Activated GTPase movement on an RNA scaffold drives co-translational protein targeting

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Approximately one-third of the proteome is initially destined for the eukaryotic endoplasmic reticulum or the bacterial plasma membrane. The proper localization of these proteins is mediated by a universally conserved protein-targeting machinery, the signal recognition particle (SRP), which recognizes ribosomes carrying signal sequences and, through interactions with the SRP receptor, delivers them to the protein-translocation machinery on the target membrane. The SRP is an ancient ribonucleoprotein particle containing an essential, elongated SRP RNA for which precise functions have remained elusive. Here we used single-molecule fluorescence microscopy to show that the Escherichia coli SRP–SRP receptor GTPase complex, after initial assembly at the tetraloop end of SRP RNA, travels over 100 Å to the distal end of this RNA, where mutations disrupt GTPase activation (Fig. 1a, distal state). This posited an attractive hypothesis in which the Ffh–FtsY GTPase complex, after initial assembly near the tetraloop, can re-localize to the distal site of the SRP RNA ~100 Å away. Nevertheless, no functional evidence for the re-localization is available, nor are the importance, timing, mechanism and regulation of such a large-scale movement understood.

To address these questions, we used single-molecule fluorescence resonance energy transfer (smFRET) and total internal reflection fluorescence (TIRF) microscopy to detect conformational dynamics of individual SRPs. Migration of the SRP–FtsY GTPase complex on the SRP RNA was tracked using FRET between a donor (Cy3) and an acceptor (Quasar 670) labelled near the RNA distal end (Fig. 1a). Stable SRP–FtsY complexes, formed with the non-hydrolysable GTP analogue 5′-guanylyl-imidodiphosphate (GMPPNP), displayed rapid transitions among multiple FRET states (Fig. 1b, c). A low FRET state (~0.1; L) was assigned to the proximal state in which the GTPase complex resides near the SRP RNA tetraloop. A high FRET state (~0.8; H) was attained ~20% of the time and assigned to the distal state in which the GTPase complex stably docks at the distal site, as verified below. Cy3 attached to the Ffh NG domain showed similar transitions but with a lower FRET value in the H state (Supplementary Fig. 2a, b), consistent with Ffh being further from the distal site than FtsY. These results directly demonstrate dynamic movements of the SRP–FtsY GTPase complex on the SRP RNA that span over 100 Å.

We used hidden Markov modelling (HMM)-based statistical analyses to determine the most likely sequence of FRET transitions. This revealed an ensemble of additional states with intermediate FRET values (0.3–0.6; M1 and M2) and extremely short lifetimes (Fig. 1b–d and Supplementary Figs 2b–g and 3a–c), representing alternative binding modes of the GTPase complex on the SRP RNA. The transition information was pooled into a transition density plot that describes the number of distinct FRET states, their FRET values, and their transition frequencies (Fig. 1e and Supplementary Fig. 2h). In addition, the kinetics of FRET transitions were obtained from dwell-time analyses (Fig. 1f, g and Supplementary Figs 2i–o and 3d–h). Whereas molecules leaving the L state rapidly transitioned to all the other states, the H state (Supplementary Fig. 2a, b) consistent with Ffh being further from the distal site than FtsY. This result directly demonstrates dynamic movements of the SRP–FtsY GTPase complex on the SRP RNA that span over 100 Å.

To test whether the H state is responsible for GTPase activation, we isolated mutant RNAs that specifically perturb the distal docking site. The 82-nucleotide RNA lacking this site reduced GTPase activation sixfold, whereas a ‘super-active’ mutant, 99A, enhanced GTP hydrolysis 2.5-fold (Fig. 2a, green bars and Supplementary Figs 1b and 4a). The 82-nucleotide RNA lacking this site reduced GTPase activation sixfold, whereas a ‘super-active’ mutant, 99A, enhanced GTP hydrolysis 2.5-fold (Fig. 2a, green bars and Supplementary Figs 1b and 4a).

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Figure 1 | smFRET–TIRF microscopy reveals dynamic movements of the SRP–FtsY complex on the SRP RNA. a, smFRET setup for the SRP–FtsY complex. FtsY Cys 345 is labelled with Cy3. The 5′-end of the DNA splint (2 nucleotides from the 3′-end of SRP RNA) is labelled with Quasar 670. b, Fluorescent signals (top) and FRET trajectory (bottom) of the SRP–FtsY complex on the SRP RNA. a, smFRET setup for the SRP–FtsY complex. FtsY Cys 345 is labelled with Cy3. The 5′-end of the DNA splint (2 nucleotides from the 3′-end of SRP RNA) is labelled with Quasar 670. c, Magnification of the grey box in b to depict the four FRET states resolved by HMM. d, smFRET histogram depicting the distribution of molecules in different states. In M state, the M1 and M2 states are binned together. e, Transition density plot for the GTPase movements. f, g, Analysis of the transition kinetics between L and H states (f). Exponential fits of the data gave the transition-rate constants (k) given in g.

Translocation of pPL results in cleavage of its signal sequence, allowing the targeting and translocation to be efficiency quantified (Supplementary Fig. 4b). Furthermore, the specificity of targeting was tested using pPL variants in which the signal sequence is systematically varied (Supplementary Fig. 4d). Mutant 82-nucleotide RNA substantially reduced the targeting of correct substrates (wild type, 8L-pPL and 7L-pPL; Fig. 2b, c and Supplementary Fig. 4c, e). By contrast, the superactive 99A RNA targeted these substrates more efficiently than wild-type SRP, without compromising the discrimination against incorrect substrates (Fig. 2b, c and Supplementary Fig. 4c, e). Thus the SRP RNA distal site, although reportedly not essential for cell survival, does enhance efficient and accurate co-translational protein targeting.

SRP and FtsY undergo an unusual GTPase cycle, driven by multiple conformational rearrangements in their heterodimer that culminate in GTPase activation (Fig 3a–d, cartoons). We questioned how these rearrangements within the GTPase complex drive its global movements on the SRP RNA, using conditions that block the GTPase cycle at distinct stages. SRP by itself exhibited no movements on the RNA (Fig. 3a and Supplementary Fig. 7c, d). Although GTPase movements were observed in the open complex, which lacks optimal positioning of the catalytic loops and hence can be isolated by leaving out GTP analogues. No GTPase movement was observed at this stage either (Fig. 3b and Supplementary Fig. 7c, d). Although GTPase movements were observed in the open complex, most of them only reached M1 and M2 but did not extensively populate the H state (Fig. 3c–e and Supplementary Fig. 7c, d). Thus, GTP-induced rearrangements within the NG domain complex drive its global movements on the SRP RNA. Moreover, stable GTPase docking at the RNA distal site requires optimal positioning of the catalytic loops, explaining why mutants that block GTPase activation, such as FtsY(A335W), severely impair protein targeting.

If the GTPase complex only transiently reaches the SRP RNA distal site where GTPase activation occurs, previous ensemble measurements would have considerably underestimated the hydrolysis rate. 635-nm laser to confirm the presence of the complex. A.U., arbitrary units.
the RNA distal end (Fig. 4a and Supplementary Fig. 10a, b), but is still unaffected by mutations in cargo, as RNC carrying luciferase, which contains no signal sequence, (Fig. 4b and Supplementary Fig. 9a–e). This is specific to the correct SecYEG complex, completely abolished the GTPase movements on the RNA distal site. RNC FtsQ, which carries an obligate SRP substrate mechanism of this event is no longer affected by mutations in SecYEG complex to the RNC–FtsQ–SRP–FtsY complex (Supplementary Fig. 11a). SecYEG restored the high FRET state (Fig. 4d, e). It also prevents premature GTP hydrolysis and is essential for ensuring the SRP–FtsY complex in the proximal state and prevent its re-localization to the efficiency and specificity of the SRP pathwaya. We wanted to know whether (early) and activated (late) states. Conditions for isolating each conformational state are described in the text and Methods. e. Summary of the FtsY(A335W) (Supplementary Fig. 11c). Thus, SecYEG drives productive docking of the GTPase complex at the RNA distal site and thus re-activates GTP hydrolysis.

We therefore performed real-time GTPase assays using the smFRET setup. If GTP hydrolysis at the distal site, which drives irreversible SRP–FtsY dissociation, occurred faster than their return to the proximal state, we would observe high FRET ‘bursts’ with GTP instead of the reversible transitions with GMPPNP. This was indeed observed (Fig. 3f). The duration between these bursts has a rate constant (0.59 s⁻¹; Supplementary Fig. 8b) expected for rearrangement to the activated complex (−1 s⁻¹) and is similar to the ensemble GTPase rate (0.7 s⁻¹), strongly suggesting that the latter is rate limited by GTPase movement to the RNA distal site. The duration of the high FRET bursts includes GTP hydrolysis and subsequent SRP–FtsY disassembly and exhibits a rate constant of 7.1 s⁻¹ (Supplementary Fig. 8a), providing a lower limit for the actual hydrolysis rate and is at least tenfold faster than ensemble measurements.

These results also show that GTP drives almost irreversible movement of the GTPases to the RNA distal site, necessitating accurate control of the timing of this movement. Indeed, ribosome-nascent chain complexes (RNC or cargo) delay GTPase activation in the SRP–FtsY complexa, which activates or SRP substrate FtsQ, completely abolished the GTPase movements on the RNA (Fig. 4b and Supplementary Fig. 9a–e). This is specific to the correct cargo, as RNC carrying luciferase, which contains no signal sequence, exerted no effects (Fig. 4c and Supplementary Fig. 9f). Further, GTP hydrolysis in the presence of RNC is no longer affected by mutations in the RNA distal end (Fig. 4a and Supplementary Fig. 10a, b), but is still reduced by a mutation in FtsY active site (Supplementary Fig. 10d, e). These results demonstrate that correct cargo stabilizes the GTPase complex in the proximal state and prevent its re-localization to the RNA distal site, thus exerting the pausing effect.

On the target membrane, RNC must be transferred from the targeting to the translocation machinery. Both the mechanism of this transfer and its timing have remained long-standing challenges. To test whether the translocon helps to regulate these events, we added the SecYEG complex and ATP, which could represent a transient intermediate in the targeting and translocation reaction. These results also suggest that SecYEG drives the GTPase movement through two mechanisms: (1) displacing the GTPase complex from the proximal site, as indicated by the reappearance of the H state even with RNC present (compare Fig. 4d with Supplementary Fig. 9b); and (2) prolonging productive docking at the RNA distal site (Fig. 4i). Finally, non-productive movements to intermediate FRET states are minimized with RNC and SecYEG present (Fig. 4g, h). Considering the size of SRP RNA relative to the ribosome, the RNC possibly masks non-productive GTPase docking sites on the SRP RNA, which could also explain the conserved length of this RNA.

In summary, we demonstrate that the SRP RNA provides a molecular scaffold that mediates large-scale movements of the SRP–FtsY complex and ATP, which could represent a transient intermediate in the targeting and translocation reaction. These results also suggest that SecYEG drives the GTPase movement through two mechanisms: (1) displacing the GTPase complex from the proximal site, as indicated by the reappearance of the H state even with RNC present (compare Fig. 4d with Supplementary Fig. 9b); and (2) prolonging productive docking at the RNA distal site (Fig. 4i). Finally, non-productive movements to intermediate FRET states are minimized with RNC and SecYEG present (Fig. 4g, h). Considering the size of SRP RNA relative to the ribosome, the RNC possibly masks non-productive GTPase docking sites on the SRP RNA, which could also explain the conserved length of this RNA.
complex, which is tightly regulated by the GTPase cycle of SRP and FtsY, the translating ribosome and the SecYEG translocon. Together with previous studies, we propose a molecular model for the SRP–FtsY complex in the presence of SecYEG, with the wild-type (step 1), the SRP RNA tetraloop is optimally positioned adjacent to the translating ribosome and FtsY, the translating ribosome and the SecYEG translocon. Together with previous studies, we propose a molecular model for GTP hydrolysis, thus minimizing abortive reactions due to premature SRP–FtsY disassembly or non-productive loss of cargo.

Nucleic-acid-mediated protein movement is a widespread phenomenon and has been observed with the spliceosome, helicases and different factors and precise timing of molecular events in a cellular scaffold to coordinate multiple protein interactions and large-scale protein rearrangements, thus enabling productive exchange of nascent chains.

**METHODS SUMMARY**

**Materials.** Protein, SRP RNA, messenger RNA, RNC and SecYEG were prepared as described in Methods. Ffh and FtsY were labelled with Cy3 at a unique cysteine residue, Cys153 and Cys345, respectively, using maleimide chemistry as described in ref. 12. Labelled DNA splint was purchased from Biosearch Technologies. Translation extracts and salt-washed, trypsin-digested microsomal membranes were prepared as described in ref. 21.

**Single-molecule instrumentation and imaging.** Olympus IX-81 microscope was modified as described previously in ref. 19. SRP was immobilized by extending the 5′-end of SRP RNA, which hybridizes to a complementary oligonucleotide (DNA splint) 3′-labelled with biotin and thus coupled to PEGylated streptavidin-coated coverslip surface through biotin–streptavidin interaction. Fluorescent signals were split by DualView (Photometrics) and recorded with an EMCCD camera (Andor). The data were extracted and analysed by scripts written in IDL and MATLAB. HMM was carried out using the HaMMy program, and the transition heat-map was plotted with TDP software.

**Biochemical assays.** Assays for GTPase activity and for co-translational protein targeting and translocation were performed and analysed as described previously in refs 8 and 21, and are summarized in Methods.

**Full Methods** and any associated references are available in the online version of the paper.

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METHODS
Plasmids. Plasmids for in vivo expression of Ffh, full-length FtsY and SRP RNA, and for in vitro transcription of FtsQ, luciferase and pPL and its signal sequence variants have been described previously25,31. The P/EK20 construct for SecYEG expression was a gift from A. Driessen25. Plasmids for mutant SRP RNAs and mutant proteins were constructed using the QuikChange mutagenesis protocol (Stratagene) following manufacturer’s instructions. The plasmid for in vitro transcription of hammerhead ribozyme-SRP RNA-hepatitis delta virus (HDV) ribozyme was a gift from A. Ferre-D’Amare26. The hammerhead coding sequence was removed and the 5'–end of SRP RNA was extended using the QuikChange mutagenesis protocol to make in vitro transcription constructs for single-molecule RNA (smRNA) constructs.

Protein preparations. Wild-type and single-cysteine mutants of Ffh and FtsY were expressed and purified as described previously4. In brief, Ffh expression was induced in logarithmically growing BL21(DE3) pLySE cells with 1 mM isopropyl-β-D-thiogalactoside (IPTG). The soluble fraction from lyzed cells was purified by cation-exchange chromatography on SP Sepharose Fast Flow resin (GE Healthcare) using a gradient of 0.25–1 M NaCl, and was further purified by gel-filtration chromatography on the Superose12 column (Amersham Biosciences). His-tagged full-length FtsY was expressed in BL21(DE3)pLyS cells by induction with 0.5 mM IPTG in logarithmically growing cells. The soluble fraction from lyzed cells was purified by anion-exchange chromatography using Q Sepharose Fast Flow resin (GE Healthcare) with a gradient of 150–500 mM NaCl, followed by affinity purification using nickel-nitritolactric acid (Ni-NTA) resin (Qiagen). For GT-Pase assays, FtsY was further purified by anion-exchange chromatography on the MonoQ column (Amersham Biosciences) using a gradient of 150–350 mM NaCl. All proteins were exchanged into SRP buffer (50 mM potassium-HEPES, pH 7.5, 150 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 0.1% (w/v) BSA) before use.

Detergent-solubilized SecYEG was expressed in BL21(DE3) cells with 0.5 mM IPTG and was purified following published procedures25,28,32,33. Cells were lysed by sonication and the membranes were collected by ultracentrifugation. SecYEG was extracted and purified by cation-exchange chromatography on the SP Sepharose Fast Flow resin (GE Healthcare) followed by affinity purification with Ni-NTA (Qiagen). DDM (Affinitrax) was used for purification of solubilized SecYEG, which has been shown to be fully functional in binding RNC (D.A. & S.S., manuscript in preparation)28, in mediating nascent peptide translocation32, and stimulating SecA ATPase activity33.

Fluorescence labelling. Single-cysteine mutants of Ffh and FtsY were labelled with Cy3-maleimide (GE Healthcare) as described in ref. 12. Protein concentration during labelling was 50–100 µM, and the dye was in tenfold molar excess. Labelling reaction was carried out in buffer A (50 mM potassium-HEPES, pH 7.0, 300 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol) with gentle shaking at room temperature (22 °C) for 2 h. Unconjugated dyes were removed by gel-filtration chromatography using Sephadex G-25 resin (Sigma). Mass spectrometry confirmed >95% labelling efficiencies. Fluorescence labelling and modifications of the SRP RNA for surface immobilization (Fig. 1a and Supplementary Fig. 1b) were prepared by coupling Cy5-maleimide (Invitrogen) for 1 h at 37 °C. Excess dyes were removed by gel-filtration chromatography and quantified by autoradiography.

SRP complexes were assembled in SRP buffer under the following conditions. Basic–FtsY complex with labelled Ffh: 1 µM DNA–smRNA hybrid, 2 µM Ffh–Cy3, 5 µM FtsY, 100 µM GMPNP. SRP–FtsY complex with labelled FtsY: 1 µM DNA–smRNA hybrid, 2 µM Ffh, 3 µM FtsY–Cy3, 100 µM GMPNP. RNC–SRP–FtsY complexes: 200 nM DNA–smRNA hybrid, 400 nM Ffh–Cy3, 500 nM RNCfrag or 10 µM RNC lactosylceramidase 1 µM FtsY, 100 µM GMPNP. SecYEG solubilized in 0.02% DDM was added to RNCfrag–SRP–FtsY complex at 10 µM. The samples were then diluted to 50 µM in imaging buffer (SRP buffer supplemented with 0.4% glucose and 1% Gloxy in Trolox), flowed on the sample chamber and incubated for 5 min before imaging. Movies were recorded at 30-ms intervals for up to 5 min until most fluorescent molecules were photobleached. A red laser was used to initiate the translocation event at the end of the movie to confirm the presence of immobilized SRP. Data analysis. Single-molecule data were pooled out by scripts written in IDL and MATLAB. In brief, fluorescent peaks in the images were identified and tracked throughout the trajectory. Traces that showed a single-donor bleaching event were used for data analysis. HMM was calculated using the HaMMy program21. Transition density map was generated by the TDP program29 using the output from HaMMy. FRET histograms were generated using home-written script in MATLAB30. Transition kinetics between different states was obtained by exponential fits to dwell-time histograms. Two-dimensional scatter plots of the average dwell time of individual molecules during transitions were generated using the home-written script in MATLAB.
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