Supplementary Note 1: Fluorescence measurements and kinetic simulations

Fluorescence measurements

Equilibrium titrations were performed using a Fluorolog-3-22 spectrofluorometer (Jobin Yvon) at room temperature in Assay buffer (50 mM KHEPES, pH 7.5, 150 mM KOAc, 10 mM $Mg(OAc)_2$, 2 mM DTT, 0.1 mg/ml BSA). Unless otherwise specified, experiments used an excitation wavelength of 360 nm and an emission wavelength of 455 nm. The FRET efficiency was calculated based on equation 1:

$$\mathrm{FRET} = 1 - \frac{F_e}{F_0} \qquad (1)$$

in which F_0 is the fluorescence intensity at 455 nm for Cm-labeled RNC alone. F_e is the fluorescence intensity at 455 nm when the Cm-labeled RNC is incubated with saturating amount of BDP-labeled SecA.

Equilibrium titrations used 10 nM Cm-labeled RNC, and indicated concentrations of SecA or SecYEG nanodisc as the titrant. The data were fit to equation 2:

$$\Delta F_{norm} = 1 \times \frac{[\mathrm{RNC}] + [\mathrm{titrant}] + K_d - \sqrt{\left([\mathrm{RNC}] + [\mathrm{titrant}] + K_d\right)^2 - 4 \times [\mathrm{RNC}][\mathrm{titrant}]}}{2 \times [\mathrm{RNC}]} \qquad (2)$$

in which "Normalized fluorescence change" (ΔF_{norm}) was calculated by dividing the observed fluorescence change at each titrant concentration over the fluorescence change at saturating titrant concentration, so that all titration curves start at 0 and plateau at 1, and the curvature of the titration curves directly reflect the K_d value.

Dissociation rate constants of SecA from RNC were measured using a Kintek stopped flow apparatus at room temperature as described previously[1]. 10 nM Cm-labeled RNC and 30 nM BODIPY-FL-labeled SecA were preincubated in Assay buffer, followed by addition of unlabeled SecA at indicated concentrations as the chase to initiate dissociation of the preformed complex. The time course of observed fluorescence (F) was fit to a double exponential function (equation 3):

$$F = F_e + \Delta F_a \times e^{-k_a t} + \Delta F_b \times e^{-k_b t} \qquad (3)$$

in which F_e is the fluorescence when the reaction reaches equilibrium, ΔF_a and k_a are the magnitude and rate constant of the fast phase, and ΔF_b and k_b are the magnitude and rate constant of the slow phase. The magnitude and rate constants of the slow phase are consistent with fluorescence bleaching of the Cm dye determined in parallel measurements. Hence, the first phase was assigned to SecA dissociation from RNC, and k_a represents the dissociation rate constant (k_1). Normalized fluorescence was calculated by dividing the observed fluorescence change at each time point over the fluorescence change when the reaction is complete, so that all the traces start at 0 and plateau at 1.

Measurements of RNC_{RodZ} transfer from SecA to SecYEG-Nd or empty nanodisc were performed using a Kintek stopped flow apparatus at room temperature in Assay buffer supplemented with 0.5 mM AMP-PNP. 10 nM Cm-labeled RNC was preincubated with 30 nM unlabeled SecA followed by addition of SecYEG nanodisc or empty nanodisc at indicated concentrations. The time course of observed fluorescence (F) was fit to Eq. 3, in which k_a represents the apparent rate constant of RNC transfer. Normalized fluorescence was calculated by subtracting the fluorescence at the end of the time course (1500 sec) from the observed fluorescence at each time point and then dividing over the total change in fluorescence over the reaction time course, so that all the traces start at 1 and plateau at 0.

Kinetic simulations

Simulations in Fig. 6a,b, Supplementary Fig. 7c-f were performed using the Berkeley Madonna software.

For the passive model in Fig. 6a and Supplementary Fig. 7c-d, the following reactions were modeled:

$$RNC_{RodZ} \bullet SecA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RNC_{RodZ} + SecA \quad (4)$$

$$RNC_{RodZ} + SecYEG \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RNC_{RodZ} \bullet SecYEG \quad (5)$$

For the active model in Fig. 6b and Supplementary Fig. 7e-f, the passive pathway (equations 4 and 5) was included in the simulation of the active model for completeness. The following reactions were modeled:

$$RNC_{RodZ} \bullet SecA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RNC_{RodZ} + SecA \quad (4)$$

$$RNC_{RodZ} + SecYEG \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RNC_{RodZ} \bullet SecYEG \quad (5)$$

$$RNC_{RodZ} \bullet SecA + SecYEG \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} RNC_{RodZ} \bullet SecYEG + SecA \quad (6)$$

The initial concentrations of all species were set based on experiment conditions as described under "Fluorescence measurements":

$[RNC_{RodZ}\bullet SecA]_0 = 10$ nM

$[SecA]_0 = 20$ nM

$[RNC_{RodZ}]_0 = 0$ nM

$[RNC_{RodZ}\bullet SecYEG]_0 = 0$ nM

$[SecYEG]_0$: varied concentrations as indicated

The SecA dissociation rate constant, k_1, was experimentally determined for RNC_{RodZ91} (0.00309 s^{-1}, Fig. 5c). To measure the SecA association rate constant (k_{-1}), we monitored the association of RNC_{RodZ} with varying concentrations of SecA using the FRET assay (Supplementary Fig. 7a). The observed rate constant of SecA binding to RNC_{RodZ} (k_{obsd}) was plotted as a function of SecA concentration and fit to equation 7:

$$k_{obsd} = k_{on}[\text{titrant}] + k_{off} \quad (7)$$

in which SecA is the titrant, k_{on} is the SecA association rate constant (k_{-1}) and was determined to be $1.48 \times 10^6\ M^{-1}s^{-1}$. The dissociation constant (K_{d1}) of the $RNC_{RodZ91}\bullet SecA$ complex was calculated to be 2.2 nM ($K_{d1} = k_1/k_{-1}$).

To obtain the dissociation constant (K_{d2}) of the RNC_{RodZ91}•SecYEG formed in the transfer reaction, we titrated SecYEG-Nd during the transfer. We preformed a complex of 10 nM RNC^{Cm}_{RodZ} with 30 nM BDP-labeled SecA, and added increasing amounts of SecYEG-Nd; the increase in Cm fluorescence due to the loss of FRET was used to monitor the transfer reaction (Supplementary Fig. 7b). The data were fit to equation 8:

$$F = F_0 + \left(F_e - F_0\right) \times \frac{[\text{SecYEG}]}{[\text{SecYEG}] + K_{1/2}} \quad (8)$$

in which F is the observed Cm fluorescence, F_0 and F_e are the Cm fluorescence at the beginning and end of the titration, respectively, and $K_{1/2}$ is the concentration of SecYEG-Nd required for 50% complete transfer. $K_{1/2}$ was determined to be 45 nM for RNC_{RodZ91}. At this concentration, we have:

$$[\text{RNC}\cdot\text{SecA}] = [\text{RNC}\cdot\text{SecYEG}] \quad (9)$$

$$[\text{RNC}\cdot\text{SecYEG}] + [\text{SecYEG}] = 45.4\ \text{nM} \quad (10)$$

$$[\text{RNC}\cdot\text{SecA}] + [\text{SecA}] = 30\ \text{nM} \quad (11)$$

$$[\text{RNC}\cdot\text{SecA}] + [\text{RNC}\cdot\text{SecYEG}] + [\text{RNC}] = 10\ \text{nM} \quad (12)$$

$$K_{d1} = \frac{[\text{RNC}]\times[\text{SecA}]}{[\text{RNC}\cdot\text{SecA}]} = 2.2\ \text{nM} \quad (13)$$

$$K_{d2} = \frac{[\text{RNC}]\times[\text{SecYEG}]}{[\text{RNC}\cdot\text{SecYEG}]} \quad (14)$$

$$K_{trans} = \frac{[\text{RNC}\cdot\text{SecYEG}]\times[\text{SecA}]}{[\text{RNC}\cdot\text{SecA}]\times[\text{SecYEG}]} = \frac{K_{d1}}{K_{d2}} = \frac{k_3}{k_{-3}} \quad (15)$$

Solving equations 9-15 gave K_{d2} = 3.4 nM and K_{trans} = 0.62. The association rate constant of RNC_{RodZ} binding to SecYEG (k_2) was assumed to be $1 \times 10^6\ M^{-1}s^{-1}$, which is typical for bi-molecular association. This results in a dissociation rate constant ($k_{-2} = K_{d2} \times k_2$) for the RNC_{RodZ}•SecYEG complex of 0.00338 s^{-1}, which is consistent with the previous observation that the half-life of the RNC•SecYEG complex is ~250 s^2. Varying the values of k_2 and k_{-2} while

maintaining the value of K_{d2} did not affect the outcome of the simulation (Supplementary Fig. 7c-f).

To measure the rate constant k_3, the observed transfer rate of RNC_{RodZ91} in Fig. 6e (green) was fit to equation 7, where $k_3 = k_{on}$ and was determined to be $1.4 \times 10^4\ M^{-1}\ s^{-1}$. k_{-3} was calculated to be $2.3 \times 10^4\ M^{-1}\ s^{-1}$ based on equation 15.

Because the formation of RNC_{RodZ}•SecYEG complex causes fluorescence quenching of RNC_{RodZ}, the simulated fluorescence (F_{sim}) starts at 1 at time = 0s, and is proportional to the sum of the fraction of RNC_{RodZ} and RNC_{RodZ}•SecA complex. Normalized fluorescence was simulated as $(F_{sim}\text{-}F_{sim,e})/(1\text{-}F_{sim,e})$ in which $F_{sim,e}$ is the fraction of F_{sim} when the reaction is complete, so that the traces start at 1 and plateau at 0.

To simulate the pulse-chase experiments to measure SecA dissociation from RNC_{RodZ} in Supplementary Fig. 7g, the following reactions were used:

$$RNC_{RodZ} \bullet SecA_{BDP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RNC_{RodZ} + SecA_{BDP} \quad (16)$$

$$RNC_{RodZ} + SecA \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} RNC_{RodZ} \bullet SecA \quad (17)$$

In which $SecA_{BDP}$ and SecA denote BODIPY-FL labeled and unlabeled SecA, respectively. The initial concentrations of all species were set based on experiment conditions as described above under “Fluorescence measurements”:

$[RNC_{RodZ}\text{•}SecA_{BDP}]_0 = 10$ nM

$[SecA_{BDP}]_0 = 20$ nM

$[RNC_{RodZ}]_0 = 0$ nM

$[SecA]_0$: varied concentrations as indicated

As described above, k_1 and k_{-1} were set to $0.00309\ s^{-1}$ and $1.48 \times 10^6\ M^{-1}s^{-1}$, respectively. Normalized fluorescence change during the chase is proportional to the fraction of the

RNC_{RodZ}•SecA complex and was simulated as $[RNC_{RodZ}•SecA]/[RNC_{RodZ}•SecA]_e$, in which $[RNC_{RodZ}•SecA]_e$ is the RNC_{RodZ}•SecA concentration when the reaction is complete.

References

1. Rome, M.E., Chio, U.S., Rao, M., Gristick, H. & Shan, S.O. Differential gradients of interaction affinities drive efficient targeting and recycling in the GET pathway. *Proc Natl Acad Sci U S A* **111**, E4929-35 (2014).
2. Wu, Z.C., De Keyzer, J., Kedrov, A. & Driessen, A.J.M. Competitive binding of the SecA ATPase and ribosomes to the SecYEG translocon. *J Biol Chem* **287**, 7885-7895 (2012).

Supplementary Note 2: Discussion of equilibrium titration of the transfer reaction.

Equilibrium titrations of the transfer reaction (Supplementary Fig. 7b) showed that, with 30 nM SecA present, the SecYEG concentration required to reach 50% transfer was 45 nM for RNC_{RodZ91}, yielding an estimated equilibria of the transfer reaction (Fig. 6b, $K_{trans} = k_3/k_{-3}$) of 0.62 (Supplementary Note 1). As thermodynamics is pathway-independent, K_{trams} is also the ratio of the affinities of the RNC_{RodZ}•SecA and RNC_{RodZ}•SecYEG complexes (Eq. 15 under Supplementary Note 1). This predicts that the affinity of the RNC_{RodZ}•SecYEG complex generated by the transfer is similar to that of the RNC_{RodZ}•SecA complex, in the low nanomolar range, which is ~100-fold tighter than the K_d value obtained by direct binding of RNC_{RodZ} to SecYEG-Nd (Fig. 5c). This implies that free RNC_{RodZ} was less competent in engaging SecYEG, possibly due to nonproductive conformations in the relatively long nascent chain, and hence formed a weaker complex with the translocon than that generated during transfer from SecA-bound RNC. If the transfer reaction occurred via the passive mechanism involving free RNC_{RodZ} as an obligatory intermediate (Fig. 6a), it would require ~100-fold higher concentrations of SecYEG-Nd than we observed. These considerations strongly suggest that the observed transfer reactions largely bypass the formation of free RNC_{RodZ} that could be conformationally trapped, and that the active transfer mechanism allowed the generation of an RNC_{RodZ}•SecYEG complex much more stable than that obtained from direct addition of RNC_{RodZ} to SecYEG.