

Expanded View Figures

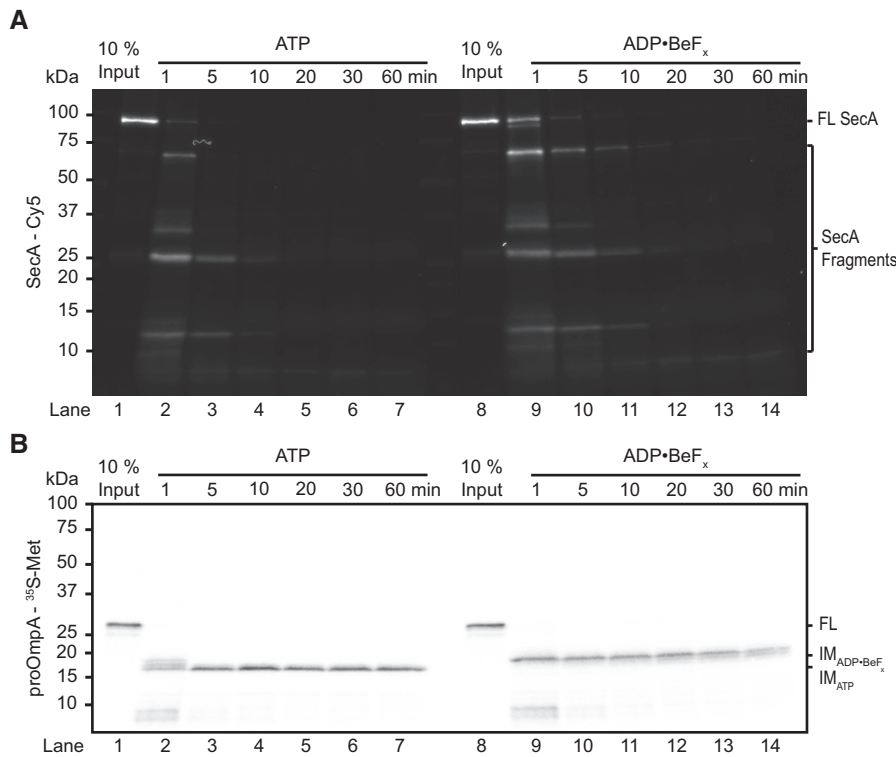


Figure EV1. Protease resistance of membrane-protected fragments.

A Translocation intermediates were generated in the presence of ATP using SecA labeled at position 285 with a Cy5 dye. To some samples, ADP•BeF_x was added, as indicated. Proteolysis was initiated by the addition of proteinase K at 23°C and quenched after incubation for the indicated times. All samples were analyzed by SDS-PAGE followed by fluorescence scanning. The gel is representative of two replicates.

B The same gel as in (A) imaged by autoradiography to visualize the radiolabeled proOmpA substrate.

Source data are available online for this figure.

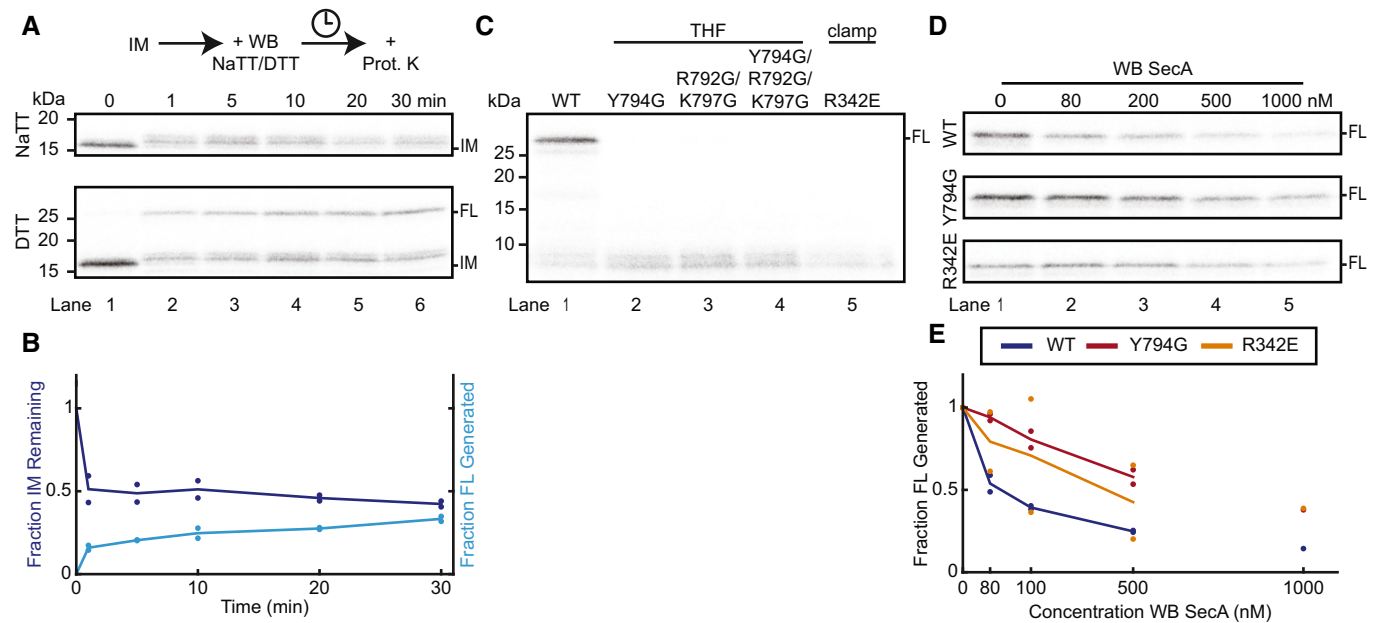
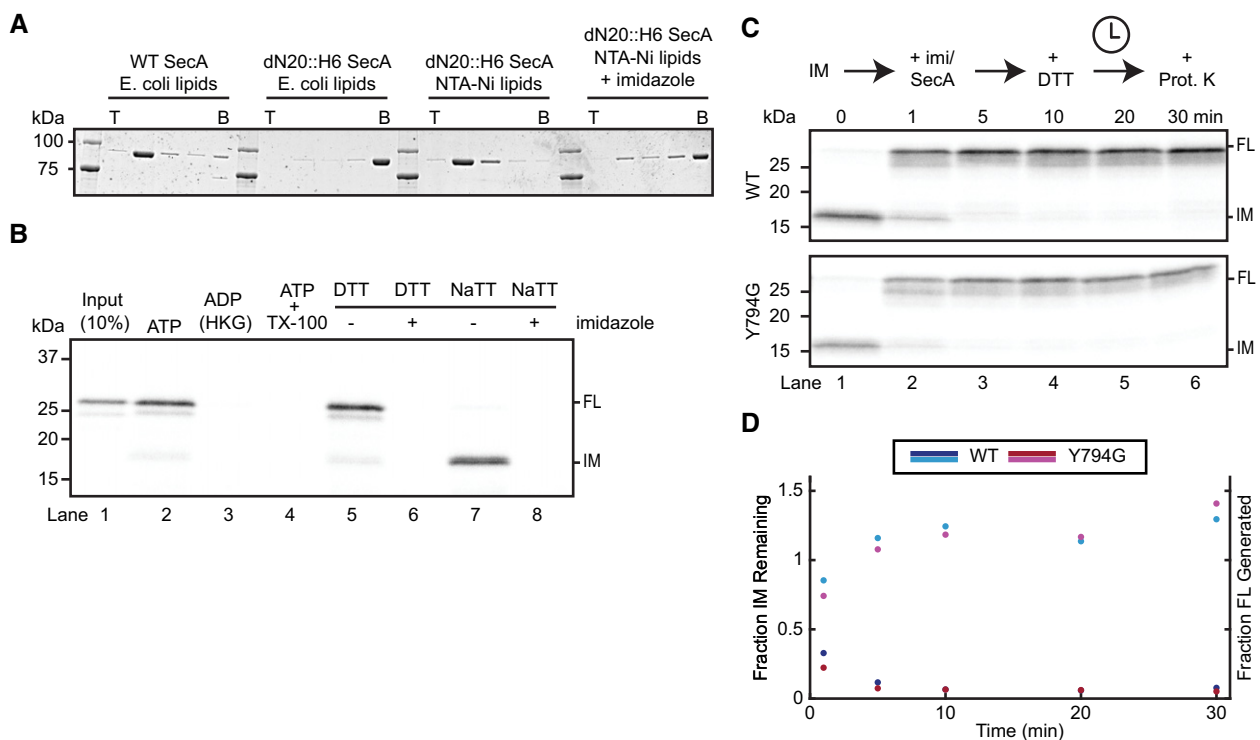


Figure EV2.

Figure EV2. Translocation activity of SecA mutants.

- A WB SecA was added to preformed intermediates (IM) in the presence of NaTT or DTT and incubated for the indicated times before proteolysis by proteinase K (Prot. K). The gel is representative of two replicates.
- B Quantification of the lower panel of (A). Points show band intensities normalized to the 0-min time point for each replicate. Lines show average of replicates. The intensities of both the IM (dark blue) and FL (light blue) bands were measured.
- C WT SecA, or the indicated mutants, was incubated with SecYEG proteoliposomes and *in vitro* translated substrate in the presence of ATP and DTT. All samples were treated with proteinase K. The gel is representative of three replicates.
- D WB SecA carrying additional mutations (WT, Y794G, R342E) was added at increasing concentrations to translocation reactions with WT SecA, SecYEG proteoliposomes, and *in vitro* translated substrate in the presence of ATP and DTT. Shown is full-length substrate protected from proteinase K treatment. The gel is representative of two replicates, except for the 1,000 nM concentration point.
- E Quantification of (D). Points show FL band intensities normalized to the value with 0 nM WB for each replicate. Lines show average of replicates. Colors correspond to WB SecA carrying additional mutations, as shown in the inset box.

Source data are available online for this figure.

**Figure EV3. dN20::H6 SecA binds to Ni-lipid-containing liposomes and translocates substrates.**

- A Binding of SecA to liposomes was determined with a flotation assay. WT or dN20::H6 SecA was incubated with liposomes containing *E. coli* polar lipids with or without Ni-NTA lipids. Imidazole was added, where indicated. The samples were loaded at the bottom (B) of a Nycodenz step gradient. After centrifugation, fractions were collected from the top (T) and analyzed by SDS-PAGE and Coomassie-blue staining. The gel is representative of two replicates.
- B ProOmpA was synthesized in reticulocyte lysate in the presence of ³⁵S-methionine and mixed with dN20::H6 SecA and SecYEG proteoliposomes containing Ni-NTA lipids. Imidazole was added to the reactions where indicated. After incubation in the presence of ATP, the samples were treated with proteinase K to degrade any non-translocated material. Where indicated, the reaction was performed in the absence of ATP and proteolysis in the presence of Triton X-100 (TX-100). Translocation reactions were performed in the presence of the reducing agent DTT or oxidizing agent NaTT, generating protease-protected full-length (FL) substrate or a translocation intermediate (IM), respectively. All samples were analyzed by SDS-PAGE followed by autoradiography. Lane 1 shows 10% of the input sample. The gel is representative of three replicates.
- C A translocation intermediate (IM) was formed with dN20::H6 SecA using substrate with a C-terminal disulfide bridge. Imidazole and WT or mutant SecA was then added, as indicated. After 10 min of incubation, DTT was added and the samples further incubated for the indicated times. All samples were treated with proteinase K (Prot. K) and analyzed by SDS-PAGE and autoradiography.
- D Quantification of (C). Points show band intensities normalized to the 0-min time point for each replicate. Lines show average of replicates. The intensities of both the IM (dark colors) and FL (light colors) bands were measured.

Source data are available online for this figure.