

SecYEG activates GTPases to drive the completion of cotranslational protein targeting

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Signal recognition particle (SRP) and its receptor (SR) comprise a highly conserved cellular machine that cotranslationally targets proteins to a protein-conducting channel, the bacterial SecYEG or eukaryotic Sec61p complex, at the target membrane. Whether SecYEG is a passive recipient of the translating ribosome or actively regulates this targeting machinery remains

unclear. Here we show that SecYEG drives conformational changes in the cargo-loaded SRP–SR targeting complex that activate it for GTP hydrolysis and for handover of the translating ribosome. These results provide the first evidence that SecYEG actively drives the efficient delivery and unloading of translating ribosomes at the target membrane.

Introduction

Correct cellular localization of proteins is essential for the proper functioning of all cells. A conserved protein-conducting channel, SecYEG in bacteria or Sec61p in eukaryotes, is the point of convergence of post- and cotranslational protein targeting pathways and mediates the translocation or integration of newly synthesized proteins (Cross et al., 2009; du Plessis et al., 2011). In bacteria, the major posttranslational pathway is mediated by the chaperone SecB and the SecYEG-interacting ATPase SecA, which deliver fully synthesized proteins to the periplasm (Hartl et al., 1990; Zhou and Xu, 2003; Driessen and Nouwen, 2008). The cotranslational route is mediated by signal recognition particle (SRP) and its receptor (SR), which target ribosome-nascent chain complexes (RNCs) to SecYEG (Walter and Johnson, 1994; Cross et al., 2009).

Extensive work on the Sec pathway showed that SecYEG and preproteins stimulate SecA's ATPase activity and activate it to drive the translocation of preproteins into the periplasm (Duong, 2003; Karamanou et al., 2007; Gold et al., 2010; Deville et al., 2011; Kedrov et al., 2011; Dalal et al., 2012). Whether SecYEG also regulates the activity of SRP and SR to drive cotranslational protein targeting is unknown. The SecY subunit of SecYEG forms a stable complex with the translating ribosome by interacting with the ribosomal exit site and the signal sequence of the nascent protein (Van den Berg et al.,

2004; Cannon et al., 2005; Frauenfeld et al., 2011). It is unclear, however, whether SecYEG simply binds RNCs released from SRP or actively regulates the activity of the RNC–SRP–SR targeting complex.

The functional core of SRP is the SRP54 GTPase (Ffh in bacteria) bound to the 4.5 S RNA (Poritz et al., 1990). The bacterial SR, FtsY, has a GTPase domain highly homologous to that in Ffh (Montoya et al., 1997a). During targeting, SRP and FtsY form a heterodimer in which both proteins undergo a series of conformational changes, including the early, closed, and activated states, that culminate in reciprocal GTPase activation (Fig. 1 A; Egea et al., 2004; Shan et al., 2004, 2009; Zhang et al., 2009). To ensure efficient and faithful delivery of cargo proteins to the target membrane, these rearrangements are regulated by RNC (Zhang et al., 2009) and phospholipids (Lam et al., 2010; Braig et al., 2011). RNC stabilizes the early intermediate but disfavors its rearrangement to the subsequent states. This generates a highly stable early targeting complex in which RNC is predicted to bind SRP with picomolar affinity and GTP hydrolysis is delayed (Zhang et al., 2009). Rearrangement of this complex to the closed/activated states, however, is required for the unloading of RNC (Shan et al., 2007; Zhang et al., 2009) and activation of GTP hydrolysis. In part, these rearrangements could be driven by anionic phospholipids, which stabilize the

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Abbreviations used in this paper: DACM, *N*-(7-dimethylamino-4-methylcoumarin-3-yl) maleimide; DDM, dodecyl- β -D-maltopyranoside; FRET, Foster resonance energy transfer; GppNHp, 5'-guanylylimido-diphosphate; RNC, ribosome-nascent chain complex; SR, SRP receptor; SRP, signal recognition particle.

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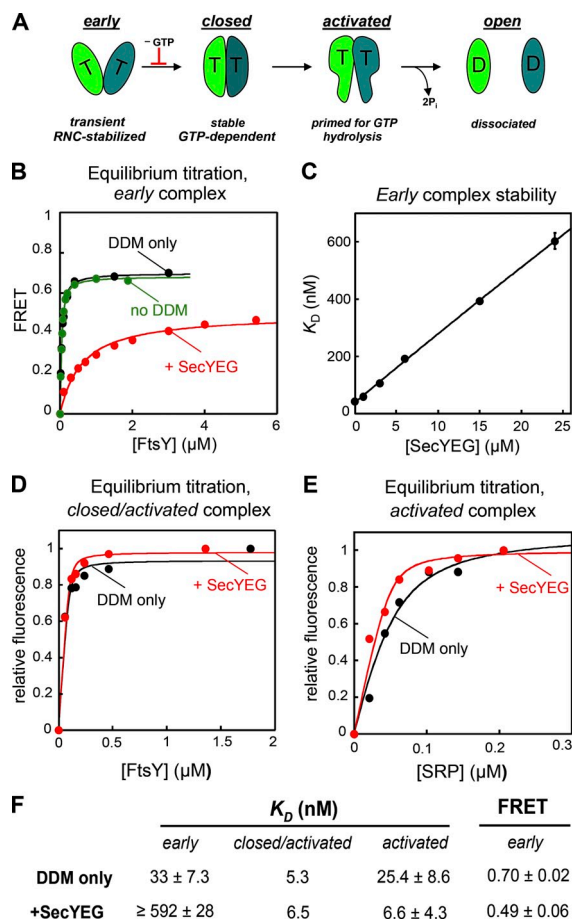


Figure 1. SecYEG destabilizes the early intermediate in the RNC-SRP-FtsY complex. (A) Conformational states of the SRP-FtsY complex. Green and blue shapes denote FtsY and Ffh, respectively. T denotes GTP and D denotes GDP. (B) Equilibrium titration of the early complex in the absence (green) and presence of 0.05% DDM (black) or 24 μM SecYEG (red). The data were fit to Eq. 1 and gave the K_D values and FRET end points shown in F. (C) Effect of SecYEG on the stability of the early complex. The K_D values of the early complex were determined as in A at the indicated concentrations of SecYEG. (D) Equilibrium titration of the closed/activated complex was performed in the presence of 0.02% DDM (black) or 12 μM SecYEG (red), using 100 nM acrylodan-labeled SRP, 230 nM RNC, and 200 μM GppNHp. The data were fit to Eq. 1 and gave the K_D values shown in F. (E) Equilibrium titration of the activated complex was performed in the presence of 0.01% DDM (black) or 10 μM SecYEG (red), using 50 nM acrylodan-labeled FtsY C356, 300 nM RNC, and 200 nM GppNHp. The data points of the two titrations overlapped at 0.2 μM SRP, concealing the data point for the -SecYEG titration. The data were fit to Eq. 1 and gave the K_D values shown in F. (F) Summary of the K_D values and FRET end points from the experiments in B-E. The values are averages of two to four experiments \pm SD. B-E show representative data from two to four experiments.

closed/activated states of FtsY (Lam et al., 2010). Membranes, however, are insufficient to drive the release of RNC (Song et al., 2000) or reverse the RNC-induced delay of GTP hydrolysis (Fig. S1). What drives these late events during the targeting reaction is unknown.

Here we show that SecYEG drives late conformational changes of the targeting complex and reactivates GTP hydrolysis. Our results demonstrate an active role of SecYEG in cotranslational protein targeting and suggest a concerted mechanism for handover of RNC from the targeting to the translocation machinery.

Results and discussion

SecYEG destabilizes the early targeting complex

We previously showed that RNCs stabilize the SRP-FtsY GTPase complex in the early conformational state, disfavor its rearrangement to subsequent conformations, and delay GTPase activation (Zhang et al., 2008, 2009). These effects prevent abortive reactions and are beneficial at early stages of the pathway. However, they pose a barrier to the cargo unloading and GTPase activation required at later stages of the pathway. To test whether SecYEG can help drive these late events, we purified SecYEG (Fig. S2 A) and first showed that it is active in RNC binding (Fig. S2 B). Furthermore, when reconstituted into proteoliposomes, SecYEG was active in SecA-dependent translocation of proOmpA (Fig. S2 C) and cotranslational translocation of a modified alkaline phosphatase bearing an SRP-dependent signal sequence derived from DsbA (Fig. S2 D). We focused on SecYEG solubilized in dodecyl- β -D-maltopyranoside (DDM) in this work, as it is fully functional in interaction with RNC, mediates proper insertion of the signal sequence (Mothes et al., 1998; Fig. S2B), and activates SecA (Duong, 2003; Gold et al., 2010; Deville et al., 2011). Further, because liposomes also exert stimulatory effects on the basal activity of FtsY and accelerate formation of the SRP-FtsY complex (Lam et al., 2010), the use of SecYEG in DDM allows us to distinguish its effects from those of phospholipids.

We asked whether and how SecYEG regulates the conformation of the SRP and FtsY GTPases in the RNC-SRP-FtsY complex. We first tested the early intermediate, which is stabilized $\sim 10^2$ -fold by RNCs bearing highly hydrophobic signal sequences such as 1A9L (Zhang et al., 2009). As this intermediate can form with or without GTP but its subsequent rearrangement is strictly GTP dependent, it can be isolated by leaving out GTP analogues (Zhang et al., 2008; Fig. 1 A). Foster resonance energy transfer (FRET) between coumarin-labeled SRP Cys235 and BODIPY-FL-labeled FtsY Cys487 was used to monitor formation of this intermediate (Zhang et al., 2008). Equilibrium titrations showed that SecYEG destabilized the RNC-SRP-FtsY early intermediate at least 15-fold, whereas DDM had no effect (Fig. 1, B and F). The destabilizing effect of SecYEG increases linearly with its concentration (Fig. 1 C), which indicates strong antagonism between SecYEG and the early complex. Importantly, SecYEG also lowered the FRET end point (Fig. 1, B and F), which suggests that it alters the conformation of the GTPases in the early complex.

We next asked whether SecYEG affects the subsequent states in the GTPase complex (Fig. 1 A). To test this, we used an environmentally sensitive dye, acrylodan, conjugated to SRP Cys235. In the presence of a nonhydrolyzable GTP analogue, 5'-guanylylimido-diphosphate (GppNHp), acrylodan at this position specifically changes fluorescence upon formation of the closed/activated complex (Zhang et al., 2009). Equilibrium titrations using this assay gave similar K_D values in the absence and presence of SecYEG (Fig. 1, D and F). As the K_D value of this complex is >20 -fold below the concentration of SRP needed for reliable titrations, a possible effect of SecYEG

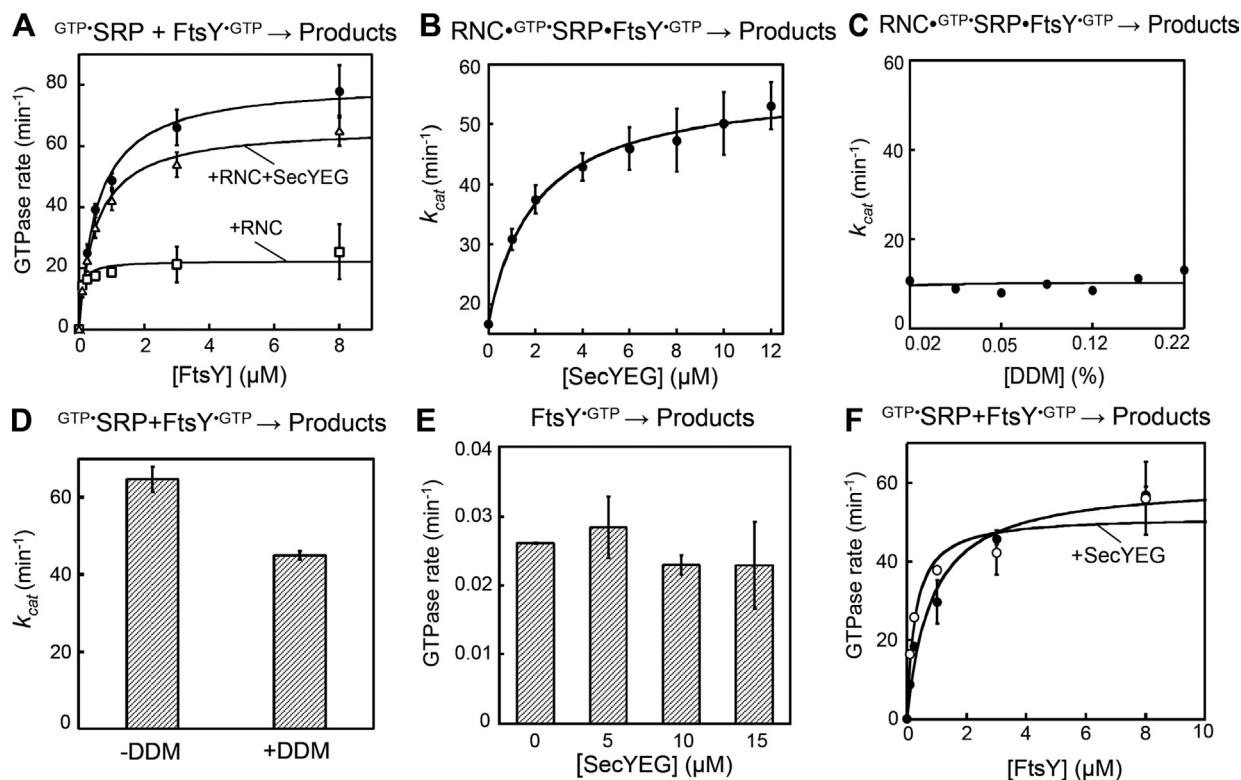


Figure 2. **SecYEG reactivates GTP hydrolysis from the RNC-SRP-FtsY complex.** (A) Stimulated GTP hydrolysis by SRP and FtsY in the absence (circles) and presence (squares) of RNC, and in the presence of both RNC and SecYEG (triangles). The data were fit to Eq. 3 and gave k_{cat} values of 80, 22, and 66 min^{-1} for the SRP-FtsY, RNC-SRP-FtsY, and RNC-SRP-FtsY-SecYEG complexes, respectively. (B and C) Effect of SecYEG (B) or DDM (C) on GTP hydrolysis from the RNC-SRP-FtsY complex. The data in B were fit to Eq. 3 and gave a K_d value of 2 μM and a k_{max} value of 57 min^{-1} . (D) DDM reduces the GTP hydrolysis rate from the SRP-FtsY complex. k_{cat} values were determined as in A in the absence and presence of 0.02% DDM. (E) SecYEG does not affect the basal GTPase activity of FtsY. Reactions were performed in the presence of 4 μM FtsY, 100 μM GTP, and the indicated concentrations of SecYEG. (F) In the absence of RNC, SecYEG does not significantly affect GTP hydrolysis from the SRP-FtsY complex. The data were fit to Eq. 3 and gave k_{cat} values of 60 and 51 min^{-1} in the absence (closed) and presence (open) of 12 μM SecYEG, respectively. The data in A, B, D, E, and F are the average of two experiments \pm SD (error bars).

on its stability might have escaped detection. To more specifically monitor the activated complex, we used acrylodan-labeled FtsY Cys356, which specifically detects movements of catalytic loops required for GTPase activation (Zhang et al., 2009). This assay revealed a two- to fourfold stabilization of the activated complex by SecYEG (Fig. 1, E and F). Together, these results demonstrate that SecYEG drives conformational changes of the targeting complex by destabilizing the early intermediate and modestly favoring the activated complex (see Fig. 5 A).

SecYEG reactivates GTP hydrolysis by the targeting complex

If SecYEG drives GTPase rearrangements to the activated state, it would reverse the RNC-induced delay of GTPase activation and reactivate GTP hydrolysis. To test this hypothesis, we determined the effect of SecYEG on the GTP hydrolysis rate of the SRP-FtsY complex (k_{cat}). As observed previously, RNC_{1A9L} delays GTPase activation, reducing the k_{cat} value from 80 min^{-1} to 22 min^{-1} . SecYEG restored the k_{cat} value to 66 min^{-1} , approaching that of the SRP-FtsY complex alone (Fig. 2 A). This stimulatory effect of SecYEG was saturable, with an apparent K_d value of ~ 2 μM (Fig. 2 B), which could represent the affinity of SecYEG for the targeting complex. The stimulation was not an artifact of DDM, as DDM concentrations up to 0.22% did

not affect GTP hydrolysis from the RNC-SRP-FtsY complex (Fig. 2 C). In comparison, the concentration of DDM in the assay was $<0.14\%$, as determined by a phenol-sulfuric acid reaction (Dubois et al., 1956). Indeed, even 0.02% DDM had an inhibitory effect on the stimulated GTPase activity in the absence of RNC (Fig. 2 D), which suggests that the actual GTPase stimulation by SecYEG may be even greater than observed here.

As SecYEG was reported to interact with FtsY (Angelini et al., 2006; Kuhn et al., 2011), we asked if SecYEG affects the basal GTPase activity of FtsY or its stimulated GTPase reaction with SRP in the absence of RNC. No stimulation was observed in either case (Fig. 2, E and F), which indicates that SecYEG specifically exerts its stimulatory effect only when the SRP-FtsY complex is bound to RNC.

SecYEG associates with the targeting complex via interaction with the ribosome

How does SecYEG reactivate GTP hydrolysis from the targeting complex? In the simplest model, SecYEG removes RNC from SRP, regenerating the SRP-FtsY complex that hydrolyzes GTP faster. Release of RNC from SRP onto the Sec61p translocon was previously observed in reconstituted eukaryotic targeting reactions (Görlich and Rapoport, 1993; Jungnickel and Rapoport, 1995; Song et al., 2000; Fulga et al., 2001). Alternatively,

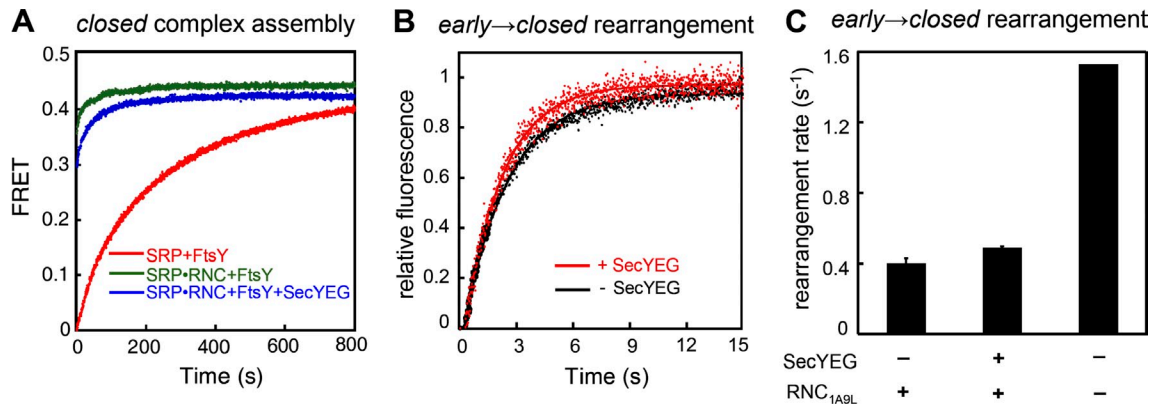


Figure 3. **SecYEG forms a quaternary complex with RNC, SRP, and FtsY.** (A) Kinetics of the closed complex assembly were measured in the absence (red) and presence of 100 nM RNC (green), and in the presence of 100 nM RNC and 7 μ M SecYEG (blue). Reactions contained 40 nM DACM-labeled SRP, 100 nM BODIPY-FL-labeled FtsY, and 200 μ M GppNHp. (B) Kinetics of the early \rightarrow closed rearrangement of the RNC–SRP–FtsY complex in the absence (black) and presence (red) of 12 μ M SecYEG. The data were fit to Eq. 2. Single exponential fits to the data gave rearrangement rate constants of 0.403 ± 0.027 and 0.489 ± 0.008 s⁻¹ with and without SecYEG, respectively. (C) Summary of the early \rightarrow closed rearrangement rate constants. The value of 1.5 s⁻¹ was obtained in the absence of SecYEG and RNC. A and B show representative data from three replicates. Error bars indicate average \pm SD.

SecYEG forms a quaternary complex with RNC, SRP, and FtsY at steady-state, in which GTP hydrolysis is activated. To distinguish between these possibilities, we analyzed the effect of SecYEG on the kinetics of formation of the closed SRP–FtsY complex. RNC dramatically accelerates the assembly of this complex (Zhang et al., 2009), providing a robust diagnostic for whether it is bound to SRP (Fig. 3 A, red and green). If SecYEG did not remove RNC from SRP, then the kinetics of closed complex formation in the presence of SecYEG would remain rapid. Consistent with this prediction, in the presence of SecYEG and RNC, the rate of closed complex assembly was similar to that with RNC-bound SRP and much faster than with free SRP (Fig. 3 A). SecYEG itself does not affect complex assembly between SRP and FtsY (Fig. S3), and thus is not responsible for the fast assembly kinetics observed in Fig. 3 A. Further, in the presence of both SecYEG and RNC, the early \rightarrow closed rearrangement of the SRP–FtsY complex occurred at the same rate, within error, as that with the RNC-loaded SRP, and was significantly slower than with free SRP (Fig. 3, B and C).

Together, these results strongly suggest that restoration of efficient GTP hydrolysis by SecYEG is caused by the formation of a quaternary RNC–SRP–FtsY–SecYEG complex in which the GTPase activity of SRP and FtsY was stimulated. The K_d value of 2 μ M observed in Fig. 2 B is likely the dissociation constant of SecYEG from this complex. We speculate that this quaternary complex is transient during protein targeting. It accumulated under our reaction conditions either because the length of the nascent polypeptide used here (\sim 85 residues) is suboptimal for its transfer to SecYEG (Mothes et al., 1998; Park and Rapoport, 2011) or because phospholipid membranes may be needed further to drive the efficient transfer of cargo. Regardless of the specific model, the observation of a quaternary complex suggests that the handover of RNC from SRP to SecYEG could occur through a concerted mechanism, in which both SRP and SecYEG are associated with RNC.

How does SecYEG associate with the targeting complex? Conserved residues in the cytosolic loops of SecYEG, R255 and R256 in the C4 loop and R357 in the C5 loop, serve as

universal binding sites for cytosolic factors including the ribosome (Ménétret et al., 2007), FtsY (Kuhn et al., 2011), and SecA (Alami et al., 2007; Kuhn et al., 2011). We therefore asked whether mutation of these residues abolishes the stimulatory effects of SecYEG. To this end, we purified SecYEG containing charge reversal mutations at these three residues (mtSecYEG; Fig. S2 A). In agreement with previous reports (Ménétret et al., 2007), mtSecYEG was defective in ribosome binding (Fig. S2 B). In contrast to wild-type SecYEG (wtSecYEG), mtSecYEG could not restore efficient GTP hydrolysis from the targeting complex. Charge reversal mutations in either the C4 (R255E/R256E) or C5 (R357E) loop of SecYEG also significantly disrupted its ability to reactivate the GTPases, but their defects are not as severe as those of mtSecYEG (Fig. 4, A and B). We further tested the ability of mtSecYEG to destabilize the early intermediate and found that, relative to wtSecYEG, this activity was significantly impaired (Fig. 4, C and D). Thus, the conserved basic residues in the cytosolic loops of SecYEG are essential for its ability to drive conformational changes in the targeting complex and to reactivate GTP hydrolysis. These results also support the specificity of the stimulatory effects observed with SecYEG and suggest that the action of its cytosolic loops occurs before RNC docking, when the targeting machinery first contacts SecYEG.

SecYEG could use its basic cytosolic loops to contact either the ribosome (Ménétret et al., 2007; Becker et al., 2009) or the A domain of FtsY (Kuhn et al., 2011). To distinguish between these possibilities, we removed the N-terminal 196 residues comprising the A domain of FtsY, generating mutant FtsY-NG (Parlitz et al., 2007). FtsY-NG could mediate the formation of the RNC-stabilized early intermediate with SRP with a K_d value similar to that of the complex formed by full-length FtsY (Fig. 4, E and F). SecYEG destabilized the early intermediate formed by FtsY-NG to the same extent, within error, as that formed by full-length FtsY (Fig. 4, E and F). These results are in agreement with the poor conservation of the A domain in bacteria (Eitan and Bibi, 2004) and its dispensability in vivo (Eitan and Bibi, 2004; Parlitz et al., 2007). Our results here

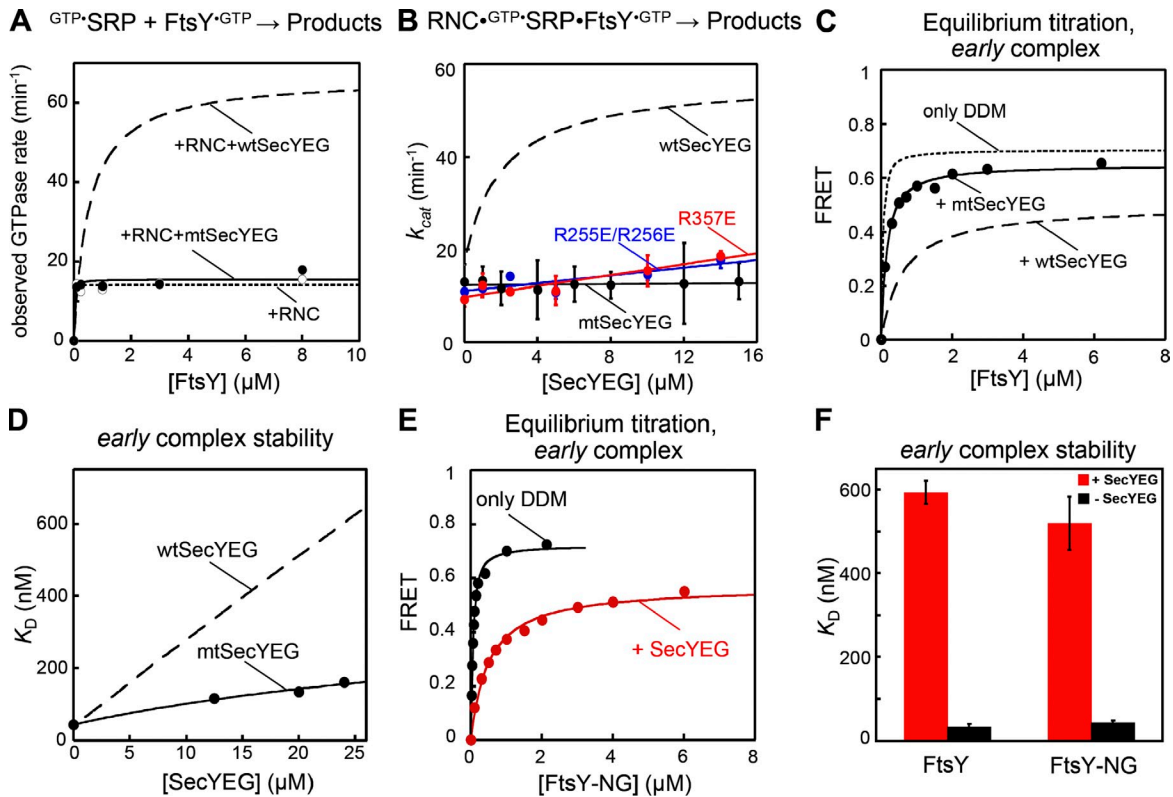


Figure 4. **Mutations in basic cytosolic loops of SecYEG abolish its stimulatory effects.** (A) Stimulated GTP hydrolysis by the RNC-bound SRP and FtsY in the absence (open circles) and presence (closed circles) of 12 μM mtSecYEG. The data were fit to Eq. 3. The broken line is the data for wild-type SecYEG from Fig. 2 A. (B) Effect of SecYEG charge reversal mutants on GTP hydrolysis from the RNC–SRP–FtsY complex. Solid black, R255E/R256E/R357E (mtSecYEG); red, R357E; blue, R255E/R256E. The broken line is the data for wild-type SecYEG from Fig. 2 B. (C) Equilibrium titration of the early complex in the presence of 24 μM mtSecYEG (closed circles). The data were fit to Eq. 1 and gave a K_d value of 162 ± 4 nM. Titration in the presence of DDM (solid line) and wild type SecYEG (broken line) are from Fig. 1. (D) Effect of mtSecYEG on the stability of the early complex. K_d values were determined as described in C. The data with wild-type SecYEG (broken line) are from Fig. 1 C. (E) Equilibrium titration of the early targeting complex formed by FtsY-NG in the presence of 0.05% DDM (black) or 24 μM SecYEG (red). The data were fit to Eq. 1 in the Materials and methods, and K_d values are given in F. (F) Summary of the effects of SecYEG on the stability of the early targeting complex formed with full-length FtsY and FtsY-NG. The data with full-length FtsY are from Fig. 1. A, C, D, and E show representative data from two to three replicates. Error bars in B and F are standard deviations from two to three experiments.

show that the A domain is not essential for the ability of SecYEG to drive conformational changes in the targeting complex. Instead, the basic cytosolic loops in SecYEG likely interact with the ribosomal protein L23 at the nascent polypeptide exit site (Ménétrez et al., 2007; Becker et al., 2009), thus exerting its stimulatory effects.

Collectively, the results here provide the first evidence that SecYEG actively modulates the conformation of the targeting complex to drive completion of the cotranslational protein targeting reaction. Combined with previous work (Song et al., 2000; Gold et al., 2010; Lam et al., 2010; Braig et al., 2011), these results suggest that SecYEG and anionic phospholipids serve overlapping yet distinct functions in mediating the delivery of the targeting complex to sites of translocation. The targeting complex preferentially localizes to regions of the membrane enriched in anionic phospholipids (Fishov and Wolfring, 1999; Vanounou et al., 2003; Erez et al., 2010; Lam et al., 2010), with which SecYEG may also preferentially associate (Campo et al., 2004; Shiomi et al., 2006; Gold et al., 2010). Although phospholipids can induce rearrangements in FtsY to favor the closed/activated states (Lam et al., 2010), they are insufficient to overcome

the stabilizing effect of RNC on the early complex (Lam et al., 2010) and reactivate the GTPases in the RNC–SRP–FtsY complex (Fig. S1). Association with SecYEG is required to overcome these “stalling” effects of RNC. Together, phospholipids and SecYEG drive the rearrangement of the targeting complex to the activated conformation, which enables the transfer of RNC to SecYEG and activates GTP hydrolysis, thus completing the targeting cycle. As shown in Fig. 3, SecYEG likely exerts these effects by forming a transient quaternary intermediate with the targeting complex, in which it displaces the SRP GTPase from the ribosomal exit site (Fig. 5 B; Halic et al., 2006). As the early complex is stabilized by interaction with the SRP RNA tetraloop (Shen and Shan, 2010), which is optimal only when the SRP NG domain interacts with L23, displacement of the GTPase complex from the ribosome exit site would also explain the destabilizing effect of SecYEG on the early complex. The questions of the fate of the signal sequence in the quaternary intermediate, what drives its transfer to SecYEG, and whether SecYEG interacts with FtsY’s GTPase domain (Angelini et al., 2005, 2006; Kuhn et al., 2011) during these events invite future investigations.

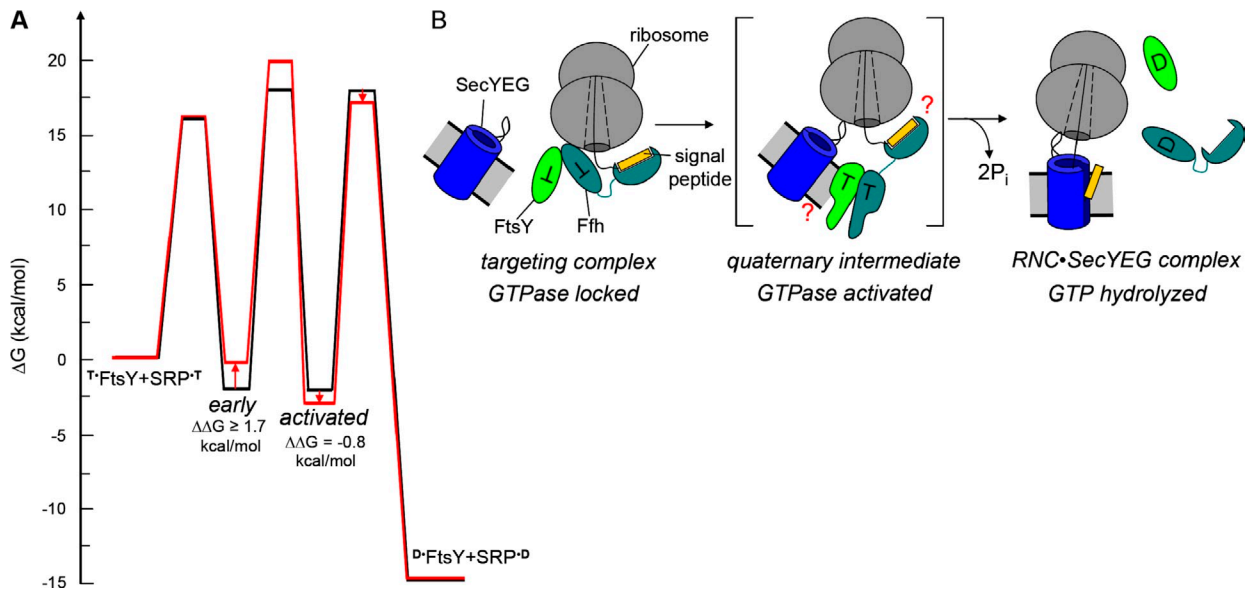


Figure 5. **SecYEG drives conformational changes in the RNC-SRP-FtsY complex.** (A) Free energy profile for the FtsY-SRP interaction in the absence (black) and presence (red) of SecYEG. The red arrows denote the effect of SecYEG. Activation energies were calculated from the rate constants using $\Delta G^\ddagger = -R\ln[kh/(k_B T)]$, where $R = 1.987 \text{ cal}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$, $h = 1.58 \times 10^{-37} \text{ kcal}\cdot\text{s}$, $k_B = 3.3 \times 10^{-27} \text{ kcal}\cdot\text{K}^{-1}$, and $T = 298 \text{ K}$. The relative free energies were calculated from the equilibrium stability of the complexes using $\Delta G = -RT\ln K$, where K is the equilibrium constant. A standard state of $1 \mu\text{M}$ was used. T denotes GTP and D denotes GDP. (B) Model for the role of SecYEG in driving GTPase rearrangements in the targeting complex and completing cotranslational protein targeting. The M domain of Ffh is also shown. The question mark denotes questions regarding the fate of the signal sequence and the interaction of SecYEG with FtsY in the quaternary complex.

Materials and methods

Materials

Full-length FtsY, FtsY-NG, Ffh, and 4.5 S RNA were expressed and purified as described previously (Montoya et al., 1997b; Peluso et al., 2001). Single cysteine mutants of FtsY and Ffh were labeled with fluorescent dyes, *N*-(7-dimethylamino-4-methylcoumarin-3-yl) maleimide (DACM), the maleimide derivative of BODIPY-FL, or acrylodan (Invitrogen) as described previously (Zhang et al., 2008, 2009). RNCs bearing a hydrophobic signal sequence 1A9L were prepared as described previously (Schaffitzel and Ban, 2007; Zhang et al., 2009). For GTPase assays, RNCs were further purified by sucrose gradient fractionation to collect monosomes free of GTPase contaminants, as described previously (Zhang et al., 2009). Liposomes were prepared from *Escherichia coli* polar lipid extract (Avanti Polar Lipids, Inc.) as described previously (de Leeuw et al., 2000; Lam et al., 2010). DDM solid was from Anatrace.

4.5 S RNA expression and purification

Expression and purification of 4.5 S RNA was performed as described in Peluso et al. (2001). In brief, DH5 α cells transformed with pSN1 were grown in Luria broth in the presence of 100 $\mu\text{g}/\text{ml}$ ampicillin and 1 mM IPTG for 10 h at 37°C. The cells were resuspended in 20 mM KOAc, pH 4.7/1 mM EDTA and extracted with acid phenol:chloroform. RNA was precipitated with isopropanol, dissolved in water, and further purified using gel filtration (TSK3000SW BAT column; Tosoh Bioscience LLC). 4.5 S RNA was precipitated with isopropanol, air dried, and stored as a pellet in ethanol at -20°C. The RNA was dissolved in the assay buffer (50 mM KHepes, pH 7.5, 150 mM KOAc, 10 mM Mg(OAc)₂, and 2 mM DTT) and quantitated using absorbance at 260 nm (1 AU = 40 $\mu\text{g}/\text{ml}$).

Fluorescence labeling

Site-specific labeling of FtsY and Ffh with fluorescent dyes was performed as described in Zhang et al. (2008, 2009). Single cysteine mutants of FtsY or Ffh were dialyzed in labeling buffer (50 mM KHepes, pH 7.0, 300 mM NaCl, and 2 mM ethylenediaminetetraacetic acid) and treated with 2 mM TCEP for 2 h. The reduced protein was incubated with five-fold molar excess of the maleimide derivative of DACM or BodipyFL for 4 h, or with a 30-fold molar excess of acrylodan for 16 h. The reaction was quenched with DTT and the dye was separated from the labeled protein by gel filtration using Sephadex G-25 (Sigma-Aldrich). Labeling efficiency was determined using the following absorption coefficients: DACM, $\epsilon_{383} = 27,000 \text{ M}^{-1}\text{cm}^{-1}$; BodipyFL, $\epsilon_{504} = 79,000 \text{ M}^{-1}\text{cm}^{-1}$; acrylodan, $\epsilon_{391} = 20,000 \text{ M}^{-1}\text{cm}^{-1}$.

Preparation of RNC

RNCs were generated and purified as described previously (Schaffitzel and Ban, 2007; Zhang et al., 2009), using in vitro translation in a membrane-free extract from *E. coli* MRE600 cells. mRNA containing a truncated mature region of PhoA with 1A9L signal sequence and SecM stalling sequence was translated at 37°C for 25 min. Stalled RNCs were bound to a Strep-Tactin Sepharose column (IBA), eluted with desthiobiotin (Sigma-Aldrich), pelleted, and redissolved in the assay buffer. For GTPase assays, monosomes were purified using a 10–50% continuous sucrose gradient and ultracentrifugation at 23,000 rpm 4°C for 15 h (SW-32 rotor; Beckman Coulter). The monosome fraction was pelleted at 55,000 rpm 4°C for 15 h (TLA-55 rotor; Beckman Coulter) and dissolved in assay buffer.

Expression and purification of SecYEG

SecYEG containing N-terminally His₆-tagged SecY was expressed from plasmid pEK20 (du Plessis et al., 2009), a gift from A. Driessen (University of Groningen, Groningen, Netherlands). SecY charge reversal mutants were generated based on pEK20 using the QuikChange mutagenesis protocol (Agilent Technologies). SecYEG was expressed in BL21 (DE3) cells and purified using previously described protocols (Van den Berg et al., 2004; Dalal and Duong, 2010) with modifications. All steps were performed at 4°C. Cells were lysed by sonication in KS300G buffer (50 mM KHepes, pH 7.5, 300 mM NaCl, and 10% glycerol). After removal of intact cells (12,000 g, 20 min), membranes were collected by ultracentrifugation at 42,000 rpm for 45 min (Ti45; Beckman Coulter), and extracted for 1 h in KS100G buffer (50 mM KHepes, pH 7.5, 100 mM NaCl, and 10% glycerol) containing 1% DDM per 10 mg/ml total protein. The suspension was clarified by ultracentrifugation at 42,000 rpm for 32 min (Ti70 rotor; Beckman Coulter). The supernatant was purified by cation exchange on SP-Sepharose Fast Flow resin (GE Healthcare; 12 ml per 6 liters of cells) in KS100G/0.02% DDM, and eluted using a gradient of 100–1,000 mM NaCl. Elution fractions containing SecYEG were further purified by affinity chromatography on Ni-NTA Agarose (Qiagen; 2 ml of resin per 6 liters of cells). Protein was loaded and washed with KS300G/0.02% DDM/20 mM imidazole, and eluted with KS300G/0.02% DDM/300 mM imidazole. Purified SecYEG was dialyzed against 50 mM KHepes, pH 7.5, 150 mM KOAc, 10% glycerol, 2 mM DTT, and 0.02% DDM for 12 h. The concentration of SecYEG was determined using absorbance at 280 nm and an extinction coefficient of 71,000 $\text{M}^{-1}\text{cm}^{-1}$ (Kedrov et al., 2011).

The single and double charge reversal mutants of SecYEG were purified as the wild-type SecYEG. The triple charge reversal mutant of SecYEG was purified by two rounds of immobilized metal affinity chromatography using Ni-Sepharose resin (GE Healthcare), following the procedure similar to that described for wild-type SecYEG except that 40 mM imidazole was used during binding and washing, and a 50–500-mM imidazole gradient was used during elution.

Fluorescence measurements

Fluorescence assays were performed as described previously (Zhang et al., 2008, 2009; Lam et al., 2010). All measurements were performed at room temperature in assay buffer (50 mM KHepes, pH 7.5, 150 mM KOAc, 10 mM Mg(OAc)₂, 2 mM DTT, and 0.01% Nikkol) supplemented with 0.02% DDM when necessary. Stability of the early complex was determined using FRET between DACM-labeled SRP Cys235 and BODIPY-FL-labeled FtsY Cys487. Equilibrium titrations were performed with 40 nM SRP, 110 nM RNC where applicable, and 100 μM GDP, with FtsY as a titrant. The data were fit to Eq. 1:

$$F_{obsd} = F_1 \times \frac{[SRP] + [FtsY] + K_d - \sqrt{([SRP] + [FtsY] + K_d)^2 - 4 \times [SRP][FtsY]}}{2 \times [SRP]}, \quad (1)$$

where F_{obsd} is the observed FRET, F_1 is the maximum FRET value at saturating FtsY concentrations, and K_d is the equilibrium dissociation constant of the early complex. Scattering due to SecYEG was subtracted before calculating the FRET values.

The stability of the closed/activated complex was determined using acrylodan-labeled SRP Cys235, with FtsY as the titrant. The stability of the activated complex was determined using acrylodan-labeled FtsY Cys356, with SRP as the titrant. Reactions were supplemented with 0.02% DDM. The data were fit to a quadratic equation similar to Eq. 1. When fluorescent FtsY was used, the denominator in Eq. 1 was replaced with $2 \times [FtsY]$.

The assembly kinetics of the closed complex from free SRP and FtsY was determined in the presence of GppNHp using FRET between DACM-labeled SRP Cys235 and BODIPY-FL-labeled FtsY Cys487, on a Fluorolog 3–22 (Horiba Jobin Yvon) as described previously (Zhang et al., 2009). The rate constant for association of SRP and FtsY (k_{on}) in the absence and presence of SecYEG was determined by measuring the observed rate of association (k_{obsd}) at varying FtsY concentrations as described previously (Zhang et al., 2009). The FtsY concentration dependence of k_{obsd} was fit to the equation $k_{obsd} = k_{on}[FtsY] + k_{off}$, in which k_{on} is the rate constant for complex assembly and k_{off} is the rate constant for complex disassembly.

The rate of early \rightarrow closed rearrangement was determined by preforming the early complex with 50 nM acrylodan-labeled SRP C235, 100 nM RNC, and 5 μM FtsY in the absence or presence of SecYEG. Rearrangement to the closed complex was initiated by addition of 200 μM GppNHp and monitored as an increase in acrylodan fluorescence on a stopped-flow apparatus (KinTek). The data were fit to Eq. 2:

$$F_{obsd} = F_0 + (F_{max} - F_0)(1 - e^{-kt}), \quad (2)$$

in which F_0 and F_{max} are the initial and final fluorescence values, respectively, F_{obsd} is the observed fluorescence, and k is the rearrangement rate constant.

GTPase assay

GTP hydrolysis reactions were performed in assay buffer, and were performed and analyzed as described previously (Peluso et al., 2001). Stimulated GTP hydrolysis of SRP with FtsY was determined using 40 nM SRP, 100 nM RNC where applicable, and increasing concentrations of FtsY as indicated. Wherever applicable, 8–12 μM SecYEG was added last and incubated with the reaction mixture for 10 min before initiation of reaction. The data were fit to Eq. 3:

$$k_{obsd} = k_{cat} \times \frac{[FtsY]}{K_m + [FtsY]}, \quad (3)$$

in which k_{obsd} is the observed rate constant, k_{cat} is the rate constant at saturating FtsY, and K_m is the concentration of FtsY required to reach the half-maximal rate.

Dose-dependent effects of SecYEG on GTP hydrolysis were measured using a preincubated mixture of 40 nM SRP, 100 nM RNC, and 3 or 8 μM of FtsY, to which an increasing concentration of SecYEG was

added before initiation of reaction. The data were fit to an equation analogous to Eq. 3, except that $[FtsY]$ is replaced with $[SecYEG]$, k_{cat} is replaced with k_{max} , and K_m is replaced with K_d .

Co-sedimentation assay

Interaction of SecYEG with RNC was determined using a cosedimentation assay as described previously (Ménétré et al., 2007; Frauenfeld et al., 2011) with modifications. 200 nM RNC was incubated with 1 μM SecYEG in assay buffer supplemented with 0.05% DDM for 35 min at room temperature. 35 μl of the solution was layered onto 200 μl of 30% sucrose solution in assay buffer supplemented with 0.05% DDM, and ultracentrifuged at 100,000 rpm for 12 min at 4°C (TLA-100 rotor; Beckman Coulter). The pellet was dissolved in SDS gel loading buffer and resolved on a 15% SDS gel.

Reconstitution of SecYEG into proteoliposomes

Purified SecYEG was reconstituted into *E. coli* liposomes as described previously (Brundage et al., 1990; van der Does et al., 1998, 2003) with modifications. Before use, liposome suspension was activated in a bath sonicator until clear, and diluted with 0.5% Triton X-100 to 4 mg/ml. 200 μg of SecYEG (0.2 mg/ml in 10 mM TrisHCl, pH 8.0, 10% glycerol, 0.1% DDM, and 100 mM KCl) was mixed with 4 mg of liposomes and incubated for 30 min at 4°C with gentle tumbling. 200 mg of Biobeads SM-2 (Bio-Rad Laboratories), equilibrated in buffer A (50 mM TrisHCl, pH 8.0, 50 mM KCl, and 1 mM DTT), was incubated with the SecYEG/liposome mixture with gentle stirring for 2 h at 4°C. The beads were removed by centrifugation at 500 g. The procedure was repeated with 200 mg of Biobeads and 4 h of incubation in the second round, and 400 mg of Biobeads and overnight incubation in the third round. SecYEG proteoliposomes were collected by ultracentrifugation at 100,000 rpm for 30 min (TLA-100.3; Beckman Coulter) and dissolved in buffer A containing 10% glycerol. The concentration of SecYEG in proteoliposomes was determined using Coomassie staining on SDS-PAGE along with SecYEG standards of known concentration.

Posttranslational translocation

Activity of SecYEG reconstituted into proteoliposomes was determined by examining SecA-dependent translocation of ³⁵S-labeled proOmpA and assayed using protection against Proteinase K, as described previously (Cunningham et al., 1989; van der Does et al., 1998, 2003). In brief, in vitro translation of proOmpA was performed in a wheat germ extract (Promega) in the presence of [³⁵S]methionine at 26°C for 30 min and stopped by transferring to ice. Translocation of the substrate into SecYEG proteoliposomes was performed in the presence of SecA and an ATP-regenerating system at 37°C for 15 min and stopped by transferring the reaction to ice. Half of the reaction was treated with 0.02 mg/ml of Proteinase K on ice for 15 min in the absence or presence of 1% Triton X-100 and quenched with PMSF. Both reactions with and without PK treatment were precipitated with TCA, resolved on a denaturing gel, and quantified using autoradiography.

Cotranslational translocation assay

The coupled transcription/translation system used for cotranslational targeting assays has been described previously (Saraogi et al., 2011). The signal sequence of PhoA was replaced with that of the SRP-dependent substrate DsbA and used as a model substrate. The coupled transcription/translation reaction containing [³⁵S]methionine was supplemented with 5 mM GTP, 1 μM SRP, 1 μM FtsY, and either *E. coli*-derived inner membrane vesicles (IMVs) or SecYEG proteoliposomes, and performed at 37°C for 30 min. The final concentration of SecYEG in the reaction was 2.2 μM. The reactions were quenched on ice, treated with 0.9 mg/ml of Proteinase K for 15 min on ice in the absence or presence of 1% Triton X-100, and quenched with PMSF. The reaction was TCA precipitated and quantified as for the posttranslational targeting reactions.

Online supplemental material

Fig. S1 shows that phospholipids do not reactivate GTP hydrolysis from the targeting complex in the presence of RNC. Fig. S2 describes purification of wild-type and mutant SecYEG, interaction of SecYEG with RNC, and in vitro targeting assays. Fig. S3 shows that SecYEG does not affect kinetics of the closed complex formation. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201208045/DC1>.

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