Signal Peptides Bind and Aggregate RNA

AN ALTERNATIVE EXPLANATION FOR GTPase INHIBITION IN THE SIGNAL RECOGNITION PARTICLE*

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The targeting of nascent secretory and integral membrane proteins to the endoplasmic reticulum of eukaryotes and the inner membrane of bacteria has been extensively studied in recent years, and many details of the pathway have been uncovered (1, 2). In eukaryotes, targeting begins when the N-terminal signal sequence of a nascent secretory protein being synthesized by the ribosome is specifically recognized by the SRP54 homologue in Escherichia coli (3). The SRP54 homologue in Escherichia coli is part of a specialized system responsible for bringing polytopic inner membrane proteins to the cell membrane for cotranslational insertion (4–8). Compared with the eukaryotic SRP, which contains six protein subunits bound to a 7SL RNA scaffold, the E. coli SRP consists of a single protein subunit (Ffh, for fifty-four homologue) that is bound to a smaller but homologous RNA termed 4.5S RNA (1). Ffh and SRP54 share similar two-domain modular structures with an N-terminal GTPase (NG) domain and a C-terminal methionine-rich M domain (9, 10) that binds to both RNA and signal sequences (11–13). The relative simplicity of the E. coli SRP makes it a tractable model for the eukaryotic system.

Signal sequences function as molecular zip codes, specifying the destination of each nascent chain. Despite a lack of consensus among the signal sequences of different proteins, they do share some very general characteristics, such as an N-terminal region that is typically positively charged (the n-region) followed by a hydrophobic core of 7–13 amino acids (the h-region) and a C-terminal polar sequence containing a cleavage site recognized by signal peptidase (the c-region) (14). How such divergent sequences are specifically recognized by SRP has remained a puzzle for many years. High resolution structures of the M domains of Ffh and SRP54 suggest that signal sequences may be bound by a hydrophobic “finger” loop that has sufficient flexibility to mold itself to a wide variety of sequences (15, 16). Based on a crystal structure of the Ffh M domain complexed with 4.5S RNA, Batey et al. (17) recently proposed that an RNA surface contiguous with the finger loop cleft might contribute to the recognition of the positively charged n-region of the signal sequence. Whether these proposed models faithfully represent signal sequence recognition will have to await a structure of SRP54 or Ffh in a complex with a signal peptide.

The structural and functional consequences of signal sequence binding to SRP have long been the subject of intensive investigation. One attractive hypothesis that has been tested by a number of laboratories is that signal sequence binding to SRP54 or Ffh regulates the binding and/or hydrolysis of nucleotides by the NG domain. In related studies, isolated peptides corresponding to several N-terminal signal sequences aggregate RNA; the peptides in general use as “nonfunctional” negative controls (e.g. those with deletions or charged substitutions within the hydrophobic core), are sufficiently different in physical character that they do not aggregate RNA and thus have no effect on the GTPase activity of the signal recognition particle. We propose that the reported effect of functional signal peptides on the GTPase activity of the signal recognition particle is an artifact of the high peptide concentrations and low salt conditions used in these in vitro studies and that signal sequences at the N terminus of nascent chains in vivo do not exhibit this activity.

N-terminal signal sequences can direct nascent protein chains to the inner membrane of prokaryotes and the endoplasmic reticulum of eukaryotes by interacting with the signal recognition particle. In this study, we show that isolated peptides corresponding to several bacterial signal sequences inhibit the GTPase activity of the Escherichia coli signal recognition particle, as previously reported (Miller, J. D., Bernstein, H. D., and Walter, P. (1994) Nature 367, 657–659), but not by the direct mechanism proposed. Instead, isolated signal peptides bind nonspecifically to the RNA component and aggregate the entire signal recognition particle, leading to a loss of its intrinsic GTPase activity. Surprisingly, only “functional” peptide sequences aggregate RNA; the peptides in general use as “nonfunctional” negative controls (e.g. those with deletions or charged substitutions within the hydrophobic core), are sufficiently different in physical character that they do not aggregate RNA and thus have no effect on the GTPase activity of the signal recognition particle. We propose that the reported effect of functional signal peptides on the GTPase activity of the signal recognition particle is an artifact of the high peptide concentrations and low salt conditions used in these in vitro studies and that signal sequences at the N terminus of nascent chains in vivo do not exhibit this activity.

In contrast, prokaryotic cells use multiple pathways to target nascent protein chains to their final destinations (3). The SRP homologue in Escherichia coli is part of a specialized system responsible for bringing polytopic inner membrane proteins to the cell membrane for cotranslational insertion (4–8). Compared with the eukaryotic SRP, which contains six protein subunits bound to a 7SL RNA scaffold, the E. coli SRP consists of a single protein subunit (Ffh, for fifty-four homologue) that is bound to a smaller but homologous RNA termed 4.5S RNA (1). Ffh and SRP54 share similar two-domain modular structures with an N-terminal GTPase (NG) domain and a C-terminal methionine-rich M domain (9, 10) that binds to both RNA and signal sequences (11–13). The relative simplicity of the E. coli SRP makes it a tractable model for the eukaryotic system.

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¶ The abbreviations used are: SRP, signal recognition particle; Ffh, fifty-four homologue.
nucleotide-binding site in SRP54 can remain empty until its high affinity interaction with SRP receptor, arguing that signal sequence binding to SRP54 has little or no effect on GTP binding. In the arena of structural effects, peptides corresponding to functional signal sequences have been shown to destabilize the tertiary structure of isolated Ffh, an effect that can be reversed by the addition of 4.5S RNA (22). These studies would seem to indicate that signal sequences inhibit, enhance, or have no effect on nucleotide binding to SRP depending upon the experimental conditions used. Here we present an explanation for this apparent contradiction.

Our work demonstrates that experiments conducted with isolated peptides corresponding to signal sequences must be carried out and interpreted with extreme caution. We initially observed aggregation of SRP by signal peptides when making concentrated samples of Ffh-4.5S RNA plus LamB signal peptide analogues for structural studies by NMR. In an attempt to find conditions to circumvent this aggregation, we discovered that signal peptide-induced aggregation of SRP occurs even at much lower concentrations, such as those used to show that signal peptides inhibit the SRP GTPase activity (18, 19). In this study, we present evidence that peptides with the features of signal sequences (e.g. a positively charged region followed by a region of substantial hydrophobicity) nonspecifically bind and aggregate RNA molecules at micromolar concentrations. In the case of 4.5S RNA complexes with Ffh, signal peptide-induced RNA aggregation results in the aggregation of the entire SRP particle, which is the likely cause for its loss of GTPase activity.

**EXPERIMENTAL PROCEDURES**

**Preparation of Proteins, Peptides, and RNA**—Ffh and 4.5S RNA were prepared as described previously (22). 5S rRNA and lysine-specific tRNA were purchased from Roche Molecular Biochemicals and Sigma, respectively. 7SII RNA from canine pancreas was a generous gift from Harris Bernstein (National Institutes of Health). KRR-LamB, DM, R1, OmpA, and K-W(AL)10 peptides were synthesized and purified according to previously published methods (23). The purity and correct mass of each peptide were verified by high pressure liquid chromatography and mass spectrometry, and the concentration of each was determined by quantitative amino acid analysis.

**GTPase Activity Assays**—GTPase assays were conducted as described previously (19), with the exception that the concentrations of Ffh protein and 4.5S RNA were 25 and 50 nM, respectively. Reactions in buffer A (50 mM triethanolamine-acetic acid, pH 7.5, 25 mM potassium acetate, 2.5 mM magnesium acetate, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% octaethylene glycol monododecyl ether (19)) were allowed to continue for 20 min before quenching. However, Ffh protein and 4.5S RNA were 25 and 50 nM, respectively. Reactions in buffer A supplemented with K acetate, 2.5 mM magnesium acetate, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, and K5W(AL)10 were allowed to continue for 20 min before quenching. However, the low basal GTPase rate of Ffh at higher salt concentrations, such as those used to show that signal peptides inhibit the SRP GTPase activity (18, 19). In this study, we present evidence that peptides with the features of signal sequences (e.g. a positively charged region followed by a region of substantial hydrophobicity) nonspecifically bind and aggregate RNA molecules at micromolar concentrations. In the case of 4.5S RNA complexes with Ffh, signal peptide-induced RNA aggregation results in the aggregation of the entire SRP particle, which is the likely cause for its loss of GTPase activity.

**Aggregation Tests**—To test for aggregation of Ffh under the conditions of the GTPase activity assay, 0.5-ml samples of Ffh ± 4.5S RNA were prepared with the indicated concentrations of signal peptides exactly as indicated for the GTPase inhibition assay. After the addition of "cold" GTP and 30 min of incubation at room temperature, samples were centrifuged for 15 min in a microcentrifuge. Supernatants were carefully removed from the resultant pellets and precipitated by the addition of 0.25 ml of 20% trichloroacetic acid, incubated for 20 min on ice, and centrifuged for 15 min as before. Pellets and trichloroacetic acid precipitates were resuspended in SDS-polyacrylamide gel sample buffer, run on 10% SDS-polyacrylamide gels, and visualized by Comassie Blue staining. To test for aggregation of RNAs, each RNA (200 nM, except where indicated otherwise) was incubated with the indicated concentrations of signal peptides in GTPase assay buffer A at room temperature for 30 min. UV absorption spectra (230–300 nm) of these solutions were recorded, samples were microcentrifuged for 15 min, and the UV absorbance of the supernatants was remeasured for comparison.

**RESULTS**

**A LamB Signal Peptide Analogue That Inhibits the Ffh-4.5S RNA GTPase Activity Also Aggregates the Complex in the Same Concentration Range**—Miller et al. (19) demonstrates that a peptide corresponding to the wild-type LamB signal sequence (Table I) causes dose-dependent inhibition of the Ffh-4.5S RNA GTPase activity. A LamB signal peptide analogue containing an insertion of three basic residues in the n-region termed KRR-LamB (Table I) behaves similarly (22). This insertion increases the aqueous solubility of the LamB signal peptide (24) but is not expected to affect its in vivo function because the insertion of two basic residues in the n-region of the LamB signal sequence does not affect its ability to target nascent LamB protein (25). KRR-LamB inhibited the GTPase activity of the Ffh-4.5S RNA complex with an IC50 of 9.4 μM and with...
GTPase activity of the Ffh KRR-LamB that caused 50% aggregation and inhibition of the when KRR-LamB was added to 4.5S RNA, the RNA absorbance of KRR-LamB tested (120 μM KRR-LamB; diamonds, 6 μM KRR-LamB; triangles, 12 μM KRR-LamB; squares, 30 μM KRR-LamB). The inset at bottom is the absorption spectrum of 6 μM KRR-LamB alone. These data are represented in graphic form in panel b in which the loss of RNA from solution with increasing KRR-LamB concentration is indicated by the ratio of absorbance at 260 nm after centrifugation to the absorbance before centrifugation. Panel c graphically represents the increase in RNA absorbance that occurs with KRR-LamB addition. The percent increase in 260 nm absorbance at each peptide concentration was calculated relative to the absorbance of 4.5S RNA alone.

**Fig. 2.** The aggregation of Ffh-4.5S RNA by KRR-LamB is mediated by a direct interaction with 4.5S RNA. a, 4.5S RNA was tested for KRR-LamB-induced aggregation by brief centrifugation after incubating 4.5S RNA with various concentrations of peptide as described under “Experimental Procedures.” Solid curves with filled symbols and dashed curves with open symbols indicate absorbance spectra recorded before and after centrifugation, respectively. Circles, no KRR-LamB added; inverted triangles, 1.2 μM KRR-LamB; diamonds, 6 μM KRR-LamB; triangles, 12 μM KRR-LamB; squares, 30 μM KRR-LamB. The inset at bottom is the absorption spectrum of 6 μM KRR-LamB alone. These data are represented in graphic form in panel b in which the loss of RNA from solution with increasing KRR-LamB concentration is indicated by the ratio of absorbance at 280 nm after centrifugation to the absorbance before centrifugation. Panel c graphically represents the increase in RNA absorbance that occurs with KRR-LamB addition. The percent increase in 260 nm absorbance at each peptide concentration was calculated relative to the absorbance of 4.5S RNA alone. 

**Aggregation of Ffh-4.5S RNA by KRR-LamB Is Mediated by a Direct Interaction with 4.5S RNA**—Surprisingly, in similar aggregation tests conducted on Ffh alone in the absence of 4.5S RNA, Ffh did not pellet even at the highest concentration of KRR-LamB tested (120 μM, Fig. 1c), demonstrating that the RNA component must be present for aggregation to occur. To determine whether this is a direct effect of KRR-LamB on 4.5S RNA, we incubated 4.5S RNA alone and with increasing concentrations of KRR-LamB and then compared the UV absorbance spectra of these samples before and after brief centrifugation. Surprisingly, the addition of KRR-LamB to 4.5S RNA resulted in a dose-dependent loss of RNA from solution after centrifugation (Fig. 2, a and b). Half-maximal aggregation of 4.5S RNA by KRR-LamB occurred at a peptide concentration of 6 μM, which was directly comparable to the concentration of KRR-LamB that caused 50% aggregation and inhibition of the GTPase activity of the Ffh-4.5S RNA complex. Interestingly, when KRR-LamB was added to 4.5S RNA, the RNA absorbance increased in a dose-dependent manner with an EC_{50} of 6–12 μM (Fig. 2, a and c). This increase was not attributable to absorbance of KRR-LamB itself because this peptide had no aromatic residues and did not absorb in this region (Fig. 2a, inset).

**KRR-LamB Aggregates RNA Nonspecifically**—To determine whether KRR-LamB aggregates 4.5S RNA via a specific binding interaction, several different RNA species were tested for aggregation by this peptide including 7SL RNA from dog pancreas (the mammalian SRP RNA), 5S rRNA from *E. coli*, and lysine-specific tRNA from *E. coli* (Fig. 3). Each RNA alone did not pellet when centrifuged (black bars); however, incubation of each RNA with 6 μM KRR-LamB resulted in significant aggregation (dark gray bars). All of the heterologous RNAs were even more sensitive to aggregation by KRR-LamB than was 4.5S RNA. In each case, the aggregation was accompanied by a significant increase in the 260 nm absorbance of each RNA (data not shown) as was observed with 4.5S RNA. These results show that KRR-LamB aggregates RNA nonspecifically.

**Other Signal Peptides Also Aggregate RNA**—A peptide corresponding to the signal sequence of the *E. coli* outer membrane porin, OmpA, as well as an idealized peptide with a longer hydrophobic core (K_{5W(AL)10})_{2} (see Table I) were tested for aggregation of 4.5S RNA. As seen in Fig. 3, incubation with 5 μM OmpA signal peptide pellets 90% of the total 4.5S RNA, and the addition of 2 μM K_{5W(AL)10} pellets 71% of the total 4.5S RNA. These results indicate that this phenomenon is not limited to LamB and may be characteristic of signal peptides in general.

**The Hydrophobic Core Plays an Important Role in Aggregation of the Ffh-4.5S RNA Complex by Peptides**—To understand
what characteristics of signal peptides are important for aggregation of the Ffh-4.5S RNA complex, we tested peptides corresponding to functional and nonfunctional analogues of the LamB signal sequence. The DM deletion mutant peptide is missing four residues from the hydrophobic core (Table I), leading to a defect in translocation of LamB nascent chains in vivo (26); the lower helical propensity of this peptide compared with wild type has been proposed to be responsible for its in vivo defect (27). When added to GTPase reactions, DM does not inhibit the Ffh-4.5S RNA GTPase activity even at the highest concentration tested (30 μM) as shown in Fig. 2a. It is noteworthy that the half-maximal increase in RNA absorption was also shifted to higher KRR-LamB concentrations compared with 6–12 μM KRR-LamB in low salt buffer (Fig. 2b, compare with Fig. 2a).

Because the signal sequence/Ffh interaction is thought to be primarily hydrophobic in nature, we initially hypothesized that increasing the ionic strength of the buffer would strengthen peptide binding to its specific site on Ffh, possibly resulting in an increased IC₅₀ for GTPase inhibition. Contrary to this idea, however, the amount of KRR-LamB required to inhibit 50% of the total Ffh-4.5S RNA GTPase activity was increased to 60–120 μM in 175 mM K acetate (Fig. 5c) compared with 9.4 μM at low salt (Fig. 1a). When the buffer ionic strength was increased, the ability of KRR-LamB to both aggregate 4.5S RNA and inhibit the GTPase activity of Ffh-4.5S RNA was reduced by similar degrees, suggesting that the aggregation and GTPase inhibition are related events. Intriguingly, however, at low KRR-LamB concentrations in which significant aggregation did not occur (<30 μM), there appeared to be a subtle increase in Ffh-4.5S RNA GTPase activity (32% increase over control at 6 μM KRR-LamB, Fig. 5d). It is tempting to speculate that the increase in ionic strength has reduced the aggregation to an extent where the true effect of signal peptide binding to its site on Ffh is uncovered.

**DISCUSSION**

In this study, we show that signal peptides do not inhibit the E. coli SRP GTPase activity directly, as originally proposed (19), but instead cause aggregation of the entire SRP particle via a nonspecific interaction with the RNA component. We propose that the SRP GTPase activity is lost secondarily because of aggregation. The “nonfunctional” signal peptides used as negative controls in these earlier studies (18, 19) are unable to aggregate RNA, which explains their lack of effect on the SRP GTPase activity. In the simplest interpretation, the Ffh protein plays a passive role and is present in aggregates only because it exists in a high affinity complex with 4.5S RNA. These data suggest that the same signal sequence characteristics that are important for SRP recognition, i.e., an N-terminal charged region, a hydrophobic core, and helical propensity, also promote efficient but nonspecific RNA aggregation. It is important to point out that we have no evidence for an in vivo
The mechanism for this aggregation, although not central to the issue at hand, still remains a puzzle. Any model for this peptide-induced RNA aggregation must take into account the electrostatic, hydrophobic, and structural elements of the interaction. Because increasing the salt concentration to a physiological level reduces peptide-induced aggregation of the RNA (Fig. 5b), we propose that positively charged side chains in the n-region of the signal peptide interact with the negatively charged phosphodiester backbone of the RNA. The hydrophobic core of the signal peptide also plays an important role as illustrated by the result that the deletion of four hydrophobic residues (DM peptide) or the insertion of a negative charge into the core of the signal peptide also plays an important role as illustrated by the result that the deletion of four hydrophobic residues (DM peptide) or the insertion of a negative charge into the core of the signal peptide also plays an important role (17).

The molecular details of the binding are a topic of some dispute; however, a Raman spectroscopy study suggests that protamine binding to DNA is accompanied by significant base unstacking (30). The molecular mechanism for this aggregation is not the central issue. What is important is that they do aggregate RNA (28), although the possibility of light scattering has not been eliminated.

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in vitro and that the discovery of this artifact forces us to rethink the generally accepted scenario of events that occurs when nascent polypeptides are targeted by SRP.

For all peptides and conditions tested, the ability of a peptide to inhibit the Ffh-4.5S RNA GTPase activity was mirrored by its efficacy for aggregating the RNA component of that complex. Therefore, we conclude that the inhibition of the Ffh-4.5S RNA GTPase activity by signal peptides is a direct result of aggregation, leading to a loss of Ffh GTPase function, and not the result of allosteric modulation of the GTPase site upon signal peptide binding to Ffh as proposed previously (19). Furthermore, because functional signal peptides also aggregate 7SL RNA (Fig. 3), we extend these conclusions to the in vitro study of signal peptide interactions with mammalian SRP as well (18). These two studies of Miller et al. (18, 19) have been interpreted as indicating that signal sequence binding to SRP causes the release of nucleotide, stabilizing an empty site form. Our results suggest that this interpretation is unwarranted and that any real effect of signal peptides on SRP is obscured by an artifactual interaction with the SRP RNA. In fact, the increase in GTPase activity at low KRR-LamB concentrations in which aggregation is reduced (Fig. 5f) provides a tantalizing hint that signal peptides may enhance the GTPase activity. A recent study by Zheng and Nichitta (34) demonstrates that euakaryotic SRP has limited affinity for the wild-type LamB sequence, but a variant in which several hydrophobic core residues are mutated to leucine is efficiently targeted. Perhaps choosing a signal peptide sequence with higher affinity for Ffh or SRP54 combined with careful control of aggregation will finally allow determination of the true effect, if any, of signal sequence binding on the nucleotide-binding domain.

If signal peptides inhibit the Ffh-4.5S RNA GTPase activity artifactualy, by what mechanism do they inhibit the GTPase activity of the isolated Ffh protein as reported previously (22)? The interaction between LamB signal peptides and RNA is only one example of the unusual chaotropic behavior these peptides exhibit. It has been demonstrated that the binding of functional signal peptides to the isolated Ffh protein destabilizes the Ffh tertiary structure as observed by limited proteolysis (22). In our current thinking, inhibition of the Ffh GTPase activity by functional signal peptides is just a symptom of this overall loss of structural integrity.

This work demonstrates the extreme care that must be taken in conducting and investigating experiments with isolated signal peptides. Signal peptides are known to spontaneously insert into membranes (35–39) and have been shown here to bind and aggregate RNA nonspecifically; they are also prone to self-association at high concentrations. These propensities must carefully be considered in the experimental design of in vitro experiments involving peptides with the characteristics of signal sequences.

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