Selective SecA Association with Signal Sequences in Ribosome-bound Nascent Chains

A POTENTIAL ROLE FOR SecA IN RIBOSOME TARGETING TO THE BACTERIAL MEMBRANE*

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Andrey L. Karamyshev and Arthur E. Johnson

From the 1Department of Medical Biochemistry and Genetics, Texas A & M University System Health Science Center, College Station, Texas 77843-1114 and 2Departments of Chemistry and of Biochemistry and Biophysics, Texas A & M University, College Station, Texas 77843

The role of SecA in selecting bacterial proteins for export was examined using a heterologous system that lacks endogenous SecA and other bacterial proteins. This approach allowed us to assess the interaction of SecA with ribosome-bound photoreactive nascent chains in the absence of trigger factor, SecB, Ffh (the bacterial protein component of the signal recognition particle), and the SecYEG translocon in the bacterial plasma membrane. In the absence of membranes, SecA photocross-linked efficiently to nascent translocation substrate OmpA in ribosome-nascent chain (RNC) complexes in an interaction that was independent of both ATP and SecE. However, no photocross-linking to a nascent membrane protein that is normally targeted by a signal recognition particle was observed. Modification of the signal sequence revealed that its affinity for SecA and Ffh varied inversely. Gel filtration showed that SecA binds tightly to both translating and non-translating ribosomes. When purified SecA-RNC complexes containing nascent OmpA were exposed to inner membrane vesicles lacking functional SecA, the nascent chains were successfully targeted to SecYEG translocons. However, purified RNCs lacking SecA were unable to target to the same membranes. Taken together, these data strongly suggest that cytosolic SecA participates in the selection of proteins for export by co-translationally binding to the signal sequences of non-membrane proteins and directing those nascent chains to the translocon.

Protein trafficking in bacterial cells is very complex with both Sec-dependent and -independent modes of translocating proteins across the plasma membrane and integrating proteins into the bilayer (1–5). The distribution of newly synthesized proteins into the appropriate processing pathway is therefore a critical issue for bacterial cells, and they have evolved multiple mechanisms for selecting and properly directing proteins into the correct pathway.

Proteins destined to be translocated or integrated via the Sec pathway are currently thought to be identified and targeted to the SecYEG translocon in two different ways (1–3, 6, 7). Nascent membrane proteins with a hydrophobic (ultimately transmembrane) signal-anchor sequence at their N termini are recognized and bound by the signal recognition particle (SRP), which then interacts with the SRP receptor (FtsY) and GTP to target the ribosome-nascent chain (RNC) complex to the translocon for co-translational integration of the substrate (the nascent membrane protein). Proteins that need to be translocated across the bacterial membrane have a less hydrophobic signal sequence at their N-terminal ends and do not bind to SRP. Instead, such proteins are thought to bind post-translationally to a SecA that is itself bound to a translocon. This is generally thought to occur by the binding of translocation substrates to SecB. However, SecB is a nonspecific chaperone that binds to unfolded cytosolic proteins as well as sorting substrates. SecB does not bind specifically to signal sequences and hence does not function as a selective sorting factor (8, 9). Yet SecB does bind tightly and specifically to SecA (10, 11) and hence facilitates the association of some substrates with the translocon. SecA does bind signal sequences (12–15), and this interaction is generally thought to occur at the membrane when the translocation substrate arrives at the translocon-bound SecA (1–3, 6, 7). However, SecA association with RNCs and the signal sequences of translocation substrates has been noted periodically (16–18).

Another protein, trigger factor (TF), has been shown to act as a sorting factor by associating with nascent cytosolic proteins at the ribosome to ensure that they are not mistakenly directed to the translocon by SRP (2, 3). The cell has therefore evolved a sorting factor that does not positively direct the substrate protein into a different compartment but instead simply ensures that the substrate stays in the same compartment in which it was synthesized. This observation is very significant, because it emphasizes how critical it is for the cell to maintain accuracy in protein sorting.

Hence, it is not surprising that the TF and SRP proteins actively scan and select nascent proteins for their substrates during translation. However, it is surprising that there is apparently no protein that recognizes and binds to a signal sequence early in translation to identify and target it to the translocon. SecB only binds to slowly folding proteins late in their translation or after synthesis is complete (10). Therefore SecB cannot select and direct signal sequence-containing proteins to the translocon early in translation. Thus, although essentially every process in the cell, including protein sorting, is mediated or catalyzed by a protein, each protein destined for translocation across the bacterial membrane is apparently synthesized completely before diffusing passively into the cytosol, sometimes in association with SecB, until it encounters and engages an unoccupied SecA-SecYEG complex at the membrane. Because no apparent active and positive recognition of the signal sequence by a protein appears to occur to regulate this important aspect of cell metabolism, we decided to re-examine the interactions of signal sequences with potential sorting factors.

One direct approach for detecting the association of a signal sequence with a sorting factor is photocross-linking. A photoreactive probe can
be incorporated into a ribosome-bound nascent chain using an *in vitro* approach we originated (19) that involves translation in the presence of a chemically modified aminoacyl-tRNA, here N⁵-(5-azido-2-nitrobenzoyl)-Lys-tRNA (eANB-Lys-tRNA). When illuminated, the probe will react covalently with any protein positioned adjacent to the probe, as was seen when a probe in the signal sequence of a pre-prolactin RNC reacted covalently with the SRP54 subunit of eukaryotic SRP (20, 21). This approach has since been used to determine the proximity of nascent chains in bacterial RNCs to various factors postulated to be involved in protein sorting, including SRP and TF (18, 22–24). Here we have used photocross-linking and other approaches to characterize the association of signal sequences with SecA in the absence of any competing factor binding. These experiments strongly suggest that cytosolic SecA is involved in the selection and targeting of nascent translocation substrates to the SecYEG translocon.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The Escherichia coli strains AD202 (25) and CJ105 (26) were used to prepare wild-type IMVs and SecA⁺ IMVs, respectively. The *E. coli* strains BL21/pZ52 (27) and W3110 with plasmid pTRC-OmpA9 (28) were generous gifts of Dr. W. Wickner and were used for SecA overproduction and as a source of the *ompA* gene, respectively. *E. coli* BL21(DE3)-pLysS (Invitrogen) was used for overexpression of Ffh from pDMF6. A plasmid (p146) containing *E. coli* leader peptidase (signal peptidase I) gene (*lepB*) in vector pGEM1 and a plasmid (pDMF6) containing the *ffh* gene under T7 promoter (29) were generous gifts of Drs. I. Nilsson and P. Walter, respectively. The *E. coli* 4.5 S RNA gene was obtained directly from *E. coli* MC1061 by PCR. Site-directed mutagenesis was performed by a QuikChange PCR technique using PfuTurbo DNA polymerase (Stratagene). Hybrid genes were constructed by overlapping PCR. DNA fragments for *in vitro* transcription were generated by PCR under standard conditions using Ex Taq DNA polymerase (Takara Bio, Inc.).

**mRNA, 4.5 S RNA, tRNA, Canine SRP, and IMVs**—Truncated mRNAs were prepared as described previously, as were [¹⁴C]Lys-tRNA and eANB-¹⁴C]Lys-tRNA (20, 30). To prevent incorporation of probes into the N terminus of OmpA, the two lysesins in the signal sequence of wild-type OmpA were replaced with Arg, and that construct is here referred to as wild-type OmpA. 4.5 S RNA was transcribed from the above PCR fragment using T7 RNA polymerase. Canine SRP was purified as described previously (31), except that the sucrose density sedimentation was omitted.

*E. coli* IMVs were prepared according to Tai *et al.* (32) with modifications. *E. coli* AD202 was grown at 37 °C in LB medium to an *A*₅₉₀ = 1, whereas the SecA⁺ mutant strain CJ105 was grown at 27 °C to an *A*₅₉₀ = 1.3 and then incubated at 43 °C for another 3.5 h. The sedimented cells from a 1-liter culture were resuspended in 25 ml of buffer A (50 mM HEPES, pH 7.5, 40 mM KOAc, 5 mM MgCl₂) containing 250 mM sucrose and 1 mM dithiothreitol. The suspension was passed twice through a French press cell at 10,000 pounds/square inch. After unbroken cells and large debris were removed by sedimentation (6,000 × *g*, 5 min, 4 °C), the cleared lysate was layered over a two-step gradient (1.2 ml each of 0.5 and 1.4 M sucrose in buffer A) and centrifuged in a Beckman SW 41 rotor (37,000 rpm, 3 h, 4 °C). The visible interface band, containing mostly crude inner membranes, was collected, diluted, and layered over a four-step sucrose gradient (3 ml of sample, 2 ml each of 0.8, 1.0, 1.2, and 1.4 M sucrose in buffer A). After centrifugation (SW 41, 37,000 rpm, 17 h, 4 °C), the visible band at the 1.0–1.2 M interface was collected and then diluted in buffer A containing 2 mM glutathione, and IMVs were purified by sedimentation (Beckman Ti-50.2 rotor, 40,000 rpm, 1 h, 4 °C). The IMV pellet was resuspended in buffer A containing 250 mM sucrose and 2 mM glutathione, and IMVs were stored in small aliquots at −80 °C.

**SecA and Ffh**—SecA and Ffh were purified from *E. coli* BL21/pZ52 and BL21(DE3)-pLysS/pDMF6 strains, respectively. Cells in buffer B (25 mM HEPES, pH 7.5, 1 mM dithiothreitol) containing 50 mM KCl and 3 mM MgCl₂ were disrupted using a French press, and the lysates were cleared by centrifugation. SecA was purified by chromatography on 5-ml Heparin HP and MonoQ HR5/5 columns (Pharmacia) using linear gradients of 50–1250 mM and 50–850 mM KCl, respectively, in buffer B. Ffh was purified by chromatography on Heparin HP and SP-Sepharose columns (Pharmacia) using linear gradients of 50–1250 mM and 150–1050 mM KCl in buffer B. The Ffh-containing fractions were concentrated using Centricon 10 (Amicon). Purified SecA and Ffh were stored in small aliquots at −80 °C.

**Translations and Photocross-linking**—Typical *in vitro* wheat germ translations (50 μl, 26 °C, 30 min in the dark) contained 50 mM HEPES, pH 7.5, 100 mM KOAc, 3.5 mM Mg(OAc)₂, 7.5 μg [³⁵S]Met, 30 pmol of eANB-¹⁴C]Lys-tRNA (or unmodified [¹⁴C]Lys-tRNA), and other components as described previously (20, 30, 33). SecA (0.02–4 μM; usually 0.4 μM), SecB (0.8–25 μM), *E. coli* SRP (Ffh/4.5 S RNA, 0.25 μM), canine SRP (0.02, 0.04, or 0.06 μM), or 0.2 A₅₀₀ units of IMVs were added as indicated. Samples were photolyzed on ice for 15 min using a 500-watt mercury arc lamp as described previously (33) and then sedimented through a 130-μl sucrose cushion (0.5 M sucrose in buffer A) in a Beckman TLA-100 rotor (100,000 rpm, 5 min, 4 °C) for those samples containing IMVs. The pellets were resuspended in SDS sample buffer or in immunoprecipitation buffer (see “Immunoprecipitation”). Samples were analyzed by 10–15% gradient SDS-PAGE (33). In all experiments, photocrosslinks and nascent chains were detected and quantified using a Bio-Rad FX Molecular Imager.

For the experiments shown in Fig. 3A, some samples were treated with apyrase as described previously (34). After translation in the dark as described above, apyrase (Sigma) was added to a final concentration of 0.1 or 0.2 units/μl and incubated for 10 min at 26 °C to hydrolyze ATP in the sample. SecA was then added to 0.4 μM, after which the samples were incubated for another 10 min before being photolyzed, and then immunoprecipitated by SecA-specific antibodies and analyzed by SDS-PAGE.

**Separating Ribosome-bound SecA from Free SecA**—Translutions (250 μl) for the experiments shown in Fig. 6 were conducted as described above, except that 150 pmol of Lys-tRNA replaced the eANB-Lys-tRNA. SecA was at 0.4 μM when present, and procedures were done in the light. RNCs were purified on a Sepharose CL-6B column (50 × 0.7 cm inner diameter) as described previously (35, 36). Fractions were analyzed by absorbance at 260 and 280 nm, liquid scintillation counting to determine [¹⁴C] counts/min, agarose gel electrophoresis to detect rRNA, and SDS-PAGE. SecA was detected in the 10% SDS-PAGE gels by Western blotting using antibodies specific for SecA and the ECL Plus detection system (Amersham Biosciences).

For the experiments shown in Fig. 7C, SecA-RNC complexes were separated from free SecA by sedimentation as described above. After resuspension in a translation mix lacking ribosomes (removed by sedimentation), the SecA-RNCs were incubated (20 min, 26 °C) in the presence of IMVs. For the ATP-free experiments, the ribosomal resuspension solution was incubated with apyrase (0.7 units/μl; Sigma) for 10 min at 26 °C before addition to the ribosomal pellet. Following photolysis, samples were immunoprecipitated prior to SDS-PAGE.

**Immunoprecipitation**—RNC or IMV pellets were resuspended in 55 μl of 1% (w/v) SDS, 100 mM Tris-HCl, pH 7.5, and placed at 55 °C for a
minimum of 30 min. After 5 μl were removed for direct analysis by SDS-PAGE, the remaining 50 μl were brought up to 500 μl with 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2% (v/v) Triton X-100, and 0.2% (w/v) SDS. Samples were then pre-cleared by rocking with 40 μl of protein A-Sepharose (Sigma) at room temperature for 1 h, after which the Sepharose beads were removed by sedimentation. SecA- or SecY-specific antibodies (generous gifts of Drs. W. Wickner and J.-W. de Gier, respectively) were added to the supernatant, and the samples were rocked overnight at 4 °C. Thereafter, 40 μl of protein A-Sepharose were added, and the incubation was continued for 2 h. The immunoprecipitate was recovered by centrifugation, washed twice with 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2% (v/v) Triton X-100, 0.2% (w/v) SDS, and one time with 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5, prior to SDS-PAGE.

RESULTS

Experimental Rationale—Although it is generally best to establish the mechanisms of a process using macromolecular components that originate from the same organism, it is sometimes advantageous to use heterologous components. Such an approach may allow one to characterize an interaction between two components from an organism without interference from homologous components with real or potential overlapping activities (i.e. in the complete absence of other components from that organism that may modulate or compete with the interaction being investigated). Here, by preparing RNCs in a wheat germ translation system that is devoid of SecA, SecB, and TF and contains only a trace amount of wheat germ SRP, we can examine the association of a bacterial signal sequence with purified bacterial SecA, SecB, SRP, or membranes in the absence of any other bacterial proteins and membranes. Because this approach eliminates any chance of interference with ribosome-associated bacterial components that would be present in a bacterial RNC preparation, interpretation of the results is more straightforward. We have therefore chosen to assess the interaction of bacterial signal sequences with SecA and bacterial SRP in a background that completely lacks endogenous bacterial components.

To detect signal sequence association with SecA, SecB, and Ffh, we decided to monitor the extent of photocross-linking to these proteins by probes located adjacent to the signal or signal-anchor sequence in the nascent chain. Although this method only detects proximity and the absence of a cross-link does not necessarily mean the absence of an interaction, the existence of photoadducts between two proteins generally does correlate closely with complex formation between the two proteins. For example, we recently used a fluorescent probe in different experiments to detect the association of ribosome-associated bacterial components with nascent OmpA (37). After translation in the dark to prevent cross-linking, radioactive bands were observed that had apparent molecular masses consistent with OmpA-85 photoadducts to SecA, TF, and an unidentified 60-kDa protein (Fig. 1A, lane 1). The presumed SecY photoadduct had an apparent molecular mass much lower than predicted, because the electrophoretic mobility of SecY is unusually high (22, 39). As expected, translation of pre-prolactin remained unaffected by addition of purified SecA or other OmpA-binding proteins in the presence of added IMVs (Fig. 1A, lane 3 and 6) probe-dependent (Fig. 1A, compare lane 1 with lane 3 and 6 and lane 3 and 5 with lane 6) probe-dependent (Fig. 1A, compare lane 1 with lane 3 and 5 and lane 6 and lane 5 with lane 6). The identities of the SecY and SecA photoadducts were confirmed by immunoprecipitation with SecY- and SecA-specific antibodies under denaturing conditions (Fig. 1A, lanes 7 and 5, respectively). Most important, because the nascent OmpA was adjacent to SecY in the membrane, the OmpA signal sequence targeted the wheat germ RNCs to the bacterial translocon. The wheat germ ribosome in the RNC therefore did not detectably interfere with the initial stages of translocation, including selection of the nascent chain for processing by the SecYEG translocon.

SecA Interacts with Certain Ribosome-bound Nascent Chains in the Absence of Membranes—Although expected, the absence of a SecA or other OmpA-binding protein was present in the wheat germ translation system. However, if purified SecA was added to the translation incubation in the absence of IMVs, an OmpA-85 photoadduct with SecA was detected by immunoprecipitation (Fig. 1B, lane 3). In fact, nascent OmpA photocross-linking to SecA occurred with nascent chains as short as 60 residues (Fig. 1C, lane 1), which indicates that SecA association with the OmpA signal sequence occurs soon after it emerges from the ribosome. Upon lengthening the OmpA-nascent chain, the extent of its photocross-linking to SecA is reduced (Fig. 1C). The reason for this decline in photoadduct yield is not yet clear. In addition, multiple photoadducts are immunoprecipitated by the antibodies to SecA as the nascent chain lengths (Fig. 1C). This heterogeneity may be due to nascent chain photocross-linking to SecA at different locations, similar to the differing electrophoretic mobilities reported for different signal sequence photoadducts with Sec61p (40).

SecA-RNC Association Requires a Signal Sequence—The observed OmpA-nascent chain photocross-linking to SecA is signal sequence-dependent. When a photocross-reactive RNC containing an OmpA derivative sequence functions as a signal sequence in targeting but is not cleaved and ends up integrated into the bilayer because the longer hydrophobic core constitutes a transmembrane sequence. The presence of a modified εANB-Lys probe in the nascent chain should have little effect on its affinity for various proteins, because an even larger fluorescent probe had no significant effect on the affinity of the pre-prolactin signal sequence for the mammalian SRP (37). After translation in the dark to create the photocross-reactive wheat germ RNCs in the presence of purified bacterial proteins, samples were illuminated with UV light to initiate photocross-linking.

Wheat Germ OmpA RNCs Are Targeted to the SecYEG Translocon—To ascertain whether a bacterial signal sequence in a RNC was able to target to the bacterial membrane despite the presence of a wheat germ ribosome, photocross-reactive RNCs were prepared with a [35S]Met-labeled 85-residue OmpA-nascent chain (here termed OmpA-85). Photoreactive probes were incorporated at positions 24 and 33 in the OmpA-nascent chain, although most nascent chains contained only a single probe, because the added εANB-Lys-tRNA had to compete for incorporation with the full complement of endogenous Lys-tRNA in the translation and only about one in four incorporated lysines was εANB-Lys (38). After incubation with inverted vesicles of E. coli inner membranes (IMVs) during translation and then photolysis, radioactive bands were observed that had apparent molecular masses consistent with OmpA-85 photoadducts to SecA, TF, and an unidentified 60-kDa protein (Fig. 1A, lane 1). The presumed SecY photoadduct had an apparent molecular mass much lower than predicted, because the electrophoretic mobility of SecY is unusually high (22, 39). As expected, formation of these radioactive species was both IMV-dependent (Fig. 1A, compare lane 1 with lane 3 and 5 with lane 6) and probe-dependent (Fig. 1A, compare lane 1 with lane 2). The identities of the SecY and SecA photoadducts were confirmed by immunoprecipitation with SecY- and SecA-specific antibodies under denaturing conditions (Fig. 1A, lanes 7 and 5, respectively). Most important, because the nascent OmpA was adjacent to SecY in the membrane, the OmpA signal sequence targeted the wheat germ RNCs to the bacterial translocon. The wheat germ ribosome in the RNC therefore did not detectably interfere with the initial stages of translocation, including selection of the nascent chain for processing by the SecYEG translocon.
lacking the signal sequence (Fig. 2A, Omp-ss(0)) was incubated with purified SecA, no photoadducts to SecA were detected at either of two nascent chain lengths (Fig. 1B, lanes 1 and 2). Under the same conditions, the wild-type OmpA was photocross-linked very efficiently to SecA (Fig. 1B, lane 3). To assess whether the absence of photoadduct was due simply to a reduced SecA-nascent chain affinity, we increased the concentration of SecA in the translation. The yield of photoadduct with wild-type OmpA increased with the SecA concentration until the latter reached 1 μM, and the yield then remained constant up to 4 μM SecA (data not shown). In contrast, no photoadducts were observed with OmpA-nascent chains lacking the signal sequence even at 4 μM SecA (data not shown). Thus, a signal sequence is apparently an obligatory requirement for SecA photocross-linking to, and hence association with, the nascent chain of an OmpA RNC.

A Signal-Anchor Sequence in a RNC Does Not Photocross-link to SecA—
To assess further the signal sequence dependence of the SecA interaction with a RNC, we prepared photoactive RNCs with two different lengths of LepB. Because LepB had been shown earlier to chemically cross-link to SecA during integration into the membrane (41), this choice seemed reasonable for an alternative signal sequence. LepB has a hydrophobic H1 domain at its N terminus that serves as a signal-anchor sequence, and two of the six natural Lys residues in the first 100 amino acids are close to the H1 domain (positions 24 and 30) in positions very similar to the two natural lysine positions in OmpA (24 and 33). Although the ANB probes were positioned in similar locations in OmpA and LepB, no LepB was photocross-linked to SecA (Fig. 2B, lanes 2, 3, 11, and 12). Yet as shown above, a strong photoadduct band was seen with OmpA (Fig. 2B, lanes 1 and 10). Thus, SecA appears to distinguish between RNCs containing the signal sequence of OmpA or the signal-anchor sequence of LepB.

The observed difference in OmpA and LepB photocross-linking to SecA could be due to the differences in the signal/signal-anchor sequences themselves and/or to the flanking sequences. To address this issue, we constructed a LepB-OmpA hybrid protein in which the signal sequence of OmpA was replaced by the first 19 residues of the LepB signal-anchor sequence (Fig. 2A, L-Omp). The probes were in the same positions in OmpA and L-Omp, and we found that the L-Omp-nascent chain could be efficiently photocross-linked to SecY in IMVs (data not shown). Although this photocross-linking to SecY showed that the LepB signal-anchor sequence was able to target the L-Omp RNC to the translocon and position itself next to SecY, a L-Omp hybrid nascent chain was only inefficiently photocross-linked to SecA (Fig. 2B, compare lanes 1 and 4, as well as 10 and 13). The 4-fold difference in SecA photocross-linking to L-Omp and OmpRNCs (Fig. 2E) indicates that the affinity of SecA-nascent chain binding is significantly higher for wild-type OmpA than for the L-Omp chimera. Thus, the identity of the signal sequence dramatically influences the extent of its interaction with SecA in solution.

On the other hand, the fact that the difference in photocross-linking yields was only 4-fold reveals that the SecA interaction with the nascent chain involves both the signal/signal-anchor sequence and its flanking sequence. If SecA binding was mediated solely by the signal sequence, then replacing the OmpA signal sequence with the LepB signal-anchor sequence should have completely eliminated nascent chain photocross-linking to SecA (cf. Fig. 2B, lanes 11 and 12). Alternatively, if SecA binding was mediated solely by the flanking sequence, then the chimera should have photocross-linked to SecA as efficiently as wild-type OmpA (cf. Fig. 2B, lane 10). However, because the chimeric nascent chain did not behave as either the wild-type OmpA or the wild-type LepB (Fig. 2B, lanes 4 and 13), it appears that the interaction of the OmpA flanking region with SecA is sufficient to provide significant binding energy. This conclusion is also consistent with the fact that probes positioned in the OmpA flanking sequence at positions 24 and 33 are able to photocross-link to SecA. Of course, because SecA catalyzes the translocation of polypeptides through the translocon, it is not surprising that it has some affinity for unfolded protein sequences (see Ref. 6).

Signal Sequence Recognition by SecA—What structural elements of the RNC-nascent chain signal sequence are recognized by SecA in solution? Although signal sequences do not have extensive sequence homology, most contain three characteristic regions: a positively charged N-terminal domain (n-region), a hydrophobic core ( h-domain), and a polar C-terminal domain (c-region) with the signal peptide cleavage site (42). The OmpA signal sequence has this organization, whereas the H1 domain of LepB lacks a positive charge at the N terminus, has a more hydrophobic core sequence, and also has no signal peptidease cleavage site (Fig. 2A).

To determine the dependence of SecA association with RNCs in solution on the positive charge at the N terminus of the signal sequence, we
prepared a series of L-Omp derivatives that had N-terminal charges ranging from −2 to +4 (Fig. 2A). SecA-nascent chain photocross-linking was greatest for L-Omp-nascent chains with a +2 charge in the n-region (L-Omp(+2)) (Fig. 2B, lanes 8 and 17, and 2E); in fact, a L-Omp RNC-nascent chain with two positive amino acid residues in the n-region photocross-linked to SecA with the same efficiency as the wild-type OmpA-nascent chain (Fig. 2B, lanes 18 and 2E). Replacing the positive charges with negative charges essentially eliminated SecA photocross-linking to, and presumably association with, the L-Omp signal sequence (Fig. 2B, lanes 1 and 10). All L-Omp derivatives (8, lanes 4–9 and 13–18; C, lanes 1–16) were truncated after codon 85 of the wild-type ompA gene. LepB RNCs contained nascent chains of 81 (B, lanes 2 and 11) or 111 (B, lanes 3 and 12) residues. The RNCs in D contain OmpA-85 (lane 1) or OmpA-85 with an arginine substituted into position 11 (lane 2). E, the photocross-linking efficiencies of various nascent chains are shown relative to that of OmpA-85 (taken as 100%). The average data and standard deviations from 2–4 independent experiments are presented. M, apparent molecular mass.

However, positive charges in the n-region are not sufficient by themselves to elicit SecA binding. The introduction of two positively charged amino acid residues at the N terminus of the mature OmpA sequence (i.e. lacking a signal sequence) did not elicit photocross-linking to SecA (Fig. 2C, lanes 7 and 15, and 2E).

In contrast, SecA association with a RNC signal sequence is absolutely dependent upon the presence of a stretch of nonpolar amino acids in the h-domain. Mutated proteins with a significantly reduced h-domain length did not photocross-link to SecA, even when the n-region was positively charged (Fig. 2C, lanes 5, 6, 13, and 14). Systematically decreasing the length of the h-domain from 15 to 7 amino acid residues reduced the extent of SecA-nascent chain photocross-link formation and finally resulted in the disappearance of SecA-RNC photocross-linking when the h-domain reached 7–9 amino acids in length (Fig. 2C and 2E). The hydrophobic core of the signal sequence is therefore a primary recognition element for SecA binding.

Furthermore, the h-domain must consist of a contiguous sequence of hydrophobic amino acids. If a positively charged Arg is positioned in the
middle of the OmpA signal sequence h-domain, photocross-linking to SecA disappears (Fig. 2D, compare lanes 1 and 2). The introduction of polar amino acid residues into the hydrophobic core of the signal sequence inhibits the translocation of many secretory proteins through the bacterial membrane (43), and these mutations have been thought to disrupt the interaction of the secretory protein with the SecYEG translocon. Yet the results shown in Fig. 2D suggest that charged residues in the h-domain may also, or instead, interfere with SecA binding to the nascent chain prior to its insertion into the SecYEG translocon.

Taken together, these data demonstrate that SecA photocross-linking to the RNC-nascent chain depends upon both the hydrophobicity of the h-domain and the charge of the n-region in the bacterial signal sequence. Consistent with these results, a very recent study of the conformation of a signal peptide bound to SecA identifies direct interactions between SecA and these regions of the signal sequence (44). SecA recognition of a RNC-bound signal sequence therefore involves at least two different elements of its structure, specifically its hydrophobic core and the electrostatic charge at its N terminus, in addition to its flanking sequence.

Early SecA-nascent Chain Interactions Are Independent of Both ATP and SecB—Because SecA is an ATPase (3, 6), SecA may need to be in the ATP-bound conformation to bind to the RNC-nascent chain. RNCs were therefore prepared in the presence of ATP and then treated either with apyrase to hydrolyze ATP (34) or with sodium azide to block ATP binding to SecA (45) prior to SecA addition. Neither treatment significantly affected the yield of SecA-nascent chain photoproduct (Fig. 3A). Thus, SecA binding to the signal sequence in a RNC-nascent chain occurs in the absence of ATP and is not ATP-dependent.

To determine whether SecB enhances or interferes with SecA binding to the nascent chain early in translation, increasing concentrations of SecB (up to 25 μM monomer) were incubated with photoreactive OmpA-85 RNCs in the absence of IMVs prior to photolysis, and the extent of SecA-nascent chain photocross-linking was not affected by SecB. We also observed no photocross-linking of SecB to ribosome-bound OmpA-nascent chains as long as 165 residues (Fig. 3B, lanes 1–7) because SecB binds weakly to RNCs (37). Does SecA bind to a ribosome or RNC, and if so, is such binding nascent chain-dependent?

SecA and Canine SRP Compete for Nascent Chain Binding—The bacterial Lep signal-anchor sequence has been shown previously to target a RNC to the eukaryotic translocon in a SRP-dependent manner (46). Because SecA binds to the Lep signal sequence when it has a +2 charge in the n-region (Fig. 2E), we assessed whether SecA and canine SRP would compete for binding to nascent L-Omp(+2)-85. As shown in Fig. 4, the addition of canine SRP reduced the extent of SecA photocross-linking to the nascent chain, thereby demonstrating that SecA and canine SRP compete for binding to an exposed RNC signal sequence. Do SecA and SRP also compete for binding to the same site on the ribosome? Because canine SRP binds to RNCs and even non-translating ribosomes with very high affinity (37) and because SecA also binds to ribosomes, the SecA and SRP binding sites may overlap or coincide.

Nascent Chain Photocross-linking to Ffh—Based on the extent of nascent chain photocross-linking (Fig. 2E), SecA interacts poorly with signal-anchor sequences but associates strongly with signal sequences. In contrast, bacterial SRP (Ffh + 4.5 S RNA) is thought to preferentially associate with membrane protein signal-anchor sequences (23, 47–52). We therefore compared the relative efficiencies of nascent chain photocross-linking to Ffh using some of the same constructs that we had used with SecA. As expected, essentially no photocross-linking to Ffh was observed for an Omp-ss(0) RNC lacking a signal sequence (Fig. 5). Hybrid L-Omp-nascent chains with the Lep signal-anchor sequence photocross-linked very efficiently to Ffh, whereas OmpA and L-Omp(n+2) photocross-linking to Ffh were less efficient, as shown in Fig. 5. A comparison of the data in Figs. 2E and 5 reveals that SecA and Ffh are notably complementary in their recognition of signal sequences. Although SecA associates more tightly with the n = +2 version of L-Omp than the n = 0 version, Ffh associates more tightly with the latter than with the former. Thus, bacterial SRP binds preferentially to membrane protein signal-anchor sequences, as others have noted (23, 47–52). But SecA binds more tightly to the nascent chain signal sequences that are poorly recognized by Ffh.

SecA binds to ribosomes in the absence of a nascent chain—Factors involved in nascent protein sorting often bind to the ribosome so as to interact with the appropriate nascent chain as it emerges from the ribosomal nascent chain tunnel (18, 24, 53–56). In fact, canine SRP binds significantly more tightly to translating than to non-translating ribosomes, and the affinity of SRP binding to the ribosome increases tremendously (but differentially) when SRP is exposed to different nascent chain signal sequences (37). Does SecA bind to a ribosome or RNC, and if so, is such binding nascent chain-dependent?

SecA association with ribosomes was evaluated by including SecA and [14C]Lys-tRNA in a wheat germ translation and then using gel filtration chromatography to separate SecA bound to ribosomes from free SecA. Ribosome elution was detected by absorbance at 260 and 280 nm (Fig. 6, A–D), [14C]Lys incorporation into nascent chains (Fig. 6, A–D), and agarose gel electrophoresis to detect ribosomal RNAs (data not shown), whereas SecA elution was detected by Western blotting (Fig. 6,
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**FIGURE 4.** Canine SRP competes with SecA for nascent chain binding. *A*, photoactive L-Omp(+2)-85 RNCs were prepared and analyzed as in Fig. 1, except that the translation was conducted in the presence of 200 nM SecA and increasing concentrations of canine SRP as indicated. The photoadducts contained radioactive nascent chains and SecA (closed circle) or SRP54 (open triangle). *B*, the average reduction and standard deviation in the number of SecA-nascent chain photoadducts formed is shown as a function of SRP concentration for three independent experiments, where 100% is the extent of photoadduct formation in the absence of SRP. Mr, apparent molecular mass.

**FIGURE 5.** Nascent chain photocross-linking to Ffh. RNCs were prepared in the presence or absence of 250 nM *E. coli* SRP, photolyzed, and analyzed as in Fig. 1 using εANB-Lys-tRNA and various truncated mRNAs as follows: OmpA-85, L-Omp, L-Omp(+2), and Omp-ss(0). OmpA derivatives were truncated at the site corresponding to codon 85 in wild-type *ompA* gene. The photocross-linking efficiencies of these nascent chains are shown relative to that of L-Omp (taken as 100%) taking into account the number of methionines in each nascent chain and the total nascent chains synthesized. Average data and standard deviations from at least three independent experiments are shown.

**FIGURE 6.** SecA binds to ribosomes. Translations that included SecA were fractionated by Sepharose CL-6B gel filtration (*A*–*D*), and the SecA content of individual fractions was determined by Western blotting (*E*–*H*). Samples contained L-Omp(+2)-85 mRNA (*A* and *E*), Omp-ss(0) mRNA truncated after codon 85 of the wild-type *ompA* gene (*B* and *F*), no mRNA (*C* and *G*), or no mRNA and wheat germ extract lacking ribosomes (ribosomes were removed by sedimentation of the extract in a Beckman TLA-100 rotor at 100,000 rpm at 4 °C for 30 min) (*D* and *H*). The fractions in which ribosomes elute are boxed.

E–H). Translations contained L-Omp(+2)-85 mRNA (Fig. 6, *A* and *E*), Omp-ss(0) mRNA (Fig. 6, *B* and *F*), or no mRNA (Fig. 6, *C* and *G*). A fourth sample lacked both mRNA and ribosomes after the latter were removed from the wheat germ extract by sedimentation prior to its being added to the sample (Fig. 6, *D* and *H*). The coincidence of A260–A280, rRNA, and nascent chain [14C]Lys (in the samples with mRNA) showed that the ribosomes eluted in fractions 12–14 (Fig. 6, *A–C*), the void volume of this chromatography system. Western blot analysis of the elution of SecA revealed that significant binding of SecA to ribosomes occurs (Fig. 6E) when the RNCs contain a signal sequence to which SecA binds (Fig. 2E). Yet surprisingly, SecA binding to ribosomes did not disappear when the RNCs either lacked a signal sequence (Fig. 6F) or had no nascent chain (Fig. 6G); SecA was absent in fractions 12–14 only when the sample lacked any ribosomes (Fig. 6H). SecA
imms (and hence protected from protease) either with or without added protease. As shown in Fig. 7 by the amount of radioactive OmpA protected from digestion by added temperature to inactivate the SecA (26). The absence of functional SecA could only translocate OmpA if functional SecA was added to the samples (compare lanes 3 and 4), whereas IMVs prepared from the SecAts strain were completely deficient in functional SecA (Fig. 7A, lane 6) but were otherwise completely functional in translocation despite the exposure to high temperature (Fig. 7A, lane 5).

To examine the initial stages of nascent chain interaction with the translocon, photoreactive OmpA-85 RNCs were translated in the presence of either wild-type IMVs or SecAts IMVs with or without added SecA. As shown in Fig. 7B, OmpA-85-nascent chains photocross-linked to SecY in wild-type IMVs whether or not additional SecA was present (lanes 1, 2), whereas SecY-OmpA-85 photocrosslinks were observed with SecAts IMVs only when functional SecA was added to the sample (lanes 3, 4). Because OmpA-85 photocrosslinks to SecY only when the nascent chain is in close proximity to the translocon protein, the residual SecA present in the purified wild-type IMVs was sufficient to effect targeting (Fig. 7B, lane 2). However, RNCs were unable to target to SecAts IMVs in the absence of added SecA (Fig. 7B, lane 4), thereby demonstrating that the IMVs purified from the SecAts strain had no SecA activity.

The SecAts IMVs were therefore used to assess nascent chain targeting to the SecYEG complex using preformed SecA-RNC complexes. Upon incubation with IMVs from SecAts cells that lacked functional SecA, the SecA-RNC complexes were able to target to the translocon and photocross-link to SecY (Fig. 7C, lane 3). However, no SecY-OmpA-85 photocrosslink was detected when photocrossreactive RNCs lacking SecA were incubated with the SecAts IMVs (Fig. 7C, lane 4). Thus, the SecA that was stably bound to RNCs (Fig. 6) effected proper targeting of the OmpA-85 RNC to the bacterial translocon, a result that strongly suggests that SecA is directly involved in targeting nascent chains cotranslationally to the SecYEG translocon.

In contrast to SecA binding to the signal sequence, which is ATP-independent (Fig. 3A), the SecA-dependent (Fig. 7C) targeting of RNCs to the translocon is ATP-dependent. When SecA-bound RNCs are exposed to SecA-depleted IMVs in the presence of ATP, the RNCs are targeted to the translocon, as evidenced by nascent chain photocrosslinking to SecY (Fig. 7C, lane 6). However, when the purified RNC-SecA complexes were resuspended into an apyrase-treated solution containing the same IMVs, no nascent chain photocrosslinking to SecY was observed (Fig. 7C, lane 7).
DISCUSSION

SecA in solution exhibits most of the same functional properties as mammalian SRP, the cytosolic component that identifies and directs nascent secretory and membrane proteins to the translocon in the endoplasmic reticulum membrane (57). Specifically, SecA binds and photocross-links to ribosome-bound nascent chains with an appropriate signal sequence but not to nascent chains lacking a signal sequence (Fig. 1). This SecA association with RNCs occurs early in translation, when the nascent chains are short (Fig. 1C), and is unaffected by the presence of either ATP or SecB (Fig. 3). SecA binds tightly to ribosomes whether or not they are translating an mRNA (Fig. 6), and the SecA binding site on ribosomes either coincides with or overlaps the SRP binding site (Fig. 4). Finally, SecA is required to target RNCs to SecYEG translocons in the membrane (Fig. 7). These combined data are therefore consistent with SecA in the cytosol functioning to select and target nascent secretory proteins in RNCs to translocons in the bacterial plasma membrane.

Yet, although mammalian SRP is released from the ribosome and membrane and recycled after the nascent chain is transferred to the eukaryotic translocon (57), SecA is intimately and actively involved in the translocation process at the SecYEG translocon (1–3, 6, 7). Because 50–75% of the SecA in the cell are found in the cytosol and are not membrane-bound (16, 58), the total cellular concentration of SecA is apparently sufficient to allow it to function both in events at the membrane and in selecting RNCs and targeting them to the translocon. Another functional difference between SecA and mammalian SRP is that the latter targets both nascent secretory and membrane proteins to the endoplasmic reticulum translocon (57). In contrast, SecA does not bind or photocross-link to nascent LepB, a bacterial membrane protein (Fig. 2, B and E); instead, signal-anchor sequences of membrane proteins preferentially associate with Ffh (23, 47–52). In fact, a systematic variation of the number of hydrophobic residues and N-terminal positive charges in the LepB signal-anchor sequence reveals that the nascent chains bound most tightly by SecA are the nascent chains bound most weakly by Ffh (Fig. 5), an observation that strongly suggests that these two sorting factors function in a complementary manner in the cell.

The observation that SecA in solution binds to the ribosome near its nascent chain exit site and appears to function as a sorting factor exacerbates an already-crowded environment at the exit site. When RNCs containing a photoactive probe in the signal sequence of OmpA were exposed simultaneously to SRP, TF, and SecA, a fraction of the nascent chains were photocross-linked to each of these components (18). One interpretation of these results is that the nascent chain is proximal to multiple cofactors when it emerges from the ribosomal nascent chain exit site.
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It therefore seems reasonable to focus on the dynamics of sorting factor interactions with the ribosome, in which the presence of a sorting factor at the exit site is dictated by its affinity (i.e. its association and dissociation rates) for the ribosome and its cellular concentration. In this situation, the frequency and duration of occupancy of a site near the exit site by the various factors would depend on their relative affinities and concentrations. Such a scheme is depicted in Fig. 8, where SecA, TF, and SRP each establish a binding equilibrium with free ribosomes that then determines the extent of nascent chain exposure to each of the sorting factors. Of course, two (or more?) of these factors could bind simultaneously to the ribosome; Fig. 8 shows them binding separately simply to avoid complicating the schematic. A particular pathway is selected when a sequence in an emerging nascent chain is recognized by and binds to its cognate sorting factor. The increased affinity arising from the binding of a sorting factor to its cognate nascent chain sequence (cf. the 20–160-fold lower K_d values for SRP-RNC complexes when the nascent chain contains a signal sequence (37)) would greatly slow the dissociation of the sorting factor from the ribosome, thereby directing the RNC into the appropriate pathway. If a non-cognate sorting factor happens to be bound at the exit site when a sorting sequence of the nascent chain (a signal sequence, a signal-anchor sequence, or neither) first emerges, the nascent chain would not bind to a sorting factor until the cognate factor bound to that RNC.

This model is odds with the current view of protein sorting in bacteria. Although SRP and TF have been shown to interact directly with nascent cytosolic and membrane proteins co-translationally to direct them into the appropriate sorting pathway, secretory proteins are thought to be fully translated and released from the ribosome before diffusing post-translationally and passively in the cell until they encounter and bind to an unoccupied SecYE translocon and SecA at the membrane (1–3, 6, 7). Some secretory proteins associate with SecB, but this interaction does not depend upon SecB binding to the signal sequence (8–10). Although SecA is known to bind to signal sequences (12–15), why has it not been previously thought to function in the sorting process prior to RNC arrival at the translocon?

One possible explanation is related to the competition for RNC binding described in the above model and depicted in Fig. 8. If TF, SRP, and SecA each bind to RNCs, then the amount of a particular sorting factor-RNC complex present in an in vitro experiment will be dictated by the relative concentrations and affinities of the sorting factors, as well as the presence or absence of a cognate sorting sequence in the nascent chain. It was for this reason that we chose to examine nascent chain interactions with SecA using an otherwise eukaryotic system that lacks TF, Fh, and SecA. The association of SecA with RNCs could then be examined without interference from unknown amounts of Fh and TF that co-purify with the ribosomes, IMVs, and other components. As has been noted previously (2), it is important to assess the functional role of a sorting factor in the complete absence of species that might compete with or modulate its activity. Because studies focusing on TF or Fh usually add an excess of those proteins to the in vitro incubations, any effects that SecA might elicit could be overwhelmed and hence invisible.

There are also at least two other possible explanations for cytosolic SecA not being recognized as a sorting factor for secretory protein RNCs. The first is that SecA has been shown by multiple laboratories to play an active and critical role at the membrane in facilitating protein export by binding to SecYEG and catalyzing protein transport through the translocon by an ATP-dependent mechanism (1–3, 6, 7). Translocon-bound SecA binds tightly to the signal sequence of a protein destined to be translocated through the plasma membrane (1–3, 6, 7), as well as to the SecB that binds to many transport substrates and maintains them in a translocation-competent state (10, 11). For this reason, most experiments that address the mechanisms of protein export in bacteria have, quite reasonably, focused on membrane-bound SecA.

The second is that bacterial secretory proteins can be translocated post-translationally into IMVs containing SecA (2). This has been interpreted to mean that no sorting factors are required for post-translational translocation, because as noted above, SecA must act at the membrane to effect translocation. Yet the fact that 50–75% of cellular SecA is found in the cytosol (16, 58) suggests that it does not function solely at the membrane. Instead, there may be two alternatives for secretory protein targeting to the translocon, one that occurs post-translationally and without any sorting factor assistance and one that is mediated cotranslationally by SecA. The choice of pathway is then presumably regulated by the physiological state of the cell.

Virtually every cellular process is regulated and/or catalyzed by a protein cofactor or enzyme, a statement that is also true for protein sorting, as evidenced by the actions of SRP and TF. Given the importance to the cell of accurate and timely protein sorting, it is therefore surprising that no bacterial protein has so far been identified as a positive sorting factor that actively directs secretory proteins to the SecYEG translocon. However, the experiments presented here in the absence of competing sorting factors strongly suggest that cytosolic SecA selects secretory protein RNCs by associating with RNC signal sequences that are not recognized or are recognized only poorly by Fh and TF, and SecA then targets the nascent chains to SecYEG translocons for export.

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REFERENCES

1. Mori, H., and Ito, K. (2001) Trends Microbiol. 9, 494–500
2. Müller, M., Koch, H.-G., Beck, K., and Schäfer, U. (2001) Prog. Nucleic Acid Res. Mol. Biol. 66, 107–157
3. de Keyzer, J., van der Does, C., and Driessen, A. J. M. (2003) Cell. Mol. Life Sci. 60, 2034–2052
4. Dalbey, R. E., and Kuhn, A. (2004) J. Cell Biol. 166, 769–774
5. van der Laan, M., Bechthold, P., Kol, S., Nouwen, N., and Driessen, A. J. M. (2004) J. Cell Biol. 165, 213–222
6. Vrontou, E., and Economou, A. (2004) Biochim. Biophys. Acta 1694, 67–80
7. Veenendaal, A. K. J., van der Does, C., and Driessen, A. J. M. (2004) Biochim. Biophys. Acta 1694, 81–95
8. Randall, L. L., Topping, T. B., and Hardy, S. J. S. (1990) Science 248, 860–863
9. Knoblauch, N. T. M., Rudiger, S., Schonfeld, H.-J., Driessen, A. J. M., Schneider-Mergener, J., and Bukau, B. (1999) J. Biol. Chem. 274, 34219–34225
10. Randall, L. L., and Hardy, S. J. S. (2002) Cell. Mol. Life Sci. 59, 1617–1623
11. Randall, L. L., Crane, J. M., Lilly, A. A., Liu, G., Mao, C., Patel, C. N., and Hardy, S. J. S. (2005) J. Mol. Biol. 348, 479–489
12. Akita, M., Sasaki, S., Matsuyama, S., and Mizushima, S. (1990) J. Biol. Chem. 265,
Selective SecA Association with Signal Sequences

8164–8169

13. Lill, R., Dowhan, W., and Wickner, W. (1990) Cell 60, 271–280
14. Miller, A., Wang, L., and Kendall, D. A. (1998) J. Biol. Chem. 273, 11409–11412
15. Kehr, M. O., and Kendall, D. A. (2002) Biochemistry 41, 5573–5580
16. Chun, S.-Y., and Randall, L. L. (1994) J. Bacteriol. 176, 4197–4203
17. Behrmann, M., Koch, H.-G., Hengelage, T., Wieseler, B., Hoffschulte, H. K., and Miessler, M. (1998) J. Biol. Chem. 273, 13889–13904
18. Eiser, G., Koch, H.-G., Beck, K., Brunner, J., and Muller, M. (2003) J. Cell Biol. 163, 35–44
19. Johnson, A. E., Woodward, W. R., Herbert, E., and Menninger, J. R. (1976)
20. Krieg, U. C., Walter, P., and Johnson, A. E. (1986)
21. Kurzchalia, T. V., Wiedmann, M., Girshovich, A. S., Bochkareva, E. S., Bielka, H., and Wintermeyer, W., Oudega, B., and Luirink, J. (1998) EMBO J. 7, 304–312
22. Beck, K., Wu, L.-F., Brunner, J., and Muller, M. (2000) EMBO J. 19, 134–143
23. Ullers, R. S., Houben, E. N. G., Raine, A., ten Hagen-Jongman, C. M., Oudega, B., Harms, N., and Luirink, J. (2003) J. Mol. Biol. 320, 679–684
24. Akiyama, Y., and Ito, K. (1990)
25. Akiyama, Y., and Ito, K. (1990)
26. Akiyama, Y., and Ito, K. (1986)
27. Buskiewicz, I., Deuerling, E., Gu, S.-Q., Jo¨ckel, J., Rodina, M. V., Bukau, B., and Wintermeyer, W. (2002) J. Biol. Chem. 277, 21675–21681
28. Nilsson, I., Whitley, P., and von Heijne, G. (1994) J. Cell Biol. 126, 1127–1132
29. MacFarlane, J., and Muller, M. (1995) Eur. J. Biochem. 233, 766–771
30. de Gier, J.-W. L., Mansournia, P., Valent, Q. A., Phillips, G. J., Luirink, J., and von Heijne, G. (1996) FEBS Lett. 399, 307–309
31. Ulbricht, N. D., Newitt, J. A., and Bernstein, H. D. (1997) Cell 88, 187–196
32. Koch, H.-G., Hengelage, T., Neumann-Haefelin, C., MacFarlane, J., Hoffschulte, H. K., Schimz, K.-L., Mecherl, B., and Muller, M. (1999) Mol. Biol. Cell 10, 2163–2173
33. Valenti, Jr., A. de, Gier, J.-W. L., von Heijne, G., Kendall, D. A., ten Hagen-Jongman, C. M., Oudega, B., and Luirink, J. (1997) Mol. Microbiol. 15, 187–196
34. Lee, H. C., and Bernstein, H. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3471–3476
35. Kramer, G., Rauch, T., Rist, W., Vorderwülbecke, S., Patzelt, H., Schulze-Specking, A., Ban, N., Deuerling, E., and Bukau, B. (2002) Nature 419, 171–174
36. Gu, S.-Q., Peske, F., Wieden, H.-J., Rodina, M. V., and Wintermeyer, W. (2003) RNA (N. Y.) 9, 566–573
37. Buskiewicz, I., Deuerling, E., Gu, S.-Q., Jo¨ckel, J., Rodina, M. V., Bukau, B., and Wintermeyer, W. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7903–7906
38. Raine, A., Ivanova, N., Wikberg, J. E. S., and Ehrenberg, M. (2004) Biochimie (Paris) 86, 495–500
39. Walter, P., and Johnson, A. E. (1994) Annu. Rev. Cell Biol. 10, 87–119
40. Cabelli, R. J., Dolan, K. M., Qian, L. P., and Oliver, D. B. (1991) J. Biol. Chem. 266, 24420–24427