer that contains a physiological concentration (150 mM K+) of monovalent cations. RNCs that were assembled by translating a truncated mRNA that encodes the NH2-terminal 86 residues of preprolactin (pPL86) were separated from cytosolic GTPases by centrifugation through a high salt–sucrose step gradient. In agreement with previous reports (Bacher et al., 1996, 1999), the GTPase activity of the pPL86 RNCs was increased slightly by the addition of SR proteoliposomes and markedly by the addition of SRP plus the SR proteoliposomes (Fig. 2 A). Assays that lacked the pPL86 RNCs confirmed the low basal GTPase activity of the SRP–SR complex in a physiological ionic strength buffer (not depicted in Fig. 2 A, but see Fig. 3 A).

The robust GTPase activity observed in Fig. 2 A appears to contradict our previous conclusion that GTP hydrolysis by the SRP–RNC–SR complex is blocked in the absence of the Sec61 complex (Song et al., 2000). To address this discrepancy, we assembled pPL86 RNCs in the absence or presence of SRP. Because SRP does not dissociate from the signal sequence of RNC complexes that are isolated by centrifugation through high salt–sucrose gradients (Powers and Walter, 1996; Raden and Gilmore, 1998), we can test whether these preassembled SRP–RNC complexes obviate the requirement for additional SRP in the GTPase assay. Very similar GTPase rates were obtained when the two RNC preparations were assayed in the presence of the SR proteoliposomes, demonstrating that the prebound SRP does not satisfy the SRP requirement for GTP hydrolysis (Fig. 2 B). The addition of free SRP plus the SR proteoliposomes to both RNC preparations resulted in a GTPase rate that was equivalent to that observed when the SR proteoliposomes were incubated with SRP in a hypotonic buffer that promotes formation of the SRP–SR complex (Fig. 2 B).
The preceding experiment suggested that RNC-bound SRP was not the active component in the GTPase assay. To address this possibility, RNCs were assembled by translation of truncated mRNAs that encode the NH$_2$-terminal 64 residues of the G protein of vesicular stomatitis virus (pG64) and the NH$_2$-terminal 77 residues of firefly luciferase (ffluc77). Unlike pPL86 or pG64, ffluc77 lacks a signal sequence for protein translocation across the endoplasmic reticulum; hence, SRP does not bind to ffluc77 RNCs. The GTPase activity of the SRP–SR complex was stimulated by both RNC preparations (Fig. 2 C). Less than 20% of the ribosomes in a wheat germ translation reaction are assembled into RNC complexes, so the RNC preparations obtained by centrifugation contain a mixture of RNCs and nontranslating ribosomes. The GTPase assays were conducted using mock RNCs that were prepared from a translation reaction that lacked mRNA (Fig. 2 D). The GTPase activity was stimulated using 0-, 3-, 6-, and 10-min time points.

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A significant fraction of the SR in a proteoliposome faces the lipidic interior and is inaccessible to the SRP and the RNCs. As observed previously (Connolly and Gilmore, 1993), the GTPase activity of the SRP and SR is low when assayed in a physiological ionic strength buffer containing detergent micelles (Fig. 3 A, open squares). The GTPase activity was stimulated roughly eightfold by the addition of pG64 RNCs (Fig. 3 A, filled squares). Deletion of SRP (Fig. 3 A, triangles) or the SR (not depicted) reduced the GTPase activity to that shown by the RNC preparation alone (circles). The GTPase activity in assays containing detergent micelles (Fig. 3 A) was higher than in assays containing the SR proteoliposomes (Fig. 2 A). Subsequent experiments used the detergent micelle assay because the GTPase activity was not influenced by experimental variations in the efficiency of proteoliposome formation or in the asymmetry of SR reconstitution.

SRP binds to nontranslating 80S ribosomes (Walter et al., 1981; Powers and Walter, 1996) in addition to polysomes.
synthesizing secretory proteins (Walter et al., 1981). Although earlier, nonequilibrium methods indicated that SRP binds the ribosome with a relatively low affinity ($K_{d}/H33360 50/9262M$; Walter et al., 1981), a recent analysis indicates that the binding affinity is substantially higher ($K_{d}/H33360 8 nM$; Flanagan et al., 2003). Purified 80S ribosomes were added to the GTPase assays of SRP and the SR to determine whether the ribosome is the active component in the RNC preparation (Fig. 3 B, squares). Notably, half-maximal stimulation of the GTPase activity was achieved when the concentration of ribosomes exceeded the concentration of the SRP and the SR. A saturable, but much lower, stimulation of GTP hydrolysis was observed in the absence of SRP (Fig. 3 B, circles). The binding site for SRP54 on the ribosome has been mapped to ribosomal proteins L23a and L35 (Pool et al., 2002), which are located in the vicinity of the polypeptide exit site on the large ribosomal subunit (Ban et al., 2000). If the ribosome stimulates the GTPase activity of the SRP–SR complex in a specific manner, one would predict that the stimulatory activity would reside on the large ribosomal subunit. Indeed, the isolated 60S subunits were almost as effective as the intact ribosome (Fig. 3 C). In contrast, 40S ribosomal subunits were comparatively ineffective even when present at a higher concentration. Neither 40S nor 60S subunits stimulated the GTPase activity of the SR in assays that were not supplemented with SRP (Fig. 3 C).

Two classes of mechanisms could explain how the ribosome could accelerate GTP hydrolysis by SRP and the SR. The addition of ribosomes could promote formation of hydrolytically active SRP–SR complexes, or the ribosome could accelerate a rate-limiting step in the hydrolysis cycle without affecting the equilibrium between SRP, the SR, and the SRP–SR complex. For example, a significant increase in the binding affinity of SRα for GTP would increase hydro-
GTPase activities of the SR and the SR subunits

The subunits of the SR (Fig. 4 A) were expressed in *Escherichia coli* to investigate the mechanism of the ribosome-stimulated GTPase activity. To facilitate purification of SRβ, the lumenal and transmembrane domains of SRβ were replaced with a 13-kD domain that is biotinylated in vivo to obtain bt-SRβ (Fig. 4 B, lane b) or a hexahistidine sequence to obtain His-SRβ (Fig. 4 B, lane e). Biotinylation domain fusion constructs were also used to express bt-SRα and bt-SRαΔN (Fig. 4 B, lanes c and d). SRαΔN lacks the NH₂-terminal 151 residues of SRα and corresponds to the COOH-terminal fragment of SRα that can be produced by limited digestion of microsomes with elastase (Meyer and Dobberstein, 1980). Coexpression of bt-SRβ and SRα allowed the isolation of bt-SR (Fig. 4 B, lane a). Coexpression of His-SRβ with NH₂-terminal fragments of SRα (SRαΔ151 or SRαΔ319) allowed purification of His-SRβΔ319 (Fig. 4 B, lane f) and His-SRβΔ319 (Fig. 4 B, lane g). In vivo formation of the SR heterodimers (bt-SR, His-SRβΔ319, and His-SRβSRαΔ151) provides evidence that SRβ and the SRX domain of SRα are correctly folded.

Limited proteolysis of rough microsomes (RMs) with trypsin cleaves SRα near residue 150 to liberate a COOH-terminal fragment that comigrates with SRαΔN. The translocation activity of the trypsin-inactivated microsomes can be restored by adding purified SRαΔN (Meyer and Dobberstein, 1980), in vitro–translated SRα (Andrews et al., 1989), or the recombinant SR heterodimer (Filga et al., 2001). Translocation of pPL into the lumen of the undigested microsomes (K-RM) was accompanied by signal sequence cleavage to yield prolactin (PL; Fig. 4 C, lane b). Translocated prolactin was not observed in the absence of microsomes (Fig. 4 C, lane a) or when the protease-digested microsomes (T₁K-RM) were tested (lane c). The translocation activity of the SRα-deficient microsomes could be reconstituted with bt-SR (Fig. 4 C, lanes d and e), bt-SRα (lanes f and g), or bt-SRαΔN (lane h). These results provide additional evidence that the recombinant proteins are folded and functional.

The GTPase activity of the SR purified from canine pancreas was compared with the recombinant proteins using either a hypotonic (50 mM K⁺) assay buffer to maximize the interaction between SRP and the SR (Fig. 4 D) or a physiological ionic strength (150 mM K⁺) buffer (Fig. 4 E). The *E. coli*–expressed proteins and the canine SR have barely detectable GTPase activities in the absence of SRP (Fig. 4, D and E). Likewise, SRP has a very low intrinsic GTPase activity (Fig. 4 E). When assayed using the low ionic strength assay buffer (50 mM K⁺), the bt-SR and bt-SRα form complexes...
with SRP that hydrolyze GTP at a rate that is comparable to the SR purified from canine pancreas (Fig. 4 D). Consistent with Fig. 3 D, the GTPase activity of complexes between SRP and the SR, or SRα, was reduced in the physiological ionic strength buffer (Fig. 4 E). SRβ did not hydrolyze GTP at a significant rate in the absence or presence of SRP (Fig. 4 E). The GTPase activities for SRP plus bt-SRαΔN showed an additive, rather than synergistic, response, indicating that active complexes were not formed between SRP and the COOH-terminal fragment of SRα (Fig. 4 E).

The E. coli–expressed SR and SR subunits were assayed for GTPase activity in the presence of 80S ribosomes (Fig. 4 F). The ability of 80S ribosomes to activate the GTPase activity of the SRP–SR complex was confirmed using the E. coli–expressed bt-SR and bt-SRα. Purified ribosomes did not stimulate GTP hydrolysis by bt-SRβ or bt-SRαΔN in the presence or absence of SRP. Assays of His-SRβ, His-SRβSRα151, and His-SRβSRα319 yielded results that were similar to bt-SRβ (unpublished data).

### The rate of SRP-SR complex formation is not accelerated by GTP

Having established that the E. coli–expressed proteins are functional by several criteria, we investigated the kinetics of SRP–SR complex formation using the IAsys optical biosensor. The immobilization strategy for the SR or the SR subunits was to coat a biotin-modified biosensor cuvette with streptavidin. After removing unbound streptavidin, the sensor surface was completed by the addition of bt-SR, bt-SRα, or bt-SRβ, or bt-SRαΔN. Binding of SRP to the SR or the SR subunits was initially analyzed in a hypotonic assay buffer (50 mM K⁺) in the absence of GTP (Fig. 5 A). SRP binds to biosensor cuvettes containing immobilized bt-SR (Fig. 5 A, a) and bt-SRα (b). SRP did not bind to bt-SRαΔN (Fig. 5 A, c), bt-SRβ (d), or to cuvettes that contained streptavidin alone (e). The dissociation of bound SRP was monitored when applicable (Fig. 5 A, a and b). The kinetics of SRP binding to the SR was analyzed by linear regression analysis of the association curves to determine the change in refractive index caused by SRP binding (extent) and to determine the initial rate of SRP binding (k on). Hyperbolic saturation curves for binding of SRP to the SR were obtained (Fig. 5 B). The Kd value derived from the saturation curve for SRP binding to the SR in the presence of GTP is 7.6 nM, which is in reasonable agreement with the value of 15 nM that was estimated using a GTPase assay (Connolly and Gilmore, 1993). Plots of k on versus SRP concentration were linear (Fig. 5 C, filled squares). The slope and y intercept of the k on plot correspond to the rate constants for association (k on) and dissociation (k diss), respectively, and yield a Kd value of 6.5 nM (Table I). Assay points obtained in the absence of GTP (Fig. 5 C, open squares) were adequately fit by very similar kinetic parameters (Table I). The binding kinetics of SRP to the SR was also examined in a physiological ionic strength buffer (150 mM K⁺) in the presence or absence of GTP (Fig. 5 C, filled and open triangles, respectively). The increase in ionic strength dramatically reduces the rate constant for complex formation, without significantly altering the rate of dissociation. The association rates for complexes formed in the absence of GTP were not significantly different from association rates obtained in the presence of GTP (Table I). The rates of dissociation were likewise not significantly influenced by the guanine nucleotide. The observation that GTP does not decrease the apparent rate constant

### Table 1: Kinetic parameters for the SRP–SR complex and the ribosome–SR complex

<table>
<thead>
<tr>
<th>Ligate</th>
<th>KOAc</th>
<th>GTP</th>
<th>k on</th>
<th>k diss</th>
<th>Kd</th>
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<tr>
<td>SRP</td>
<td>50</td>
<td>25</td>
<td>$1.1 \pm 0.1 \times 10^6$</td>
<td>$6.9 \pm 1.8 \times 10^{-3}$</td>
<td>6.5</td>
</tr>
<tr>
<td>SRP</td>
<td>50</td>
<td>0</td>
<td>$1.2 \pm 0.3 \times 10^6$</td>
<td>$6.5 \pm 3.5 \times 10^{-3}$</td>
<td>5.3</td>
</tr>
<tr>
<td>SRP</td>
<td>150</td>
<td>25</td>
<td>$4.8 \pm 1.8 \times 10^4$</td>
<td>$6.7 \pm 0.5 \times 10^{-3}$</td>
<td>140</td>
</tr>
<tr>
<td>SRP</td>
<td>150</td>
<td>0</td>
<td>$2.9 \pm 0.7 \times 10^4$</td>
<td>$5.0 \pm 0.5 \times 10^{-3}$</td>
<td>175</td>
</tr>
<tr>
<td>80S</td>
<td>150</td>
<td>0</td>
<td>$1.4 \pm 0.2 \times 10^4$</td>
<td>$1.2 \pm 0.2 \times 10^{-2}$</td>
<td>0.9</td>
</tr>
<tr>
<td>60S</td>
<td>150</td>
<td>0</td>
<td>$1.9 \pm 0.2 \times 10^4$</td>
<td>$2.0 \pm 0.5 \times 10^{-2}$</td>
<td>1.1</td>
</tr>
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### Figure 6: Kinetics of ribosome binding to immobilized SR.

- **A-C** Binding of ribosomes or ribosomal subunits to the SR in buffer D (150 mM K⁺). (A) Association curves for binding of 1 nM 80S ribosomes to the following immobilized proteins: (a) bt-SR, (b) bt-SRα, (c) bt-SRβ, (d) bt-SRβ + 25 μM GTP, and (e) bt-SRαΔN. Binding of (f) 1 nM 80S ribosomes, (g) 1.7 nM 60S subunits, or (h) 2.4 nM 40S subunits to immobilized bt-SR. Ribosomes or ribosomal subunits were added at the 1-min time point. (B and C) Equilibrium binding of ribosomes (B) or 60S ribosomal subunits (C) to immobilized bt-SR. The insets show plots of k on versus ligate concentration. (D) Co-sedimentation of SR, SR subunits, and SRP with 80S ribosomes. Binding assays in buffer D were incubated for 5 min at 25°C and contained 50 nM SRP and/or 40 nM SR (or SR subunits) and 160 nM ribosomes as indicated. Individual assays contained the following proteins: (a) SRP plus bt-SR, (b) bt-SR, (c) SRP, (d) SRP plus bt-SR, (e and f) bt-SRαΔN, (g and h) bt-SRα, (i and j) bt-SRβ, (k and l) His-SRβ, (m and n) His-SRβSRα151, or (o and p) His-SRβSRα319. The assays were layered onto 50-μl cushions of 500 mM sucrose in buffer D and centrifuged for 10 min at 279,000 g using a TLA100 rotor. After centrifugation, the samples were separated into supernatant (S) and pellet (P) fractions and resolved by PAGE in SDS, and the SR and SRP were detected using antibodies to SRP54, SRα, or SRβ. Images from sequential immunoblots using polyclonal and monoclonal antibodies were combined in the bottom panel.
for complex dissociation is explained by the fact that the rate of GTP hydrolysis \( (k_{\text{cat}} = 3.5 \times 10^{-2} \text{ s}^{-1}) \) is more rapid than \( k_{\text{dis}} \) for assays conducted in the absence or presence of GTP. Consequently, GTP hydrolysis is not the rate-limiting step in the dissociation reaction. The calculated \( K_d \) values for the SRP–SR complex in a physiological ionic strength buffer are not significantly different from each other (Table I) and are in reasonable agreement with the \( K_d \) value \( (K_d + \text{GTP} = 125 \text{ nM}) \) estimated using the GTPase assay (Connolly and Gilmore, 1993).

The nonhydrolyzable GTP analogue Gpp(NH)p has been used to stabilize the interaction between SRP and the SR (Connolly et al., 1991; Rapiejko and Gilmore, 1992). Extensive biosensor experiments conducted in the presence of the Gpp(NH)p are not feasible because the biosensor surface cannot be completely regenerated between data points by the high salt wash procedure used to dissociate the SRP–SR complex. However, several single point experiments were conducted to compare the dissociation of SRP from the SR in the presence of GTP, GDP, or Gpp(NH)p. Dissociation of SRP from the SR was greatly reduced when GTP hydrolysis was prevented (Fig. 5 D, compare curves a and b with c).

**Binding of ribosomes to the SR**

A ternary complex between the ribosome, the SRP, and the SR might involve direct contact between the ribosome and the SR. Possible interactions between the ribosome and the SR or the SR subunits were explored using the biosensor (Fig. 6 A). Ribosomes bind to immobilized bt-SR (Fig. 6 A, a) and bt-SR\(_{\alpha} \) (b) but not to bt-SRB\(_{\alpha} \) (c) or to bt-SR\(_{\alpha} \)N (e) in the physiological ionic strength buffer (150 mM K\(^+\)). The addition of GTP did not increase binding of ribosomes to SRB\(_{\alpha} \) (Fig. 6 A, d). Large ribosomal subunits (Fig. 6 A, g) but not small subunits (h), recapitulate the binding to the SR that is observed for the intact ribosome (f). Hyperbolic binding curves for the interaction between the ribosome and immobilized SR (Fig. 6 B) yielded a \( K_d \) value of \( 1.9 \pm 0.6 \) nM. The rate constants for the association \( (k_{\text{ass}}) \) and dissociation \( (k_{\text{dis}}) \) reactions were calculated from the \( k_{\text{ass}} \) plot (Fig. 6 B, inset) and are shown in Table I. Similar kinetic parameters were obtained for binding of the 60S subunit to bt-SR (Fig. 6 C and Table I).

Analyzing the kinetics of ternary complex formation using the biosensor was not feasible because the ligate solution \( (0.87 \pm 0.02 \text{ nM}) \) is in good agreement with the \( K_d \) value obtained using the biosensor. More importantly, the stoichiometry of binding was found to be roughly 1:1 based upon the experimentally determined \( B_{\text{max}} \) value and the concentration of SR in the proteoliposomes. Binding of 80S ribosomes to Sec61 proteoliposomes was analyzed as a control (Fig. 7 B), and the \( K_d \) value we obtained \( (5.4 \pm 0.9 \text{ nM}) \) was in good agreement with the previous literature (Kalies et al., 1994).

**Discussion**

**Dual recognition of SRP and ribosomes by the SR**

Despite the relatively low affinity between the SR and the SRP in a physiological ionic strength buffer, the SRP–RNC complex is efficiently targeted to the SR. Here, we have

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**Figure 7. Binding of ribosomes to SR and Sec61 proteoliposomes.** Increasing amounts of \(^{125}\text{I}-\)labeled 80S ribosomes \((0.03–0.65 \text{ pmol})\) were incubated with the SR proteoliposomes \((A)\) or Sec61 proteoliposomes \((B)\) in 50 mM TEA, 150 mM KOAc, 2.5 mM Mg(OAc)\(_2\), 1 mM DTT. Proteoliposome-bound ribosomes were separated from free ribosomes by gel filtration chromatography as described in the Materials and methods.
tested several possible mechanisms that could contribute to an enhanced affinity between the SR and an SRP–RNC that would promote the targeting reaction in isotonic or hypertonic buffers.

Previous studies using canine microsomes or SR proteoliposomes have indicated that GTP is not required for targeting of SRP–RNCs to the SR (Rapiejko and Gilmore, 1994; Song et al., 2000), and that the targeting step precedes cooperative, stable binding of GTP to SRP54 and SR\(\alpha\) (Rapiejko and Gilmore, 1997). Nonetheless these previous studies do not eliminate the formal possibility that the affinity of the SRP–RNC for the SR could be enhanced by rapidly reversible low-affinity binding of GTP to the empty site forms of SRP54 and SR\(\alpha\). As shown here, biosensor experiments designed to monitor the binding affinity between SRP and the SR demonstrated that the addition of GTP did not significantly increase the rate constant for formation of the SRP–SR complex in either isotonic or hypertonic buffers. As reported previously (Connolly et al., 1991), the nonhydrolyzable GTP analogue Gpp(NH)p stabilizes the SRP–SR complex by reducing the dissociation rate.

Could signal sequence–specific binding of SRP to an RNC cause a conformational change in SRP that enhances the affinity between SRP and the SR? This hypothesis was based upon the report that RNCs assembled in an in vitro translation system activate the GTPase activity of the SRP–SR complex (Bacher et al., 1996, 1999). Our analysis of this experimental system disclosed the remarkable finding that nontranslating ribosomes activate the GTPase activity of the SRP–SR complex. Furthermore, bona fide SRP–RNC complexes assembled using a secretory mRNA did not hydrolyze GTP when targeted to the SR proteoliposomes, consistent with our previous observation that both GTP and Gpp(NH)p stabilize the SR–SRP–RNC complex (Song et al., 2000).

The third hypothesis we considered was that the ribosome forms a platform for assembly of the SRP–SR complex. Purified 60S ribosomal subunits, but not 40S ribosomal subunits, stimulate the GTPase activity of the SRP–SR complex, consistent with the evidence that SRP54 binds to the L23a and L35 proteins in the large ribosomal subunit (Pool et al., 2002). Here, we obtained evidence that the SR has a high binding affinity for purified ribosomes or 60S ribosomal subunits. Notably, the rate constant for association of a ribosome–SR complex is 300-fold faster than the rate constant for formation of the SRP–SR complex. Consequently, the kinetics of targeting of the SRP–RNC complex to the SR should be dominated by the SR–ribosome interaction. The SR–ribosome interaction is also characterized by a relatively fast dissociation rate \((k_{\text{diss}} = 1.2 \times 10^{-7} \text{ s}^{-1})\). The rate of dissociation of SRP from the SR is less rapid, and this rate should decrease for the SRP–RNC complex. Binding of GTP to SRP54 and SR\(\alpha\) substantially increases the stability of the SRP–RNC–SR complex because GTP hydrolysis by SRP54 and SR\(\alpha\) is delayed until a vacant Sec61 complex is identified as an acceptor for the RNC complex (Song et al., 2000). We propose that the SR, by dual recognition of the ribosome and the SRP, will reject ribosomes that lack bound SRP.

The affinity between the SR and the ribosome appears to be conserved between eukaryotic and prokaryotic organisms. Depletion of either the translocon subunit SecE or the bacterial SRP (Ffh) leads to the in vivo accumulation of membrane-bound ribosome–FtsY complexes (Herskovits et al., 2002). The NH\(_2\)-terminal acidic (A) domain of FtsY, which is involved in membrane binding (de Leeuw et al., 1997), is not homologous to the NH\(_2\)-terminal 319 residues of SR\(\alpha\). Further work will be required to define the structural basis for the evolutionarily conserved interaction between the ribosome and the SR.

Roles for the SR subunits

The SR\(\beta\) subunit of the SR was dispensable for the GTPase activity of the SRP–SR complex. Complexes formed between FtsY, the prokaryotic equivalent of SR\(\alpha\), and Ffh–4.5S RNA, the prokaryotic equivalent of SRP54 and the 7S RNA, hydrolyze GTP in a cooperative manner that has been investigated as a paradigm for the SRP–SR complex (Powers and Walter, 1995; Jagath et al., 2000; Peluso et al., 2000), hence it was not surprising that SR\(\beta\) was dispensable for the GTPase cycles of SRP54 and SR\(\alpha\).

The observation that SR\(\beta\) does not hydrolyze GTP when assayed alone was not unexpected, as most GTPases have very low hydrolysis rates in the absence of GEFs and GTPase-activating proteins (GAPs) (Bourne et al., 1991). SR\(\beta\) does not hydrolyze GTP in the presence of 80S ribosomes, indicating that the ribosome cannot fulfill both the GEF and GAP functions for SR\(\beta\). Although photolabeling experiments had suggested that the ribosome acts as a GEF to stabilize a nucleotide-free form of SR\(\beta\) (Bacher et al., 1999), a more recent report does not support this conclusion (Legate and Andrews, 2003). Our GTPase assays do not address which step, or steps, in the SR\(\beta\) GTPase cycle occurs in the presence of the ribosome.

The SR\(\alpha\) domain of SR\(\alpha\) (residues 1–178) is necessary and sufficient for GTP-dependent heterodimerization with SR\(\beta\) (Young et al., 1995; Ogg et al., 1998; Legate et al., 2000; Schwartz and Blobel, 2003). The GTP-bound, but not GDP-bound, form of SR\(\alpha\) forms stable heterodimers with SRX (Schwartz and Blobel, 2003). In the absence of a currently unidentified SR\(\beta\) GAP, the SR\(\beta\) GTPase is thought to be catalytically inert when bound to SRX (Schwartz and Blobel, 2003). An alternative model for the SR\(\beta\) GTPase cycle proposes that GTP binding to SR\(\beta\) regulates the release of the signal sequence from SRP54 (Fulga et al., 2001).

SR\(\beta\) can be cross-linked to a 21-kD protein in the large ribosomal subunit (Fulga et al., 2001). Here, we observe an SR\(\beta\)-independent, high-affinity interaction between SR\(\alpha\) and the 60S subunit, suggesting that SR\(\alpha\) positions SR\(\beta\) adjacent to the 21-kD protein. As bt-SR\(\alpha\) and bt-SR have similar affinities for the ribosome, we conclude that SR\(\beta\) does not occlude the ribosome-binding site on SR\(\alpha\) nor does it enhance the affinity of the SR for the ribosome. Previous studies that analyzed the ribosome–SR\(\beta\) interaction have used either the recombinant SR heterodimer (Fulga et al., 2001) or trypsin-digested SR heterodimers that retain the NH\(_2\)-terminal fragment of SR\(\alpha\) (Bacher et al., 1999). The discrepancy between our results and these previous studies concerning the ribosome-binding and GTPase activities of
SRβ might be explained by these structural differences in the reagents.

The NH2-terminal domain of SRα that is sufficient for ribosome-binding activity is polar (64% charged or polar residues) and basic (pI = 9.16). GTase assays and biosensor experiments showed that SRP does not bind to bt-SRαΔN, despite the evidence that bt-SRαΔN is properly folded. It is unlikely that the NH2-terminal 151 residues of SRα are sufficient for the interaction of the SR with SRP, as the GTase cycle of the SRP–SR complex almost certainly requires direct contact between the N and G domains of SRP54 and SRα. A model for the Fh–FtsY complex (Montoya et al., 2000) predicts important interactions between the G domains of the two GTPases.

**Regulation of the SR–ribosome interaction**

Within the cell, a futile GTase cycle catalyzed by the SRP–SR complex is not favored due to the low affinity between the SR and free SRP. However, the discovery that the ribosome can promote assembly of the SRP–SR complex in isotonic buffers raises new questions about the in vivo regulation of the SRP–SR GTase cycle. A futile cycle involving SRP, the SR, and a ribosome would be restricted to the RER surface and would depend upon the presence of SR and SR that are not engaged in bona fide targeting reactions. The cellular concentration of SRP and the SR may be regulated to ensure that the GTPases are substoichiometric relative to membrane-bound ribosomes.

Nontranslating ribosomes do not compete with SRP–RNCs for targeting to the Sec61 complex (Raden and Gilmore, 1998). This observation strongly suggests that there must be a mechanism to prevent the SR from being saturated by 60S ribosomal subunits, or ribosomes that are not engaged in the synthesis of secretory proteins. Although the relatively rapid dissociation rate for the ribosome–SR complex may contribute to such a mechanism, we speculate that there are additional factors that destabilize the SR–ribosome complex by selectively accelerating the dissociation rate. Ribosomes bearing nascent polypeptides that are synthesized on cytoplasmic polysomes recruit ribosome-associated chaperones, including the nascent chain–associated complex and members of the Hsc70 family (Wang et al., 1995; Bukau et al., 2000). A role for the nascent chain–associated complex in preventing signal sequence–independent binding of RNCs to the translocation channel has been proposed (Lauring et al., 1995; Moller et al., 1998), but the mechanism remains a matter of controversy (Neuhof et al., 1998; Raden and Gilmore, 1998). Future experiments will address the possibility that cytosolic chaperones regulate the binding affinity between the SR and the ribosome.

**Materials and methods**

**Purification of the SRP, the SR, and the Sec61 complex**

RMs, KOAc-washed RMs (K-RM), trypsin-digested K-RM (TK-RM), SRP, and the SR were isolated from canine pancreas as previously described (Walter et al., 1981; Rapeioko and Gilmore, 1992; Connolly and Gilmore, 1993). Puromycin-high salt–extracted microsomes (PK-RM) and chymotrypsin-digested PK-RM (C-PK-RM) were characterized previously (Song et al., 2000). The canine Sec61 complex was purified from PK-RM by a modification of the method of Görlich and Rapoport (1993) using glycerol gradient centrifugation and anion and cation exchange chromatography.

**Isolation of ribosomes, ribosomal subunits, and RNC complexes and radiiodination of ribosomes**

Ribosomes were isolated from canine RM by extraction with high salt as previously described (Collins and Gilmore, 1991). Residual SRP was separated from 80S ribosomes by two sequential centrifugations through a high salt–sucrose cushion (Collins and Gilmore, 1991) followed by centrifugation through a physiological salt–sucrose cushion and resuspension of the ribosomes in buffer A (50 mM triethanolamine-acetate [TEA], pH 7.5, 150 mM KOAc, 5 mM Mg(OAc)2, 1 mM DTT). Canine 80S ribosomes were disassociated into 40S and 60S subunits by treatment with 1 mA Puromycin in buffer A, after which the sample was applied to a 14-ml 10–30% sucrose gradient in 50 mM TEA, 500 mM KOAc, 5 mM Mg(OAc)2, 1 mM DTT. The ribosomal subunits were resuspended by centrifugation for 4.5 h at 200,000 g, using a Beckman Coulter SW40 rotor.

Sucrose gradient–purified 80S ribosomes (Raden et al., 2000) were re-suspended in DTT-free buffer A and labeled with 125I Bolton-Hunter reagent (Amersham Biosciences) as previously described (Raden et al., 2000). Proteoliposomes were prepared as previously described (Song et al., 2000) using a modification of the method of Görlich and Rapoport (1993). Binding of 125I-labeled ribosomes to proteoliposomes was assayed as previously described (Raden et al., 2000). In brief, 0.03–0.6 pmol of 125I-labeled ribosomes was incubated with aliquots of the proteoliposomes in buffer A. The 25-μl sample was applied to a 1.2-ml Sepharose CL-2B column equilibrated in buffer A to resolve proteoliposome-bound ribosomes (0.3–0.6 ml of eluate from unbound ribosomes (0.6–1.3 ml of eluate). Truncated mRNAs encoding the NH2-terminal 86 residues of PK (pPL86), 64 residues of vesicular stomatitis virus glycoprotein (pG64), 77 residues of firefly luciferase (fluc77), or 156 residues of bovine opsin (op156) were prepared as previously described (Rapiejko and Gilmore, 1994). SR–RNC–opt156 complexes were assembled in a reticulocyte lysate reaction as previously described (Rapiejko and Gilmore, 1997) and adjusted to 375 μM cycloheximide to block further translation. Membrane integration and N-linked glycosylation of op156 were assayed as previously described (Rapiejko and Gilmore, 1997).

RNC complexes bearing pL86, pG64, or fluc77 were assembled by translating truncated mRNAs for 15 min in a wheat germ reaction that lacked radioisotopic amino acids and SRP, unless noted otherwise. After blocking further translation by the addition of 2 mM cycloheximide, the translation products were adjusted to 500 mM KOAc before centrifugation for 1 h at 400,000 g, at 4°C through a high salt–sucrose cushion (1 M sucrose, 25 mM Heps-KOH, pH 7.8, 500 mM KOAc, 5 mM Mg(OAc)2, 1 mM cycloheximide, 1 mM DTT). The RNCS were resuspended in half of the volume of the translation buffer B (25 mM Heps-KOH, pH 7.8, 5 mM Mg(OAc)2, 1 mM cycloheximide, 1 mM DTT) adjusted to 500 mM KOAc and resolubilized by centrifugation as described above. Finally, the RNCs were resuspended in buffer B adjusted to 150 mM KOAc at a concentration of 1 μM ribosomes.

**GTase assays**

GTase assays were conducted at 25°C in a total volume of 5 μl and contained 25–50 nM SR (canine SR, recombinant SR, or SR subunits), 50 mM SRP, 140 mM RNCs or mock RNCs, and 0.5 μM [γ-32P]GTP (410 Ci/mmol) in buffer C (50 mM TEA-OAc, 150 mM KOAc, 5 mM Mg(OAc)2, 2 mM DTT, 2 mM cycloheximide) unless noted otherwise. The detergent micelle GTase assays contained 0.1% Nidol. Aliquots of the GTase assays were removed at frequent time intervals and spotted onto PEI-cellulose thin layer plates to resolve GDP from GT (Connolly and Gilmore, 1993).

**Expression and purification of SR and SR subunits**

DNA encoding a canine SRβ derivative lacking the NH2-terminal 54 residues (SRβΔN) was obtained by PCR amplification of the SRβ plasmid pMAC455 (Young et al., 1995) using appropriate primers and standard PCR conditions. The SRβΔN coding sequence was inserted into the PinPoint vector (Promega) to obtain pbt-SRβΔN. The dicistronic plasmid pbt-SRβ–SRα encoding bt-SRβ and bt-SRα was constructed by inserting the SRα coding sequence derived from plasmid pG4N (Rapiejko and Gilmore, 1994) into pbt-SRβΔN. The plasmids pbt-SRβ and pbt-SRα encode fusion proteins between the biotinylation domain and canine SRα or canine SRα lacking the NH2-terminal 151 residues, respectively. The SRβΔN sequence was subcloned into pET11b (Novagen) to obtain pHis-SRβ, Het-
erodimers consisting of His-SR and NH2-terminal fragments of SRa (SRa121 or SRa139) were expressed from dicistronic plasmids. All constructs were verified by DNA sequencing. The biotinylated proteins were purified from the E. coli (p109) lysates by affinity chromatography (Soft-Avidin resin; Promega) and anion and cation exchange chromatography. The His-tagged proteins were purified from E. coli (Rosetta; Novagen) lysates by Ni-NTA (Qiagen) affinity chromatography and cation exchange chromatography.

**IAsys affinity sensor experiments**

Binding of SRP or ribosomes to the SR or subunits was assayed using an IAsys affinity sensor (Affinity Sensors). The binding surface was constructed by incubating saturating amounts of streptavidin (Promega) with a biotin-coated cuvette for 5 min. After a brief wash with buffer D (50 mM TEA, 150 mM KOAC, 2.5 mM Mg(OAc)2, 0.1% Nikkol) the bi-SR or a bi-SR subunit was added and incubated until equilibrium binding was observed (5 min). Preparation of the sensor surface was followed by a brief wash with buffer D. Binding time courses were performed at 25°C using a variety of ligates (SRP, ribosomes, or ribosomal subunits) in buffers D or E (buffer D with KOAc reduced to 50 mM) in either the absence or presence of 25 μM GTP. The ligate was preincubated for 2 min at 25°C (with GTP when appropriate) before the addition to a cuvette containing the immobilized bi-protein. Analysis of binding experiments showed that 6.5 min was sufficient to calculate equilibrium binding values. After binding, the cuvette was rapidly washed three times with buffer D or E (with or without GTP), and dissociation of ligate was monitored for 3 min. After binding, the surface was regenerated by dissociating residual ligate with buffer F (50 mM TEA, 300 mM KOAc, 5 mM Mg(OAc)2, 0.1% Nikkol). The cuvette was then washed and reequilibrated in buffer D or E containing GTP as indicated. The high salt wash procedure removes the ligate without damaging or detaching the bi-protein. Dissociation of Gpp(NH)p–stabilized bi-SR–SRP complexes was incomplete.

Binding of ligate to the sensor surface is measured as a response (arc seconds of change in the refractive index), which corresponds to the accumulation of mass within the optical window at the binding surface. The extent (in arc seconds) refers to the calculated maximum response (Rmax) at equilibrium for a given concentration of ligate. The rates of ligate binding (koff) and the extent (Rmax) were calculated from association curves using FASTfit software supplied with the instrument.

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**References**


