Signal Recognition Particle Binds to Ribosome-bound Signal Sequences with Fluorescence-detected Subnanomolar Affinity That Does Not Diminish as the Nascent Chain Lengthens*

Received for publication, January 8, 2003, and in revised form, March 4, 2003
Published, JBC Papers in Press, March 5, 2003, DOI 10.1074/jbc.M300173200

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The binding of signal recognition particle (SRP) to ribosome-bound signal sequences has been characterized directly and quantitatively using fluorescence spectroscopy. A fluorescent probe was incorporated cotranslationally into the signal sequence of a ribosomes-nascent chain complex (RNC), and upon titration with SRP, a large and saturable increase in fluorescence intensity was observed. Spectral analyses of SRP and RNC association as a function of concentration allowed us to measure, at equilibrium, $K_d$ values of 0.05–0.38 nM for SRP-RNC complexes with different signal sequences. Competitive binding experiments with nonfluorescent RNC species revealed that the nascent chain probe did not alter SRP affinity and that SRP has significant affinity for both nontranslating ribosomes ($K_d = 71$ nM) and RNCs that lack an exposed signal sequence ($K_f = 8$ nM). SRP can therefore distinguish between translating and nontranslating ribosomes. The very high signal sequence-dependent SRP-RNC affinity did not decrease as the nascent chain lengthened. Thus, the inhibition of SRP-dependent targeting of RNCs to the endoplasmic reticulum membrane observed with long nascent chains does not result from reduced SRP binding to the signal sequence, as widely thought, but rather from a subsequent step, presumably nascent chain interference of SRP-RNC association with the SRP receptor and/or translocon.

In mammalian cells, ribosomes are found both in the cytoplasm and at the membrane of the endoplasmic reticulum (ER). These two classes of ribosome differ in the nature of their translation products, with membrane-bound ribosomes synthesizing secretory or membrane proteins that are being translocated across or integrated into the ER membrane cotranslationally. The structural feature that distinguishes the cytoplasmic ribosomes from the membrane-bound ribosomes is the presence in the latter of a nascent chain that contains a signal sequence. When a nascent chain signal sequence emerges from the ribosome, it is recognized and bound by a ribonucleoprotein termed the signal recognition particle (SRP) (for review, see Ref. 1). The binding of SRP to the signal sequence-containing ribosome-nascent chain complex (RNC) temporarily prevents it from synthesizing protein. The resulting “elongation-arrested complex” then diffuses to the ER membrane where a GTP-dependent interaction with the SRP receptor initiates a series of events which includes the binding of the RNC to the site of cotranslational translocation and integration at the ER membrane (the translocon), the release of the signal sequence from the SRP, the release of SRP and the SRP receptor from the translocon, and the resumption of protein synthesis by the ribosome (for review, see Refs. 1–3). After targeting is complete, nascent chain translocation or integration then proceeds at the translocon (for review, see Ref. 4).

SRP therefore has a critical regulatory role in the cell because it is responsible both for the proper trafficking of newly synthesized proteins and for the conversion of a cytoplasmic ribosome to a membrane-bound ribosome. In doing so, SRP functions in multiple ways to effect RNC targeting to the ER membrane, including the selection of appropriate RNCs, the regulation of RNC translation, and specific interactions with the SRP receptor and translocon at the membrane. Although much progress has been made, and models are abundant, the molecular mechanisms that accomplish each of these functions are still largely undefined experimentally. The necessity to discriminate accurately between ribosomes with and without signal sequences is especially interesting because there is no consensus for the sequences that can serve as signal sequences (5). The signal sequence binding site on SRP is therefore promiscuous, yet it must simultaneously be exceptionally accurate to avoid improper trafficking of the nascent chains. These conflicting demands are most likely resolved thermodynamically by discrimination between satisfactory and unsatisfactory sequences which is based on the binding affinity between the SRP and the putative signal sequence. This model would predict that legitimate signal sequences in RNCs would bind much more tightly to SRP than would other nascent chain sequences.

Soon after the discovery of SRP, Walter and colleagues (6) examined the binding of SRP to ribosomes with nascent chains...
that either contained or lacked signal sequences. Their experiments yielded an estimate of $K_0$ less than 8 nM for SRP bound to an RNC with a preproactin (pPL) nascent chain, and a $K_d$ less than 50 μM for nontranslating ribosomes. Because these binding affinities were estimated using a nonequilibrium sedimentation velocity technique, the authors noted that the above values represent the minimal binding affinities. Thus, these early experiments demonstrated a high affinity interaction between SRP and RNCs which was signal sequence-dependent, but no rigorous thermodynamic analysis of these interactions has been done.

Elongation arrest was also identified early as a property of SRP when experiments revealed that SRP inhibits translation of secretory proteins, but not cytoplasmic proteins, in the absence of ER microsomes (1, 6). Because some nascent chains become translation-incompetent when they become too long, elongation arrest was proposed as a mechanism for slowing translation and thereby prolonging the time during which an RNC could be targeted successfully to the ER membrane. For example, the targeting of RNCs with pPL nascent chains to ER microsomes was shown to be abolished when the nascent chain exceeded 140 residues in length, presumably because SRP affinity for the RNC had been reduced by the lengthening and folding of the nascent chain which interfered with SRP access to the signal sequence (7).

We have here employed fluorescence techniques to monitor directly the association of SRP with various RNC complexes. This approach has allowed us to quantify for the first time the interactions of SRP with the signal sequence, the nascent chain, and the ribosome at equilibrium, and hence to obtain accurate measurements of the $K_0$ values for complexes containing SRP. The resulting data reveal that some long standing views about the mechanisms involved in SRP-dependent ribosome selection and targeting need to be revised. The following new conclusions are among those revealed by the fluorescence experiments. The RNC-SRP $K_0$ is more than 100-fold smaller than estimated previously; SRP has different affinities for different signal sequences; SRP binds more tightly to translating ribosomes than to nontranslating ribosomes because of a conformational change in the ribosome that is recognized by the SRP; and SRP binds with very similar affinity to signal sequence-containing RNCs with short and long nascent chains. Hence, in contrast to current dogma, the inability of RNCs with long nascent chains to target to the ER membrane is not the result of a failure of SRP to bind to the signal sequence.

**EXPERIMENTAL PROCEDURES**

**Plasmids and mRNA—**Plasmids coding for golden hamster BiP, rat preproinsulin I (pPI), and chimpanzee/h9251 (h9280)/NBD-Lys-tRNALys) were purified as described previously (13) using either plasmid DNA cut in the coding region with a particular restriction endonuclease or PCR-produced derivatives with a single base change in the anticodon which converted the tRNA$	extsuperscript{Lys}$ into a tRNA that recognizes the amber stop codon (generously provided by Dr. Greg Beckler, Promega Corp.; here termed tRNA$	extsuperscript{Amber}$) were synthesized in vitro using T7 RNA polymerase as described earlier for SRP RNA (16). The resulting RNA samples were purified by anion exchange chromatography using a Pharmacia FPLC equipped with a Mono Q HR 10/10 column. The RNA was eluted in 10 mM NaOAc (pH 4.5), 5 mM MgCl$	extsubscript{2}$ with a 115-mm linear gradient of NaCl from 0.48 to 1.0 M. The fractions containing functional tRNA$	extsuperscript{Amber}$ were detected by aminoclayation assays (18), except that MgCl$	extsubscript{2}$ was at 6 mM, and no KCl was added; most of the tRNA$	extsuperscript{Amber}$ eluted near 0.55 M NaCl. The fractions with the highest tRNA$	extsuperscript{Amber}$ content were aminoclayated with $[^{14}C]$$\text{Lys}$, chemically modified with NBD, and stored as described previously with the above changes (17). Using this procedure the suppressor tRNAs have exhibited excellent suppression efficiencies with a variety of mRNAs, often exceeding 50% even for amber codons located far into the coding sequence (data not shown). As seen previously (17), the NBD modification has no detectable effect on the ability of Lys-tRNA$	extsuperscript{Amber}$ to function in protein synthesis.

**Translation Intermediates—**Truncated mRNAs were translated for 25 min at 26 °C in a wheat germ extract as described previously (17, 19, 20). Translations (250 or 500 μl total volume) contained 0.60 pmol/μl of either eNBD-$[^{14}C]$$\text{Lys}$-tRNA$	extsuperscript{Amber}$ or unmodified $[^{14}C]$$\text{Lys}$-tRNA$	extsuperscript{Amber}$. Following translation, RNCs were purified by gel filtration at 4 °C using a Sepharose CL-6B column (1.5-cm inner diameter × 20 cm) and an elution buffer containing 50 mM HEPES (pH 7.5), 1.40 mM Mg(OAc)$\textsubscript{2}$, 5 mM MgCl$	extsubscript{2}$, and 1 mM dithiothreitol (Buffer A). A slow flow rate was used during gel filtration to ensure the removal of noncovalently bound fluorophores (see Ref. 17). The radioactivity and A$	extsubscript{280}$ of a 70-μl aliquot of each 550-μl fraction were used to identify those fractions containing the RNCs that elute in the void volume, and only the leading half of the void volume peak was pooled.

For competitive binding experiments, nontranslating ribosomes were purified by incubating wheat germ extract with 2 mM puromycin for 15 min at 26 °C and then purifying the ribosomes by gel filtration as above. Nonfluorescent RNCs were prepared as above, except that translations included unmodified $[^{14}C]$$\text{Lys}$-tRNA$	extsuperscript{Amber}$ instead of NBD-labeled Lys-tRNA$	extsuperscript{Amber}$. Prior to addition to NBD-RNC samples, nonfluorescent and nonfluorescent RNCs were concentrated using a Centricon YM-30 (Millipore, Bedford, MA).

**Targeting of RNCs to the ER Membrane—**Translations (25 μl) of various RNCs were performed as before without added tRNA and with $[^{35}S]$$\text{Met}$ for nascent chain detection. After 25 min at 26 °C, cycloheximide and then SRP were added to 1 mM and 40 mM, respectively. Following another 10-min incubation at 26 °C, 3-4 eq of high salt- and EDTA-washed microsomal membranes were added (14). After incubating for 5 min at 26 °C in the presence of membranes, each sample was layered on a 50-μl cushion (0.5 M sucrose in Buffer A) and sedimented for 3 min at 4 °C and 20 p.s.i. in a Beckman Airfuge (30 4680 rpm). The supernatants were resuspended in 100-μl sample buffer, whereas the supernatants were precipitated in acid before resuspension in sample buffer. The protein contents of the membrane and supernatant pellets were analyzed on a 10–15% SDS-polyacrylamide gradient gel, and radioactivity was detected and quantified using a Bio-Rad FX PhosphorImager.

**Fluorescence Spectroscopy—**Steady-state fluorescence intensity was monitored using an SLM 8100 photon-counting spectrofluorometer with a two-grating excitation monochromator, a single grating emission monochromator, and a 450-watt xenon lamp. Samples were excited at 468 nm (4-nm bandpass), and emission was detected at 530 nm (4-nm bandpass). All spectral measurements were done at 4 °C in Buffer A using 4 × 4-mm quartz microcuvettes. Sample mixing in these cuvettes was complicated as described previously (22). A 2-μl sample compartment of the fluorometer was flushed with a stream of N$	extsubscript{2}$ to minimize condensation on the cold cuvettes. After each addition of titrant and mixing, no spectral measurements were made for at least 10 min. This delay was sufficient to ensure that the samples had reached equilibrium in terms of both ligand binding and temperature and that all noncovalent association had equilibrated. To prevent RNC association with the walls of the cuvette during the titration, the interiors of the cuvettes were coated with purified phosphatidylcholine (22, 23).

For spectroscopic analysis, 250-μl samples of purified NBD-labeled RNCs (usually 1 μM) in Buffer A were titrated at 4 °C by the sequential addition of known amounts of SRP in small volumes. After each addition, the emission intensity of the NBD and blank samples were measured after reaching equilibrium. After blank subtraction and then dilution correction, the net NBD emission intensity (F) at each point in the titration was compared with the initial intensity ($F_0$) of the sample in the absence of SRP. At the end of each titration, the amount of
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[14C]Lys in each cuvette was measured to determine the actual concentration of nascent chains. To correct for the significant background signal (mostly light scattering) observed with samples lacking NBD, we initially performed two translations in parallel, one with eNBD-Lys-tRNA and one with Lys-tRNA to serve as a blank. NBD and blank samples with equivalent background signals were then created from the separate RNC pools by equating (by dilution) either their absorptions at 260 nm (because ribosomes are responsible for the observed light scattering in these samples) or their emissions at 485 nm (λex = 468 nm) (17). The signal of the blank was then routinely subtracted from the signal of the fluorescent sample to yield the net NBD emission intensity. In later experiments, blank samples contained only ribosomes that had been purified by the gel filtration of wheat germ extract (the same NBD sample used in the NBD translation) because the presence of residual tRNA, mRNA, nascent chains, and SRP after purification and titration did not detectably alter the amount of light scattering.

Data Analysis—The total concentration of NBD-RNCs in a purified sample was determined directly by counting the [14C]Lys present in the solution using a scintillation counter and experimentally determined counting efficiencies. The number of RNCs equaled the number of [14C]Lys in all but the pPLss samples because there is one eNBD-[14C]Lys/ribosome-bound nascent chain when the probe is incorporated using the amber suppressor. In the case of pPLss, the number of nascent chains was twice the number of [14C]Lys because each pPLss nascent chain contains two lysines, and only one-fourth of the incorporated lysines are eNBD-[14C]Lys because of the competition between eNBD-[14C]Lys-tRNAos and endogenous Lys-tRNAos (13). The ribosome concentration was determined by absorbance at 260 nm (1 A260 unit = 23.1 pmol (24)).

We have previously derived equations to analyze the competitive binding of a ligand to two alternate receptors, one of which is labeled with a fluorescent probe. The dissociation constant for the unlabeled competing receptor is obtained by analysis of the dependence of a spectral change on the concentrations of ligand and receptors (25–28). Here, because every sample contains more than one ribosomal species that can bind to SRP, the titration data have been analyzed using either the cubic equation (Equation 2), which describes the spectral change to two simultaneous receptor binding equilibria, or the quartic equation (Equation 10), which was derived for three competing equilibria (see Fig. 1B). In both cases, fluorescence data were expressed as the fractional change in the initial fluorescence

\[ rac{\Delta F}{F_0} = (F - F_0)/F_0 \]  

(Eq. 1)

where \( F \) is the net initial emission intensity of NBD-RNCs and \( F_0 \) is the net accurately corrected intensity observed at a given SRP concentration. Fluorescence studies of SRP binding to NBD-RNCs with nontranslating ribosomes as a competitor were performed by measuring the fluorescence change in the presence of excess nontranslating ribosomes. NBD-RNCs at a total concentration of [N], in the sample and a large excess of nontranslating ribosomes at a total concentration of [R] were titrated with SRP, and the change in NBD-RNC emission was measured as a function of the total SRP concentration ([SRP]). Data from these titrations and titrations with no excess ribosomes were analyzed using the cubic equation (Equation 2) to obtain the maximum fluorescence change (\( \Delta F_{\text{max}}/F_0 \)) and the dissociation constants for the SRP-NBD-RNC (Kc) and the SRPribosome (Kd) complexes that best fit the data. For the nonlinear least squares analyses, the parameters (Kc, Kd, and \( \Delta F_{\text{max}}/F_0 \)) were fit with the stoichiometric factors n and r, the number of SRP binding sites/NBD-RNC or nontranslating ribosome, respectively, set to 1, an assumption that fits the titration data very well.

Although the cubic equation has been published previously using different nomenclature (28), the equation with our nomenclature is shown below (Equation 2), where the fraction of sites on NBD-RNC occupied by SRP is \( \Delta F_{\text{max}}/F_0 \),

\[ \frac{D_1(\Delta F_{\text{max}})^3 + D_2(\Delta F_{\text{max}})^2 + D_3(\Delta F_{\text{max}}) + D_4 = 0}{D_1 = n[N][K_c - K_c]/K_c} \]  

(Eq. 3)

\[ D_1 = n[N][K_c - K_c]/K_c \]  

(Eq. 3)

\[ D_2 = [S][2K_c - K_c] + n[N][K_c - K_c] + r[R] \]  

(Eq. 4)

\[ D_3 = [S][2K_c - K_c] + n[N][K_c - K_c] + r[R] \]  

(Eq. 5)

\[ D_4 = [S][K_c - K_c] \]  

(Eq. 6)

For competitive ligand binding to three receptors, one of which is fluorescent, a quartic equation was derived from the equilibrium constants and conservation equations where the SRPribosome concentration is termed [RS], the SRP-nonfluorescent RNC concentration is designated [US], the SRP-NBD-RNC concentration is termed [NS], and \( \Delta F_{\text{max}}/F_0 = [NS]/[n[N]_0] \). In addition to the parameters in the cubic equation, the quartic includes Kd (Kd for SRP-nonfluorescent RNC) and u (number of SRP binding sites/nonfluorescent RNC).

\[ K_c = (n[N]_0 - [NS])/(r[S]_0 - [RS] - [US]_0)/[NS] \]  

(Eq. 7)

\[ [RS] = K_c/[NS]_0/[R]/u/[N]_0[K_c + (NS)/K_c - K_c]) \]  

(Eq. 8)

\[ [US] = K_c/[NS]_0/[u]/[N]_0[K_c + (NS)/K_c - K_c]) \]  

(Eq. 9)

Substitution of Equations 8 and 9 into Equation 7 and rearrangement gave Equation 10 for \( \Delta F_{\text{max}}/F_0 \), as a function of the total concentrations of all of the interacting species and binding parameters. For the nonlinear least squares analyses, the dissociation constants for the various SRP-ribosomal species and the maximum change in emission intensity (\( \Delta F_{\text{max}}/F_0 \)) were fit simultaneously by the quartic equation (Equation 10) with the stoichiometric factors (n, r, and u) fixed at 1.

\[ m_1(\Delta F_{\text{max}})^4 + m_2(\Delta F_{\text{max}})^3 + m_3(\Delta F_{\text{max}})^2 + m_4(\Delta F_{\text{max}}) + m_5 = 0 \]  

(Eq. 10)

\[ m_1 = n[N]_0 \]  

(Eq. 11)

\[ m_2 = n[N][C_0] + C_0 - 1 - [S]_0 - K_c + r[R]/u/[U]_0/C_0 \]  

(Eq. 12)

\[ m_3 = n[N]/[C_0]C_0 - C_0 - 1 - [S]_0/C_0 + C_0 - 1 - K_c/C_0 + C_0 \]  

(Eq. 13)

\[ m_4 = -n[N]/[C_0]C_0 + [S]_0/C_0 + C_0 - C_0/C_0 \]  

(Eq. 14)

The validity of the quartic equation was confirmed by finding indistinguishable fits to the data by a model in which the expressions for the equilibrium constants and conservation equations were solved simultaneously by SCIENTIST software (Micromath Software, Salt Lake City, UT).

The above equations provide exact solutions for the various Kc values and \( \Delta F_{\text{max}}/F_0 \) that are valid for all concentrations of the interacting species. These exact equations are necessary for the analysis of tight binding interactions such as those studied here, where simplifying assumptions regarding equivalence of free and total concentrations cannot be made. The equations are generally applicable to the analysis of interactions at equilibrium where one ligand binds independently (noncooperatively) to two (Equation 2) or three receptors (Equation 10), one of which is fluorescent. All nonlinear least squares analyses were performed with the SCIENTIST program. All reported estimates of error represent ± 2 S.E.M.

The Kd values for SR complexes with NBD-pPLss-RNC and nontranslating ribosomes were determined by combining all of the individual titration data sets in the presence and absence of excess free ribosomes and analyzing them simultaneously by nonlinear least squares fitting of the cubic equation using the SCIENTIST fitting program (28). Similarly, the data from multiple independent experiments were combined and analyzed simultaneously to determine the Kd values using the quartic equation. Many different preparations of purified SR and translation components have been used in our experiments over several years, and we observed little dependence of the Kd values on sample origin.

RESULTS

Experimental Approach

Equilibrium Kd Values Determined Using Fluorescence Spectroscopy—To determine most accurately the affinity of SRP for ribosomes with various nascent chains, it is necessary to meas-
ure the $K_d$ of each complex at equilibrium. Nonequilibrium techniques estimate the extent of complex formation in a sample by first separating the complex from the unbound species and then measuring the amount of complex. $K_d$ values calculated from such data are typically much higher than the true dissociation constants because the complex dissociates during the separation process. For example, we found that $K_d$ values for aminoacyl-tRNA-EF-Tu-GTP ternary complexes determined using nonequilibrium techniques were 10–1,000-fold higher than the actual equilibrium dissociation constant (26, 27). Because nonequilibrium methods may substantially underestimate SRP affinity for signal sequences and ribosomes, we have chosen to measure $K_d$ values at equilibrium.

The optimal approach for quantifying the amounts of bound and unbound species in a sample at equilibrium is to use a spectroscopic technique that can distinguish between the bound and free species without separating them. Here we positioned a fluorescent probe in the signal sequence with the expectation that SRP association with the signal sequence would alter the environment of the fluorophore and hence its fluorescence signal. As shown below, this expectation was realized, and we were able to measure directly the fraction of signal sequences bound to SRP in a sample. Another advantage of using a nondestructive spectroscopic technique to monitor binding is that the sample can be examined over time to ensure that the spectral signal has reached a constant value and hence that the sample is at equilibrium.

$K_d$ values are determined experimentally from the concentration dependence of complex formation, and hence it is necessary to examine samples with measurable amounts of both free and bound RNCs and SRP. Because Walter et al. (6) showed that the $K_d$ for SRP binding to signal sequence-containing RNCs was less than 8 nM, significant amounts of both free and bound species would be observed only in samples containing nanomolar concentrations of RNCs and SRP. Fluorescence is the only acceptable choice for this study because it is the only spectroscopic technique that can detect and measure probe concentrations that are nanomolar or lower.

**Selective Labeling of the Nascent Chain**—To position a fluorescent probe solely in nascent chains (which constitute much less than 1% of the total protein in our samples), a fluorescent labeled amino acid must be incorporated into the nascent chain as it is being synthesized by the ribosome. This requires a functional aminoacyl-tRNA analog whose amino acid has been chemically modified, an approach that was originated by us (29). Here we have used the well characterized $\epsilon$NBD-Lys-tRNA<sup>25</sup>* to incorporate NBD probes into nascent chains (17). $\epsilon$NBD-Lys is incorporated into polypeptide at the same rate and to the same extent as unmodified Lys, so the translation machinery does not discriminate against the abnormally large amino acid. Yet $\epsilon$NBD-Lys-tRNA<sup>25</sup>* must compete with endogenous Lys-tRNA for incorporation at Lys codons in the mRNA, and hence only about 25% of the lysines incorporated in an in vitro translation are $\epsilon$NBD-Lys (13). This competition complicates the determination of the number of nascent chains in a sample because only the $\epsilon$NBD-[<sup>14</sup>C]Lys are radioactive. Hence, we have also used a tRNA that can be aminoacylated with Lys and modified with NBD but that recognizes an amber stop codon during translation. This amber suppressor tRNA, $\epsilon$NBD-Lys-tRNA<sup>amb</sup>, incorporates its tRNA wherever an amber stop codon has been introduced into the mRNA, and only termination factors compete for decoding the stop codon. Because termination factor action releases the nascent chain from the ribosome, the total number of ribosome-bound nascent chains in an RNC sample programmed with an amber stop codon-containing mRNA is equal to the number of incorporated $\epsilon$NBD-[<sup>14</sup>C]Lys residues, and every RNC contains an NBD dye. This approach allows us to examine SRP-signal sequence interactions in the context of the ribosome using ribosome-bound nascent chains rather than free signal sequence-containing peptides. Also, because only functional ribosomes will be able to synthesize a nascent chain, every $K_d$ measurement involves only functional RNCs.

**Homogeneous RNCs**—Homogeneous RNC samples were created using mRNAs that were truncated at a specific site within the coding sequence. Translation of such an mRNA proceeds normally until the ribosome reaches the end of the truncated mRNA. Because there is no stop codon, the ribosome does not dissociate from the mRNA but instead remains associated with the peptidyl-tRNA and its nascent chain to create a translation intermediate (RNC) with a nascent chain length that is dictated by the length of the truncated mRNA (e.g., (13)). To confirm that every RNC in our samples had the same length of nascent chain, samples were examined by SDS-PAGE after gel filtration, and a single band containing 90–100% of the $\epsilon$NBD-[<sup>14</sup>C]Lys-labeled nascent chains was observed with the expected molecular mass (data not shown).

RNCs were prepared using plasmids coding for the proteins shown in Fig. 1A. In one, the pPL signal sequence was fused to a 109-residue stretch of polypeptide which did not contain any lysines; this construct was designated pPL<sub>109</sub>. When pPL<sub>109</sub> mRNAs were translated in the presence of $\epsilon$NBD-Lys-tRNA<sup>25</sup>*, $\epsilon$NBD-Lys was incorporated only into positions 4 and 9 of the nascent chain where lysines are located in the native pPL sequence. In a different construct, here termed pPL<sub>C</sub>, a codon in the middle of the hydrophobic core of the pPL signal sequence was converted into an amber stop codon. An uncharged and nonpolar $\epsilon$NBD-Lys was then incorporated into position 18 of the pPL signal sequence when mRNAs of this pPL<sub>C</sub> derivative were translated in the presence of $\epsilon$NBD-Lys-tRNA<sup>amb</sup>.

**Competitive Binding Experiments**—$K_d$ values for complexes containing SRP and an unmodified, nonfluorescent RNC can be determined spectroscopically using competitive binding experiments. In this approach, the fluorescent RNCs serve solely to quantify the distribution of SRP within the sample. When SRP is added to a sample (a cuvette) containing both unmodified RNC and NBD-labeled RNC (NBD-RNC) complexes, two competing binding equilibria are established which reflect the relative affinities of SRP for RNC and NBD-RNC. Because the amount of SRP-NBD-RNC in a sample is given directly by the magnitude of the observed spectral change, the extent of competition by the nonfluorescent RNC for binding to SRP is given by the extent to which the RNC reduces SRP-NBD-RNC complex formation (i.e., lowers the magnitude of the spectral change). After calculating the distribution of SRP in the sample (bound to NBD-RNC, bound to RNC, or free), the $K_d$ value for the nonfluorescent SRP-RNC complex is determined from this distribution and the spectroscopically determined $K_d$ for SRP-NBD-RNC. The equations representing the two equilibria for competitive SRP binding to RNC and to NBD-RNC can be solved simultaneously because the free SRP concentration is the same for each equation. This yields a cubic binding equation as an exact solution, relating the observed fluorescence change to the known total concentrations of the components and the binding parameters. This equation is required to analyze high affinity interactions where assumptions about the equivalence of free and total component concentrations cannot be made.

In the present case, the weak binding of SRP to nontranslating ribosomes (6) further complicates matters. Because many ribosomes (50–75%) in in vitro translations do not synthesize protein and because our purification procedures do not

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separate RNCs from nontranslating ribosomes, any binding of SRP to nontranslating ribosomes would reduce the amount of SRP available for binding to RNCs. Thus, there may actually be three species vying for SRP binding at equilibrium, each with its own binding affinity. This is illustrated in Fig. 1B, where the $K_d$ values for the NBD-RNC, unlabeled (nonfluorescent) RNC, and nontranslating ribosomes are given by $K_n$, $K_u$, and $K_r$, respectively. Because the free SRP concentration is the same for each of the three equilibrium equations that hold simultaneously, and the distribution of SRP between the ribosomal species is determined by their relative $K_d$ values. NBD-labeled RNCs are depicted with a fluorescent probe (small black circles) incorporated in the signal sequence (black sawtooth lines) of the nascent chain (black lines), whereas unlabeled RNCs are shown without the fluorescent dye. Nontranslating ribosomes are shown without a nascent chain. Although unlabeled signal sequence-containing RNC is shown in the figure as the competitor, the same competitive binding approach may be used to determine the dissociation constant for SRP binding to other ribosomal species (e.g., an unlabeled globin RNC that lacks a signal sequence).

Fig. 1. Protein constructs and competitive binding experiments. A, the protein constructs used to form RNCs in this study are shown schematically with signal sequences in gray. Solid lines indicate the position of either lysine codons (pPLss) or amber stop codons (pPL, pPI, pBiP, globin) used to incorporate eNBD-Lys. B, competitive binding equilibria. When SRP (light gray ovals) is titrated into a sample containing three different ribosomal species, the extent of SRP binding to each reflects the affinity of SRP for each ribosomal species. At equilibrium, the free SRP concentration will be the same for each of the three equilibrium equations that hold simultaneously, and the distribution of SRP between the ribosomal species is determined by their relative $K_d$ values. NBD-labeled RNCs are depicted with a fluorescent probe (small black circles) incorporated in the signal sequence (black sawtooth lines) of the nascent chain (black lines), whereas unlabeled RNCs are shown without the fluorescent dye. Nontranslating ribosomes are shown without a nascent chain. Although unlabeled signal sequence-containing RNC is shown in the figure as the competitor, the same competitive binding approach may be used to determine the dissociation constant for SRP binding to other ribosomal species (e.g., an unlabeled globin RNC that lacks a signal sequence).

To ascertain whether the binding of SRP to a signal sequence can be detected spectroscopically, fluorescent probes were positioned at three different locations within the pPL signal sequence. eNBD-Lys was introduced in place of the Lys residues normally found at positions 4 and 9 of the 30-residue pPL signal sequence in NBD-pPLss65-RNC complexes (the subscript indicates the length of the nascent chain), and in the middle of the hydrophobic core (position 18) of the pPL signal sequence in NBD-pPL86-RNC complexes. When each of these complexes was titrated with purified SRP, a substantial increase in NBD emission intensity was observed (Fig. 2). The SRP-dependent increase in fluorescence intensity averaged 65% for NBD-pPLss65-RNC and 60% for NBD-pPL86-RNC. Thus, binding of SRP to a signal sequence in an RNC can be detected spectroscopically. Furthermore, SRP binding is detected equally well by fluorescent probes located at different sites within the signal sequence.

A number of controls were done to eliminate the possibility that the observed fluorescence change was an artifact. First, the mammalian SRP contains one RNA of 300 nucleotides and 6 polypeptides designated SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72, where the number refers to the molecular dissociation constants, stoichiometric factors, and maximum fluorescence change as parameters. This is an exact solution applicable to any concentration of the various components, independent of assumptions regarding free and total concentrations of ligand.

**Fluorescence-detected Binding of SRP to RNCs**

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A number of controls were done to eliminate the possibility that the observed fluorescence change was an artifact. First, the mammalian SRP contains one RNA of 300 nucleotides and 6 polypeptides designated SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72, where the number refers to the molecular...
mass of the protein in kDa (1). Because photocross-linking experiments showed that the signal sequence binds to SRP54 (11, 30), we pre pared an SRP lacking SRP54 by reconstituting purified SRP and the other five SRP proteins as described previously (16). When NBD-pPL86-RNC was titrated with SRP–SRP54, no spectral change was observed (Fig. 2A). This result is fully consistent with SRP54 being the signal sequence-binding component of SRP. Second, no change in fluorescence occurred when NBD-pPLag-RNC was titrated with SRP (Fig. 2B). Because this nascent chain is too short for the signal sequence to have emerged from the ribosome, no SRP-dependent spectral change was expected. Third, no fluorescence change was detected when SRP was titrated into RNCs containing an 84-residue nascent chain of globin which lacked a signal sequence but had an NBD probe positioned in the nascent chain at nearly the same location as in NBD-pPLag (Figs. 1A and 2B). Fourth, no SRP-dependent increase in emission intensity was observed when NBD-pPLag nascent chains were released from their RNCs using puromycin prior to the addition of SRP (data not shown). Thus, the SRP-dependent increase in fluorescence shown in Fig. 2, A and B, required a fully assembled and functional SRP as well as a nascent chain that had an exposed signal sequence and was bound to a ribosome.

Photocross-linking was also used as an independent assay of SRP association with RNCs. Photoreactive probes were incorporated into the same sites occupied by fluorophores in the various nascent chains, and the resulting RNCs were photolyzed after the addition of SRP as before (11). Photocross-linking to SRP54 was observed only in samples containing nascent chains with an exposed signal sequence; no cross-linking to globin or pPLag nascent chains was observed (data not shown).

**SRP Binds to Nontranslating Ribosomes**

The extent of SRP-NBD-RNC formation can be determined directly from the magnitude of the fluorescence changes shown in Fig. 2, A and B, at different SRP concentrations. In principle, we can use this data to determine the dissociation constants for each SRP-NBD-RNC complex. However, as noted above, such an analysis is complicated by the presence of another potential SRP binding partner in the sample, the nontranslating ribosome. This potential competition must be taken into account in any calculations of $K_d$ values.

Samples containing a 30–60-fold excess of nontranslating ribosomes over NBD-pPLag-RNCs were titrated with SRP. If SRP-nascent complexes form, fewer SRPs would be available to form SRP-NBD-pPLag-RNC complexes, and the SRP-dependent fluorescence change at low SRP concentrations would be lower for samples with the large excess of ribosomes than for samples without the added ribosomes. Fig. 3 shows parallel SRP titrations of NBD-pPLag-RNC with or without a 47-fold molar excess of nontranslating ribosomes over RNCs, and it is clear from these data that the nontranslating ribosomes do compete, albeit poorly, with RNCs for binding to SRP. Thus, SRP does bind to nontranslating ribosomes, but weakly.

The $K_d$ values for SRP complexes with both NBD-pPLag-RNC and nontranslating ribosomes were determined by simultaneous nonlinear least squares fitting of the combined data of many titrations by the cubic binding equation (Equation 2) to yield a $K_d$ of 0.18 nM for SRP-NBD-pPLag-RNC and a $K_d$ of 71 nM for SRP-nascent complexes (Table 1). SRP therefore binds with very high affinity to RNCs with an exposed signal sequence, and the SRP-signal sequence interaction is responsible for a 350-fold reduction in $K_d$. As expected, this interaction is largely or solely hydrophobic because increasing the ionic strength by 0.5 M KOAc did not alter the $K_d$ (data not shown).

**FIG. 3. Competitive binding of ribosomal species to a limited amount of SRP.** Competitive binding experiments show SRP titrations of 1.3 nM NBD-pPLag-RNCs supplemented with no added ribosomes (○), a 47-fold molar excess of nontranslating ribosomes (■), or a 2.1-fold molar excess of nonfluorescent pPLag-RNCs (●). For the three titrations shown, nonlinear least squares fitting of the titration data, using either the cubic equation or the quartic equation and assuming $n = r = u = 1$, yielded the best fit lines shown. For these particular titrations, the best fit parameters were: $K_d = 0.18$ nM, $K_r = 71$ nM, and $ΔF_{\text{max}}/F_0 = 0.61$ for the cubic equation with no added ribosomes; $K_d = 0.23$ nM, $K_r = 71$ nM, and $ΔF_{\text{max}}/F_0 = 0.62$ for the cubic equation with added nontranslating ribosomes; and $K_d = 0.20$ nM, $K_r = 71$ nM, $K_r = 0.13$ nM, and $ΔF_{\text{max}}/F_0 = 0.59$ for the quartic equation with added unlabelled pPLag:RNCs. Data shown are representative of multiple independent titrations performed for each RNC.

Although SRP binds with significant affinity to nontranslating ribosomes, their ability to compete with RNCs for SRP is limited. For example, when titration data were simulated assuming $K_d = 0.21$ nM for NBD-pPL-RNCs, $K_d = 71$ nM for nontranslating ribosomes, and $ΔF_{\text{max}}/F_0 = 0.60$, a 5-fold excess of ribosomes over NBD-pPL-RNCs (the maximum expected in the usual in vitro translation; we typically observed a ratio of ribosomes to RNCs between 2:1 and 1:1 in our experiments (data not shown)) had almost no effect on the predicted SRP-dependent fluorescence change. Thus, the concentration of nontranslating ribosomes present in a typical in vitro translation does not detectably reduce SRP binding to NBD-RNCs with exposed signal sequences.

In fact, the affinity of SRP for nontranslating ribosomes is so much less than it is for RNCs with signal sequences that it is difficult to determine the $K_d$ for nontranslating ribosomes accurately even in equilibrium experiments. This is evidenced by the fact that the $K_d$ calculated for the SRP:nascent complex can be altered by even small variations in the value for the maximum $ΔF/F_0$, which explains the large uncertainty in the $71$ nM $K_d$ value.

In early experiments, the $K_d$ for SRP binding to ribosomes lacking signal sequences was estimated to be less than 50 μM (6). This value differs greatly from the above $K_d$ of 71 nM because the latter was determined at equilibrium, and the former was determined by a nonequilibrium technique. Similarly, the dissociation constant determined here for SRP binding to RNC (0.21 nM) is 40-fold lower than the previous estimate (8 nM). Because the dissociation rate is typically greater for low affinity complexes than for high affinity complexes and because a high dissociation rate will reduce the amount of complex detected in a nonequilibrium approach, it is not surprising that the difference between the equilibrium and nonequilibrium $K_d$ values is greater for the weaker SRP:nascent complex.

**The Fluorescent Probe Does Not Interfere with SRP Binding**

To determine whether the fluorescent probe in the signal sequence influenced SRP binding either positively or nega-
ribosome selection for binding to the ER membrane

Table I
SRP association with various RNCs

| Nascent chain | n \( ^{c} \) | \( K_d \) | \( \Delta F_{max}/F_0 \) |
|--------------|----------------|----------------|------------------------|
| Direct titration\(^b\) | | | |
| NBD-pPL86 | 22 | 0.21 ± 0.03 | 0.60 ± 0.04 |
| NBD-pBIP77 | 4 | 0.05 ± 0.02 | 0.44 ± 0.03 |
| NBD-pPL86 | 4 | 0.38 ± 0.10 | 0.26 ± 0.03 |
| NBD-pPL35 | 4 | ND | None |
| NBD-globin84 | 3 | ND | None |
| Competition with NBD-pPL\(^{ext}\)-RNCs\(^d\) | | | |
| pPL86 | 4 | 0.12 ± 0.03 | |
| pPL35 | 5 | 8.6 ± 2.8 | |
| Globin84 | 3 | 8.0 ± 2.4 | |
| None (empty ribosomes)\(^f\) | 3 | 71 ± 36 | |

\(^a\) No. of independent titrations.
\(^b\) Samples contained 0.5–2.3 nm NBD-RNCs. \( K_d \) values and \( \Delta F_{max}/F_0 \) were obtained by fitting the data with Equation 2 while setting \( n = r = 1 \) and \( K = 71 \text{ nm} \).
\(^c\) ND, not determined.
\(^d\) Competition experiments involving RNCs contained a 2.4–12-fold molar excess of unlabeled RNCs over NBD-pPL\(^{ext}\)-RNCs. \( K_d \) values and \( \Delta F_{max}/F_0 \) were obtained by fitting the data with the Equation 10 while setting \( n = r = u = 1 \) and \( K = 71 \text{ nm} \).
\(^e\) For competition experiments with nontranslating (empty) ribosomes (30–59-fold molar excess), the \( K_d \) values and \( \Delta F_{max}/F_0 \) for SRP-ribosome complexes were determined by fitting the data with the Equation 2 while setting \( n = r = 1 \).

Thus, SRP binds to translating ribosomes with an exposed nascent chain that lacks a signal sequence. Furthermore, SRP can also distinguish between ribosomes that are translating and ribosomes that are not translating.

This conclusion was confirmed by competitive binding experiments using RNCs that were translating a secretory protein with a signal sequence but whose nascent chains were not long enough for the signal sequence to have emerged from the ribosome. When the data from five titrations containing a 7–12-fold excess of pPL\(^{ext}\)-RNC over NBD-pPL\(^{ext}\)-RNCs were combined and analyzed simultaneously by nonlinear least squares fitting of the data with the quartic binding equation, the SRP-pPL\(^{ext}\)-RNC complex was found to have a \( K_d \) of 8.6 nm (Table I). Thus, SRP binds more tightly to translating ribosomes than to nontranslating ribosomes. But the additional binding energy does not originate from a nonspecific interaction between SRP and the nascent chain because the nascent chain is still inside the ribosome in the pPL\(^{ext}\)-RNC.

### SRP Affinity for Signal Sequences Is Not Uniform

To determine whether SRP binds every signal sequence with the same affinity, RNCs were prepared with either of two other nascent chains containing a signal sequence, pPI or pBiP. An eNBD-Lys was incorporated into the middle of the signal sequence of the pPI and pBiP derivatives shown in Fig. 1A, and the association of SRP with either NBD-pPI\(^{ext}\)-RNC or NBD-pBiP77-RNC was monitored using fluorescence. The data shown in Fig. 4 and summarized in Table I reveal that SRP binding to signal sequences other than pPL can be detected spectroscopically. The magnitude of the SRP-dependent increase in NBD emission intensity is not the same for each signal sequence, but this spectral difference is unimportant for \( K_d \) measurements because the fraction of NBD-RNC bound to SRP in a sample is given by \( \Delta F/\Delta F_{max} \), and the \( K_d \) is then determined from the SRP concentration dependence of the normalized spectral value \( \Delta F/\Delta F_{max} \). Because the \( K_d \) values for the pPI, pPL, and pBiP signal sequences differ significantly, these titrations demonstrate that SRP affinity for an RNC is dictated by its signal sequence.

### SRP Affinity for Signal Sequences Is Unaffected by Nascent Chain Length

The effect of nascent chain elongation on SRP binding to the nascent chain signal sequence was assessed by creating a set of...
best fit parameters were 

Because, as noted above, only equilibrium for the pBiP RNCs, and 

fitting of the titration data using the cubic equation and assuming 

likely 2-fold: (i) to ensure that only those RNCs with legitimate 

for such tight binding of SRP to RNCs with signal sequences is 

affinity (0.05 nM; Table I). The physiological requirement 

the rate of release of the signal sequence from the SRP 

ER lumen as part of the unfolded protein response (31). Of 

BiP must take precedence and be transported quickly into the 

sequences for which it has the highest affinity, the SRP-signal 

and these differences are presumably physiologically signifi-

DISCUSSION

The affinities of SRP for various ribosomal species were 

determined directly and at equilibrium. This is very important 

because, as noted above, only equilibrium \( K_d \) values provide an 

accurate measure of affinities and thermodynamic free energies. 

The fluorescence approach used here reveals that SRP binds to signal sequences in RNCs with exceptionally high affinity (0.05–0.38 nM; Table I). The physiological requirement for such tight binding of SRP to RNCs with signal sequences is likely 2-fold: (i) to ensure that only those RNCs with legitimate 

signal sequences are selected by SRP and targeted to the ER membrane, and (ii) to maximize the time of elongation arrest, thereby maximizing the chances of the RNC targeting successfully to the ER membrane. Because RNCs with long nascent chains are less likely to target successfully to translocons (7), a high affinity interaction between SRP and RNCs with signal sequences would slow the dissociation rate of SRP from the SRP-RNC complex and cause nascent chain elongation to be inhibited for a longer time, thereby increasing the time available for successful targeting. Thus, the high affinity SRP-RNC interaction appears to be required to select RNCs accurately for targeting to the ER membrane and also to target efficiently those RNCs to the membrane.

However, RNCs with different signal sequences bind to SRP with different affinities. The measured \( K_d \) values for the three SRP-RNC complexes examined here differ by 8-fold (Table I), and these differences are presumably physiologically significant. Because SRP will bind preferentially to the RNC signal sequences for which it has the highest affinity, the SRP-signal sequence affinity may correlate with the priority of the nascent chain for translocation within the spectrum of nascent chains that are vying for an empty translocon. The extremely high affinity of SRP for the pBiP signal sequence appears to be consistent with this idea because when the cell is under stress, BiP must take precedence and be transported quickly into the ER lumen as part of the unfolded protein response (31). Of course, the rate of release of the signal sequence from the SRP at the translocon during targeting will also be affected by the
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strength of the SRP-signal sequence interaction, so other considerations will undoubtedly influence the optimal SRP affinity for a particular signal sequence and nascent chain. Thus, the variation in SRP-signal sequence affinities presumably evolved through a balancing of different physiological or mechanistic requirements within the cell. More SRP-RNC \(K_d \) values will have to be determined and correlated with targeting efficiency, among other things, to appreciate fully the functional significance of different SRP-RNC affinities.

Early on, SRP was proposed to scan the nascent chain as it emerged from the ribosome to ascertain whether or not the nascent chain had an appropriate signal sequence (32). This mechanism of signal sequence detection requires SRP to bind to ribosomes that lack an exposed signal sequence, and Walter et al. (6) did observe weak binding of SRP to ribosomes that lacked a signal sequence (\(K_d \approx 50 \mu M\)). The competitive binding experiments reported here confirm that observation by demonstrating that SRP binds to ribosomes that lack either a signal sequence or a nascent chain. However, the equilibrium dissociation constants determined here show that SRP affinity for nontranslating ribosomes is actually quite high (\(K_d = 71 \text{ nM}\)).

Although this dissociation constant was determined with canine SRP and wheat germ ribosomes because of the difficulty of purifying mammalian ribosomes free of the relatively high concentration of endogenous SRP, the well documented compatibility of the wheat germ and canine molecules involved in targeting and translocation suggest that the dissociation constant for a mammalian SRP and ribosome would be similar. The spectroscopic data are therefore consistent with the view that SRP is typically bound to a ribosome and not free in the cytosol. This would allow the SRP to scan the emerging nascent chain for a signal sequence and detect its presence as quickly as possible, thereby minimizing the chances that the nascent chain would become too long to target to the translocon efficiently.

At first glance, the affinity of SRP for nontranslating ribosomes reported here may appear to be too high. However, the \(K_d\) for the SRP-ribosome complex is reasonable given the concentrations of these species in the cell. The SRP concentration in the mammalian cytoplasm has been estimated to be about 10 nM (7), and the total ribosome concentration has been estimated to be about 10–100-fold higher than the SRP concentration in different organisms (33). Because a significant fraction of the ribosomes in a mammalian cell (up to \(\approx 50\%\)) will be bound to the ER membrane at translocons and hence unavailable for SRP binding, the actual ribosome concentration will be lower than 100–1,000 nM and may be lower than 50–500 nM in pancreatic cells. Although these estimates are very rough, the low cytosolic concentrations of SRP and free (not bound to the ER membrane) ribosomes reveal that a \(K_d\) below 100 nM is required to ensure that most of the SRP is bound to a ribosome in the cytosol. Because the actual SRP and ribosome concentrations will vary with cell type, it seems likely that the \(K_d\) value has evolved to balance and best satisfy the needs of a variety of different cells and circumstances.

The competitive binding experiments also revealed an unexpected property of the ribosome, that of a conformational difference between translating and nontranslating ribosomes which is detected by SRP (Table I). The discovery that SRP can distinguish between translating and nontranslating ribosomes provides even more compelling evidence for the cotranslational scanning and detection of nascent chain signal sequences by SRP because the 9-fold difference in \(K_d\) values will lead to a preferential binding of SRP to ribosomes that are engaged in protein synthesis. It is also important to note that this preference exists even before the nascent chain emerges from the ribosome. This result shows that the increased affinity of SRP for an RNC over a nontranslating ribosome is caused by a change in ribosome conformation at the SRP binding site on the ribosome, not by an interaction between the SRP and nascent chain.

The inability of RNCs to target to the ER membrane when their nascent chains become too long was originally attributed to an inability of the SRP to bind to the signal sequence of RNCs with a long nascent chain, presumably because folding of the longer nascent chain interfered with SRP accessibility to the signal sequence (7). Yet by placing a fluorescent probe in the signal sequence to monitor SRP binding directly, we found that SRP can recognize and bind to a signal sequence in a long nascent chain that had plenty of time to fold into a stable conformation before SRP was added (Table II). Furthermore, the affinity of the SRP for the signal sequence in an RNC was essentially independent of nascent chain length once the signal sequence had fully emerged from the ribosome and was able to contact its binding site on the SRP optimally (Table II). We therefore conclude that a long nascent chain interferes with RNC targeting to the ER membrane at some point after the formation of the SRP-RNC complex. Because the SRP-RNC complex next interacts with the SRP receptor and the translocon, it appears that the extra polypeptide of an elongated nascent chain interferes sterically with either the interaction of SRP with the SRP receptor and/or the interaction of the RNC with the translocon. The latter possibility seems particularly reasonable because the cytosol contains many ribosomes that are synthesizing cytoplasmic proteins, and such ribosomes will undoubtedly diffuse to and collide with empty (ribosome-free) translocons at some frequency. If long nascent chains were to interfere with the binding of such ribosomes to free translocons, the cell would reduce the chances of improperly binding a ribosome to the translocon. The discrimination against an RNC with a long nascent chain may therefore constitute a significant safety mechanism to minimize improper trafficking.

A very important and controversial issue is the effect that GTP binding to SRP has on its ability to bind to a signal sequence. Because the RNCs examined here were purified by gel filtration, the spectral data reported above were obtained in the absence of nucleotides. We have since examined the nucleotide dependence of RNC selection by SRP spectroscopically and quantitatively, but those results will be reported elsewhere because they are outside the scope of this paper.

In summary, the spectroscopic detection of SRP binding to a RNC signal sequence has allowed us to characterize this interaction quantitatively, and this in turn has allowed us to reexamine the mechanism of SRP selection of RNCs for targeting to the ER membrane. Our data reveal, among other things, that SRP affinity for RNC signal sequences is both variable and exceedingly high and is also unaffected by the length of the nascent chain. Furthermore, SRP binds with nanomolar affinity to ribosomes that lack signal sequences and can distinguish between translating and nontranslating ribosomes.

Acknowledgments—We are grateful to Drs. David Andrews, Greg Beckler, Linda Hendershot, Sandra Wolin, and Veronica Worrell for providing plasmids, to Dr. Hung Do for photocross-linking experiments, and to current and former members of the Johnson laboratory for helpful discussions and advice.

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