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***In vitro* assays for targeting and insertion of tail-anchored proteins into the ER membrane**

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Abstract

Membrane proteins mediate numerous essential cellular functions. Due to the aggregation propensity of hydrophobic transmembrane domains in aqueous environments, the targeting and insertion of membrane proteins pose major challenges to cells. In the Guided Entry of Tail-anchored protein (GET) pathway, an essential class of newly synthesized tail-anchored proteins (TAs) are chaperoned and guided by multiple targeting factors to the endoplasmic reticulum (ER) membrane. Deciphering the molecular mechanism of this cellular process has benefitted from successful *in vitro* reconstitution of individual molecular events in the GET pathway with purified components. Here we describe recently developed protocols for *in vitro* reconstitution of functional complexes of TA substrates with their targeting factors, for monitoring the transfer of TAs between targeting factors, and for the insertion of TA into the microsomal membrane. These procedures are generally applicable to the interrogation of other post-translational membrane protein targeting pathways.

Keywords

protein targeting; membrane proteins; tail-anchored proteins; amber suppression; fluorescence; chaperones

INTRODUCTION

Integral membrane proteins represent over 30% of the proteins encoded by the genome and mediate numerous essential cellular functions including molecular transport, signal transduction, energy generation, and cell-cell recognition. Nuclear-encoded membrane proteins begin their biosynthesis in the cytosol and are localized to the appropriate cellular membrane via either co-translational pathways, in which the nascent protein is delivered together with the translating ribosome during its synthesis (Akopian, Shen, Zhang, & Shan, 2013; Saraogi & Shan, 2014), or post-translational pathways, in which nascent proteins are recognized and targeted after being released from the ribosome (Chio, Cho, & Shan, 2017; Hegde & Keenan, 2011; Johnson, Powis, & High, 2013). The post-translational targeting of membrane proteins poses particular challenges to both the cellular proteostasis network and the mechanistic interrogation of the pathway, since hydrophobic transmembrane domains

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(TMDs) on nascent membrane proteins are highly prone to aggregation in the cytosol. This demands that post-translational targeting factors must provide effective chaperones to maintain nascent membrane proteins in a soluble, translocation-competent state before arrival at the target membrane. The aggregation-prone nature of membrane protein substrates also poses significant challenges to the biochemical reconstitution of soluble and functional targeting complexes, which are essential for understanding the molecular mechanism of the pathway.

Recent work on the Guided Entry of Tail-anchored protein (GET) pathway provides a paradigm for the molecular basis of post-translational targeting as well as methods to interrogate this process at high resolution. A combination of genetic and biochemical reconstitutions led to the discovery of most of the required components as well as the sequence of molecular events in this pathway (Jonikas et al., 2009; Schuldiner et al., 2008; F. Wang, Brown, Mak, Zhuang, & Denic, 2010), as summarized in Fig. 1. The substrates of this pathway, ER-destined tail-anchored proteins (TAs), contain a single TMD at the C-terminus and mediate many essential cellular functions including vesicular trafficking, synaptic transmission, protein translocation, protein quality control, and apoptosis (Hegde & Keenan, 2011). The co-chaperone Sgt2 is the most upstream factor identified thus far that binds the TA substrate and, with help of the Get4/Get5 complex, transfers TAs to the Get3 ATPase. Get3 delivers TAs to a receptor complex at the ER, comprised of Get1 and Get2, which mediates TA insertion into the ER membrane.

This unit describes protocols for the reconstitution and measurement of three molecular events in the GET pathway (steps 1, 2, and 3 in Fig.1). Basic Protocol 1 describes methods for the reconstitution and purification of fluorophore-labeled Sgt2•TA complex using an *in vitro* translation (IVT) system coupled to amber suppression technology. Using the purified and fluorescently labeled Sgt2•TA complex, Basic Protocol 2 describes experimental steps to monitor TA transfer from Sgt2 to Get3 via Förster resonance energy transfer (FRET). Finally, Basic Protocol 3 describes methods for *in vitro* reconstitution of the Get3•TA complex and measurement of TA insertion into the ER membrane.

BASIC PROTOCOL 1. *IN VITRO* RECONSTITUTION OF SGT2•TA^{Cm} COMPLEXES

In this procedure, TA proteins are *in vitro* translated in a bio-orthogonal *E. coli* S30 extract supplemented with recombinant purified Sgt2, generating Sgt2•TA complexes (Fig. 2A). If the translation reaction also contains a pair of engineered amber suppressor tRNA (tRNA^{Tyr}_{CUA}) and an evolved tRNA synthetase (CmRS) that aminoacylates tRNA^{Tyr}_{CUA} with a non-natural fluorescent amino acid, L-(7-hydroxycoumarin-4-yl)ethylglycine (Cm) (J. Wang, Xie, & Schultz, 2006) (Charbon et al., 2011), the fluorophore is site-specifically incorporated four residues upstream of the TA-TMD during its synthesis (Fig. 2A) (Rao et al., 2016). The resulting fluorescent Sgt2•TA^{Cm} complex is further affinity-purified using the His₆ tag on Sgt2 (Fig. 2A).

Detailed methods for the preparation of several reagents in this protocol have been described and will not be repeated here. For preparation of *E. coli* S30 extracts, the reader is referred to

(Goerke & Swartz, 2008; Zawada, 2012). The generation of S30 extract containing an optimized tRNA_{CUA}^{Tyr}, tRNA_{CUA}^{Opt} (Young, Ahmad, Yin, & Schultz, 2010), is described in (Saraogi, Zhang, Chandrasekaran, & Shan, 2011). Purification of the RNA aptamer that inhibits release factor-1 (RF-1 aptamer) is described in (Saraogi et al., 2011). The purification of His₆-Sgt2, T7 RNA polymerase, and a mutant CmRS(D286R) with improved recognition of the anticodon loop of tRNA_{CUA} have been described in (Rao et al., 2016).

Material

E. coli S30 extract containing tRNA_{CUA}^{Opt} (Goerke & Swartz, 2009; Saraogi et al., 2011; Young et al., 2010)

250 μM RF-1 aptamer (Saraogi et al., 2011)

40–80 μM Tagless T7 RNA polymerase (Rao et al., 2016)

500–600 μM Tagless CmRS(D286R) (Rao et al., 2016)

150–200 μM His₆-tagged Sgt2 (Rao et al., 2016)

Small molecule mix (SMM) (see Recipe)

10 mM amino acid mix (–Met or +Met) (see Recipe)

40 mM 7-hydroxycoumaryl ethylglycine (Cm) (Millipore-Sigma, cat# 792551)

Plasmid encoding protein of interest (POI), such as a model TA (SUMO-Bos1-opsin) under control of the T7 promoter (pUC19-Strep₃-SUMO-Bos1-opsin or pUC19-Strep₃-SUMO-Amb-Bos1-opsin) (Rao et al., 2016)

11 mCi/mL ³⁵S-Met (Perkin Elmer, cat#NEG009T001MC)

Buffer A (see Recipe)

Fixing solution (see Recipe)

3M Potassium acetate (KOAc) dissolved in Buffer A (Millipore-Sigma, cat#P5708)

2M Imidazole dissolved in Buffer A (pH~7.0) (Millipore-Sigma, cat#I5513)

Maxi-prep kit (QIAGEN, cat#12963)

sterile 100×15mm petri dish

0.6 mL microfuge tubes (amber and clear)

15 mL Falcon tubes

Aluminum foil

Ni-NTA agarose (QIAGEN, cat# 30310)

Amicon Ultra-15 Centrifugal Filter, 10 kDa cutoff (Millipore-Sigma, cat# UFC901024)

10 mL Poly-Prep® Chromatography Column (Bio-Rad, cat#7311550)

12.5% Tris-Glycine SDS-PAGE gels

30 °C water bath
 30 °C benchtop incubator
 TLX-Optima ultracentrifuge (Beckman Coulter Inc.) or equivalent
 TLA100 rotor (Beckman Coulter Inc.) or equivalent
 Gel dryer
 Storage phosphor screen (GE Healthcare Life Sciences)
 Typhoon Phosphorimager (GE Healthcare Life Sciences)
 Refrigerated benchtop swinging bucket centrifuge (Beckman Coulter Inc.)

Note 1: All reagents and apparatus that come into contact with the IVT reaction should be nuclease-free following standard procedures.

Note 2: T7 RNA polymerase and CmRS(D286R) are stored in 50% glycerol-containing buffer at −30 °C. DNA templates are stored in 10 mM Tris-HCl pH 8.0 at −30 °C. All other reagents should be flash-frozen in liquid nitrogen and stored at −80 °C.

Note 3: Throughout the procedure, keep all materials on ice unless otherwise specified.

Test amber suppression efficiency in trial translations

Note: Follow the safety guidelines for the handling and disposal of radioactive material.

- 1 Set the water bath to 30 °C.
- 2 Prepare a master mix (total volume 30 µL) in a 0.6 mL microfuge tube with the following components at the specified final concentrations. Mix reagents by gentle pipetting.
 - a. 31 % (v/v) SMM
 - b. 28 % (v/v) *E. coli* S30 extract containing tRNA_{CUA}^{Opt}
 - c. 1 mM amino acid mix (−Met)
 - d. 2 µM T7 RNA polymerase
 - e. 5–10 % (v/v) ³⁵S-Met
- 3 Prepare three 0.6 mL microfuge tubes for the IVT reaction. Add 8 µL of the master mix in step (2) to each tube.
- 4 Add the following components to each reaction tube at the specified final concentrations.
 - a. Reaction #1: control plasmid that does not contain the amber codon (pUC19-Strep₃-SUMO-Bos1-opsin, 50 ng/µL).
 - b. Reaction #2: amber codon-containing plasmid (pUC19-Strep₃-SUMO-Amb-Bos1-opsin, 50 ng/µL).

- c. Reaction #3: amber codon-containing plasmid (50 ng/μL), 15 μM CmRS(D286R), 75 μM Cm, and 12 μM RF-1.
- d. Adjust the reaction volume to 10 μL with Buffer A and mix by gentle pipetting.

Note 1: The amber codon can be introduced into the desired position on the plasmid DNA using QuickChange mutagenesis (Agilent).

Note 2: Plasmid templates for coupled in vitro transcription-translation should be purified from a Maxi-prep kit (QIAGEN). Do not use plasmids purified from a Mini-prep kit.

- 5 Incubate the reactions at 30 °C for 90 min.
- 6 Quench the reaction by addition of SDS sample buffer. Boil the samples at 95 °C, 3 minutes.
- 7 Resolve samples by SDS-PAGE on a 12.5 % Tris-glycine gel. Fix the gels by gentle shaking in Fixing solution for 30 min.
Note: The sample loading volume can be adjusted depending on the specific activity of ³⁵S-Met.
- 8 Dry the gel using a gel dryer. Expose the dried gel in storage phosphor screen.
- 9 6–24 hours later, the screen can be scanned using a Phosphorimager. A representative gel for a successful amber suppression reaction is shown in Fig. 2B.

Generate Sgt2•TA^{Cm} via large-scale IVT

Note: For every new DNA vector, optimize translation and test amber suppression with trial translations (steps 1–9 above) before carrying out preparative scale reactions.

- 10 Set a benchtop incubator at 30 °C.
- 11 Ultracentrifuge His₆-Sgt2 at 100,000 rpm for 30 °C.
- 12 Prepare a Petri dish and aluminum foil on the bench.
100×15mm Petri dishes are optimal for 5–10 mL translation reactions. If desired, smaller Petri dishes can be used for 1 mL translation reactions.
- 13 Add the following reagents gently onto the Petri dish at the specified final concentrations.
 - a. 31% (v/v) SMM
 - b. 28% (v/v) *E. coli* S30 extract containing tRNA_{CUA}^{Opt}
 - c. 1 mM amino acid mix (+Met)
 - d. 2 μM T7 RNA polymerase
 - e. 12 μM RF-1 aptamer

f. 15 μ M CmRS(D286R)

g. 2 μ M His₆-Sgt2

- 14 Partially cover the Petri dish with aluminum foil. Add 75 μ M Cm to the reaction mixture.

Note: Cm is light-sensitive. The sample should be protected from light throughout steps 14–26. Use amber tubes or aluminum foil to cover the sample, and turn off the light in the cold room.

- 15 Add the amber codon-containing plasmid to the petri dish at a final concentration of 50 ng/ μ L. Gently swirl to mix all reagents. The reaction should cover the entire surface of the Petri dish.

- 15 Completely cover the Petri dish with aluminum foil. Incubate the translation reaction at 30 °C for 90 min.

Purify the Sgt2•TA^{Cm} complex

- 17 Transfer 5 mL of the IVT reaction to a 15 mL Falcon tube and place on ice.
- 18 Dilute 2-fold with ice cold Buffer A. Add imidazole to a final concentration of 20 mM.
- 19 Add 0.8 mL Ni-NTA agarose pre-equilibrated in Buffer A with 20 mM imidazole to the tube. Incubate with gentle rotation at 4 °C for 1 hr.
- 20 Centrifuge at 1000 $\times$ g, 4 °C for 5 min in a refrigerated benchtop swinging bucket centrifuge. Carefully discard the supernatant without disturbing the resin.
- 21 Dilute the resin with ice-cold Buffer A (5-fold). Transfer the mixture to a 10 mL Poly-Prep® Chromatography Column in a cold room (4 °C). Allow the resin to pack on the column by gravity flow.
- 22 Wash with at least 20 column volumes of ice-cold Buffer A supplemented with 20 mM imidazole and 300 mM KOAc.

Note: This high-salt wash step is important for removing proteins non-specifically bound to the TA or Sgt2.

- 23 Wash with 10 CV ice-cold Buffer A with 20 mM imidazole.
- 24 Elute proteins with ice-cold Buffer A containing 300 mM imidazole.
- 25 Concentrate the elution fractions using the Amicon Ultra-15 Centrifugal Filter (10 kDa cutoff).

Note: During the concentration step, the sample can be diluted with Buffer A multiple times to reduce the imidazole concentration.

- 26 Aliquot the sample into 0.6 mL-amber tubes, flash freeze in liquid nitrogen, and store at –80 °C.

- 27 To assess the purity and yield of the complex, run samples on SDS-PAGE gels and estimate protein concentration using standard Coomassie blue staining and quantitative western blot analyses.

BASIC PROTOCOL 2. ASSAY TO MEASURE TA TRANSFER FROM SGT2 TO GET3

This basic protocol describes experimental steps for the generation and purification of BODIPY-FL-labeled Get3 and the FRET-based assay to measure TA transfer from Sgt2 to Get3 (Fig. 3A) (Rao et al., 2016). In this assay, TA^{Cm} in the Sgt2•TA^{Cm} complex (prepared from Basic Protocol 1) serves as the FRET donor. BODIPY-FL is site-specifically conjugated onto Get3 using Sfp phosphopantetheinyl transferase (Yin, Lin, Golan, & Walsh, 2006; Yin et al., 2005) and serves as the FRET acceptor. The efficiency of TA transfer from Sgt2 to Get3 is monitored by the reduction in the fluorescence intensity of Cm. Other factors that may contribute to the fluorescence change, such as environmental sensitivity of the dye, have been excluded (Rao et al., 2016). The methods for preparation of additional reagents (BODIPY-FL-CoA, Get3 with a ybbR tag (DSLEFIASKLA) inserted between residues S110 and D111, and Sfp-His₆) have been described (Rao et al., 2016; Yin et al., 2006)

Material

Sgt2•TA^{Cm} complex (prepared from Basic Protocol 1)
 3–4 mM BODIPY-FL-CoA (Rao et al., 2016)
 100–150 μM Tagless Get3-ybbR (Rao et al., 2016)
 300–400 μM Sfp-His₆ (Rao et al., 2016; Yin et al., 2006)
 50–100 μM Get4/5 (Rao et al., 2016)
 Sfp labeling buffer (See Recipe)
 Buffer A (See Recipe)
 Buffer B (See Recipe)
 150–200 mM ATP (pH ~ 7.0)
 2M Imidazole dissolved in Buffer A (pH ~7.0)
 Sephadex G-25, fine (Millipore-Sigma, cat# G2580)
 Econo-Column®, 1.0 × 50 cm (Bio-Rad, cat# 7371052)
 1.5 mL microfuge tubes (amber)
 Ni-NTA agarose (QIAGEN, cat# 30310)
 Amicon Ultra-15 Centrifugal Filter, 10 kDa cutoff (Millipore-Sigma, cat# UFC901024)
 10 mL Poly-Prep® Chromatography Column (Bio-Rad, cat#7311550)
 Quartz microcuvettes, 6.5mm(W)×6.5mm(L)×48mm(H) (Starna Cells, Inc.)

NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific)

TLX-maxima tabletop ultracentrifuge (Beckman Coulter Inc.) or equivalent

TLA100 rotor (Beckman Coulter Inc.) or equivalent

Fluorolog-3–22 spectrofluorometer (Jobin Yvon) or equivalent

Preparation and purification of BODIPY-FL-labeled Get3 (Get3^{BDP})

Note: The samples should be protected from light until all preparation steps are done.

- 1 Hydrate Sephadex-G25 with ddH₂O and load the resin onto Econo-Column® Chromatography Columns (1.0 × 50 cm).

- 2 Equilibrate the resin with 2–3 CV of Buffer B by gravity flow.

Note: Due to the slow flow rate, start this step at least 4 hr before the labeling reaction.

- 3 Set up a labeling reaction (total volume 800 µL) in a 1.5 mL amber microfuge tube containing the following components at the specified final concentrations.

40 µM Get3-ybbR

90 µM BODIPY-FL-CoA

2 µM Sfp-His₆

Sfp labeling buffer

Note 1: For every new protein or labeling position, we recommend running a small-scale trial labeling reaction (20 µL) prior to the large-scale reaction.

Note 2: Representative data for SDS-PAGE analysis of the trial labeling reaction is shown in Fig. 3B. Fluorophore conjugation changes the electrophoretic mobility of Get3-ybbR on a 10% SDS-PAGE gel, allowing labeled and unlabeled Get3 to be distinguished. The labeling efficiency can be calculated according to $I_{\text{labeled}} / (I_{\text{unlabeled}} + I_{\text{labeled}})$, in which I is the intensity of the Get3 bands.

- 4 Incubate the reaction at RT for 1 hr with gentle rotation.
- 5 Put the reaction on ice. Supplement imidazole to a final concentration of 20 mM.
- 6 Load Ni-NTA agarose (100 µL) onto a 10 mL Poly-Prep® Chromatography Column. Allow resin to pack by gravity flow.
- 7 Equilibrate the resin with 5 CV Buffer A with 20 mM imidazole.
- 8 Load the labeling reaction onto the Ni-NTA column by gravity flow. Collect the flow-through, which contains labeled tagless Get3-ybbR and free dye.
- 9 Load the flow-through fraction onto the equilibrated Sephadex-G25 column.
- 10 Elute protein with Buffer B. Run the column by gravity flow at a rate of < 1 mL/min. Collect 0.5 mL elution fractions.

- 11 Identify Get3^{BDP}-containing elution fractions by measuring A₂₈₀ and/or A₅₀₈ using a NanoDrop.
- 12 Concentrate the elution fractions using an Amicon Ultra-15 Centrifugal Filter (10 kDa cutoff). Flash freeze in aliquots and store at –80 °C.

Validate the TA transfer assay

Note: All protein samples should be ultracentrifuged at 100,000 rpm in a TLA100 for at least 30 min at 4 °C prior to fluorescence measurements. Protect samples from light during assays (step 13–19).

- 13 Turn on the spectrofluorometer 1 hr prior to the experiment to warm up the lamp. Prepare clean Quartz microcuvettes during this time.
- 14 Set up the following reactions containing the specified components at indicated concentrations, each in a total volume of 300 µL. Incubate at RT for 10 min.
 - a. Donor only: 50 nM Sgt2•TA^{Cm} and 150 nM Sgt2 in Buffer B.
 - b. Acceptor only: 400 nM Get3^{BDP}, 400 nM Get4/5, and 2 mM ATP in Buffer B.
 - c. Donor with unlabeled Get3: 50 nM Sgt2•TA^{Cm}, 150 nM Sgt2, 400 nM Get3, 400 nM Get4/5, and 2 mM ATP in Buffer B.
 - d. Donor with Acceptor-labeled Get3: 50 nM Sgt2•TA^{Cm}, 150 nM Sgt2, 400 nM Get3^{BDP}, 400 nM Get4/5, and 2 mM ATP in Buffer B.
- 15 Collect the fluorescence emission spectra of the three samples at 390 – 600 nm using an excitation wavelength of 360 nm.

Measure the equilibrium of TA transfer from Sgt2 to Get3

- 16 Turn on the spectrofluorometer 1 hr prior to the experiment. Prepare two sets of clean quartz cuvettes.
- 17 Set the spectrofluorometer in the real time display mode. Set the excitation wavelength to 360 nm, and emission wavelength to 450 nm.
- 18 Prepare 600 µL solution containing 50 nM Sgt2•TA^{Cm}, 150 nM unlabeled Sgt2, 150 nM Get4/5, and 2 mM ATP in Buffer B.
- 19 Transfer 260 µL of the sample into each cuvette. Serially add small volumes of unlabeled Get3 or Get3^{BDP} to desired concentrations. The concentration of Get3 or Get3^{BDP} added should be identical between the two measurements. Record the Cm fluorescence intensity before and after each addition.

Note: Make sure to close the shutter in between fluorescence measurements.
- 20 Correct the recorded Cm fluorescence intensity for volume changes during the titration.
- 21 Calculate the FRET efficiency (*E*) at each Get3 concentration using Equation 1.

$$E = \left(1 - \frac{F_{DA}}{F_D}\right) \times 100 \quad (1)$$

where F_D and F_{DA} are the corrected Cm fluorescence intensities of the reaction with Get3 and Get3^{BDP}, respectively.

- 22 Plot FRET efficiency as a function of Get3 concentration and fit the data to Equation 2.

$$E_{obsd} = E_{Max} \times \left[\frac{[TA] + (Get3) + K_{transfer} - \sqrt{([TA] + [Get3] + K_{transfer})^2 - 4[TA][Get3]}}{2[TA]} \right] \quad (2)$$

where E_{obsd} is the observed FRET efficiency at a given Get3 concentration, E_{Max} is the FRET efficiency at saturating Get3 concentration, and $K_{transfer}$ is the equilibrium constant for TA transfer at the specific Sgt2 concentration.

BASIC PROTOCOL 3. MEMBRANE INSERTION OF TA FROM GET3•TA

This basic protocol describes procedures for the generation and purification of Get3•TA complexes by *in vitro* translation of the TA substrate in the *E. coli* S30 extract supplemented with Get3. Inclusion of Sgt2 and Get4/5 is optional and does not substantially affect the activity or conformation of Get3•TA (Chio, Chung, Weiss, & Shan, 2017), but lengthens the subsequent purification of the Get3•TA due to Get4/5 association with Get3. The efficiency of TA targeting and insertion from the purified Get3•TA into yeast rough ER microsomes (yRM) is assessed via glycosylation of an engineered opsin tag at the C-terminus of TA substrates (Fig. 4A), which results in a molecular weight shift on SDS-PAGE that can be visualized using autoradiography.

Materials

E. coli S30 extract (Goerke & Swartz, 2009; Saraogi et al., 2011)

SMM (see Recipe)

10 mM amino acids mix (–Met) (see Recipe)

11 mCi/mL ³⁵S-Met (Perkin Elmer, cat#NEG009T001MC)

Plasmid encoding model TA substrate under control of the T7 promoter (pUC19-Strep3-SUMO-Bos1-opsin)

40–80 μM Tagless T7 RNA polymerase

100–200 μM Get3 (tagless or His₆-tagged)

50–100 μ M Get4/5 (tagless, optional)
150–200 Sgt2 (tagless, optional)
50–100 U/mL yRM from *get3* yeast cells (Schuldiner et al., 2008)
Buffer A (see Recipe)
Buffer B (see Recipe)
Biotin (Millipore-Sigma, cat# B4501)
2M Imidazole dissolved in Buffer A (pH~7.0) (Millipore-Sigma, cat#I5513)
Strep-Tactin resin (IBA Lifesciences, cat# 2-1201-025) or Ni-NTA agarose (QIAGEN, cat# 30310)
0.6 mL microfuge tubes
Micro Bio-Spin™ Columns (Bio-Rad, cat# 7326204)
Amicon Ultra-0.5 Centrifugal Filter (30 kDa cutoff) (Millipore-Sigma, cat# UFC5030)
Scintillation fluid (RPI, cat#111195)
Scintillation vials (VWR, cat#66022-274)
12.5% Tris-Glycine SDS-PAGE gels
NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific)
30 °C water bath
TLX Optima ultracentrifuge (Beckman Coulter Inc.) or equivalent
TLA100 rotor (Beckman Coulter Inc.) or equivalent
Scintillation counter (Beckman Coulter Inc.)
Gel dryer
Storage phosphor screen (GE Healthcare Life Sciences)
Typhoon Phosphorimager (GE Healthcare Life Sciences)

Generate and purify Get3•TA from a coupled in vitro transcription-translation reaction

Note: Keep all materials on ice unless otherwise specified. Follow standard procedures to maintain a nuclease-free environment.

- 1 Ultracentrifuge Get3 in the TLA100 rotor at 100K rpm, 30 min, 4 °C to remove any aggregates.
If used, Get4/5 and Sgt2 should also be ultracentrifuged.
- 2 Collect aliquots of other necessary materials and keep frozen in liquid nitrogen.
- 3 Thaw frozen materials in water bath at RT.

Set up a 100 μL *in vitro* translation reaction in a 0.6 mL microfuge tube containing the following components at specified final concentrations:

- a. 31% (v/v) small molecule mix
- b. 28% (v/v) *E. coli* extract
- c. 1 mM amino acids (–Met)
- d. 5–10% (v/v) ^{35}S -methionine
- e. 50 ng/ μL plasmid DNA encoding opsin- and Strep₃-tagged TA
- f. 2 μM Get3
- g. 2 μM T7 RNA polymerase
- h. 2 μM Sgt2 (optional)
- i. 2 μM Get4/5 (optional)

Note: Immediately flash freeze unused reagents except for T7 RNA polymerase. All reagents can undergo at least five freeze-thaw cycles without appreciable loss of activity.

- 4 Incubate the reaction in a 30 °C water bath for 90 min.
- 5 Equilibrate 75 μL Strep-Tactin resin (resin volume) in Buffer B for purification based on Strep₃-tagged TA, or 75 μL Ni-NTA agarose in Buffer A with 20 mM imidazole for purification based on His₆-tagged Get3.
- 6 Incubate the translation reaction mix with equilibrated resin at 4 °C for 30 min with gentle rotation.
- 7 Transfer the mixture to a Micro Bio-Spin™ column over a receiving container (e.g. 15 mL conical tube). Push gently with a gloved thumb on top of the column to generate flow and allow the resin to settle (may need to manually push for additional flow).
- 8 Wash the column with an additional 1 mL of equilibration buffer.
If Get4/5 was used, include 500 mM NaCl in the wash. This is followed by an additional wash with 1 mL of equilibration buffer.
- 9 Place the washed column over an Amicon Ultra-0.5 Centrifugal Filter (30 kDa cutoff). Elute with 400 μL of the appropriate elution buffer (Buffer B supplemented with 5 mM biotin for Strep-Tactin; Buffer A supplemented with 300 mM imidazole for Ni-NTA).
- 10 Concentrate to ~75 μL . Dilute with 400 μL Buffer B.
- 11 Repeat step 10 two more times.
- 12 Concentrate to ~75 μL and transfer to a clean 0.6 mL tube.
- 13 Transfer 5 μL to scintillation vials containing 5 mL of scintillation fluid. Scintillation count using a scintillation counter following manufacturer's

instructions. The yield of ^{35}S -labeled Get3•TA is typically 50,000 – 150,000 dpm.

Monitor TA insertion into yRM from purified Get3•TA

- 14 Dilute the purified Get3•TA to approximately 50,000 – 75,000 dpm in Buffer B.
- 15 Set up a 50 μL or 100 μL insertion reaction with 10% (v/v) of the diluted Get3•TA. Additional components, such as 2 mM ATP or 0.5 μM Get4/5, can also be included to test for their effects.
- 16 Initiate the reaction by adding 20% (v/v) *get3* yRM.
- 17 At different time points, remove 6 – 12 μL aliquots of the reaction and add to quench tubes containing an equal volume of 2×SDS Buffer. Flash freeze quenched reactions in liquid nitrogen.

Note: TA substrate can be digested by endogenous proteases if quenched aliquots are not flash frozen and left at RT for too long.

- 18 After taking the last time point, boil the quench tubes at 90–95 °C for 3–5 minutes. Run 12 μL of each aliquot on a 12.5% Tris-Glycine SDS-PAGE gel.

Note: A molecular weight marker should be run alongside the reaction samples. The optimal electrophoresis time is empirically determined for best separation of the glycosylated and non-glycosylated TA bands.

- 19 Dry the gel using a gel dryer. Expose the dried gel using a phosphor screen overnight.
- 20 Scan the screen using a Phosphorimager the next day. Insertion efficiency (%) can be calculated as $I_{\text{glyc-TA}} / (I_{\text{TA}} + I_{\text{glyc-TA}}) * 100$, where $I_{\text{glyc-TA}}$ and I_{TA} are the intensities of the glycosylated and nonglycosylated TA, respectively.
- 21 Fit the time-dependence of the insertion efficiency to Equation 3.

$$\% \text{insertion} = T(1 - e^{-k_{\text{obsd}} * t}) \quad (3)$$

in which T is the maximum %insertion, and k_{obsd} is the observed rate constant of the insertion reaction.

REAGENTS AND SOLUTIONS

Filter Milli-Q double deionized water (d.d.H₂O) with 0.2 μm membrane. Use filtered water for all the procedures.

10x salt solution

Dissolve the following reagents in water at the specific final concentrations. Filter the solution with 0.2 μm membrane.

1750 mM L-Glutamic acid potassium salt monohydrate

100 mM L-Glutamic acid ammonium salt
120 mM L-Glutamic acid hemimagnesium salt tetrahydrate

10x Master Mix (2 mL)

Mix the following solutions

320 µL of 75 mM ATP, pH 7 (12 mM final concentration)
230 µL of 75 mM GTP, pH 7 (8.6 mM final concentration)
230 µL of 75 mM UTP, pH 7 (8.6 mM final concentration)
230 µL of 75 mM CTP, pH 7 (8.6 mM final concentration)
136 µL of 5 mg/mL Folinic acid (340 µg/mL final concentration)
684 µL of 5 mg/mL *E. coli* tRNA (1.7 mg/mL final concentration)
add 170 µL water.
Filter the solution with 0.2 µm membrane.
Freeze in liquid nitrogen and store at –80°C.

1M K-HEPES (pH 7.5)

Dissolve 238.3 g HEPES in 750 mL water
Adjust pH to 7.5 with KOH pellets.
Add water up to 1 L.
Filter the solution with 0.2 µm membrane and store at 4°C.

0.66 M Phosphoenol-pyruvate, monopotassium salt (PEP) (pH 7)

Dissolve 0.41 g PEP in 3 mL of 50 mM K-HEPES (pH 7.5).
Adjust pH to 7 with KOH.
Filter sterilize the solution and store at –80 °C.

0.15 M NAD

Dissolve 0.1g NAD in 10 mL of 50 mM K-HEPES (pH 7.5).
Filter sterilize the solution and store at –80 °C.

20 mM Coenzyme A, sodium salt hydrate (CoA)

Dissolve 0.06 g CoA in 4 mL of 50 mM K-HEPES (pH 7.5).
Filter sterilize the solution and store at –80 °C.

0.2 M Sodium oxalate

Dissolve 0.134 g Sodium oxalate in 5 mL of 50 mM K-HEPES (pH 7.5).

Filter sterilize the solution and store at -80°C .

0.1 M Spermidine

Dissolve 0.145 g Spermidine in 10 mL of 50 mM K-HEPES (pH 7.5).

Filter sterilize the solution and store at -80°C .

0.1 M Putrescine

Dissolve 0.088 g Putrescine in 10 mL of 50 mM K-HEPES (pH 7.5).

Filter sterilize the solution and store at -80°C .

3× Small molecule mix (SMM)

Mix the following solutions

500 μL 10× salt solution (1×)

500 μL 10× Master Mix (1×)

250 μL of 0.66 M PEP (33 mM final concentration)

110 μL of 0.15 M NAD (0.33 mM final concentration)

65 μL of 0.02 M CoA (0.26 mM final concentration)

67.5 μL of 0.2 M Sodium oxalate (2.7 mM final concentration)

75 μL of 0.1 M Spermidine (1.5 mM final concentration)

50 μL of 0.1 M Putrescine (1 mM final concentration)

8.74 μL 2-mercaptoethanol (2.5 mM final concentration)

SMM can be stored at -80°C for up to 12 months and undergo at least ten freeze-thaw cycles without appreciable loss of activity.

10 mM Amino acid mix (+Met)

Weigh 20 amino acids (0.045 g Alanine, 0.106 g arginine, 0.066 g asparagine, 0.067 g aspartic acid, 0.061 g cysteine, 0.073 g glutamine, 0.074 g glutamic acid, 0.038 g glycine, 0.105 g histidine, 0.066 g isoleucine, 0.066 g leucine, 0.092 g lysine, 0.075 g methionine, 0.083 g phenylalanine, 0.058 g proline, 0.053 g serine, 0.060 g threonine, 0.102 g tryptophan, 0.091 g tyrosine, 0.059 g valine)

Dissolve all amino acids in 50 mL of 50 mM K-HEPES (pH 7.5).

Adjust pH to 7.5 and store at -80°C .

Note: methionine is not included in 10 mM Amino acid mix(–Met).

Buffer A

50 mM K-HEPES (pH 7.5)

150 mM Potassium acetate.

5 mM Magnesium acetate tetrahydrate
2 mM 2-mercaptoethanol
10% (v/v) glycerol

Buffer B

50 mM K-HEPES (pH 7.5)
150 mM Potassium acetate.
5 mM Magnesium acetate tetrahydrate
1 mM DTT
10% (v/v) glycerol
Freeze in liquid nitrogen and store at -80°C .

Sfp labeling buffer

50 mM K-HEPES (pH 7.5)
10 mM MgCl_2

Fixing solution

7% (v/v) Methanol
7% (v/v) Acetic acid
1% (v/v) Glycerol
85% (v/v) water

COMMENTARY

Background Information

In vitro reconstitution of targeting complexes in the soluble, functional form paves the way for mechanistic dissection of the molecular mechanisms underlying membrane protein targeting to and insertion into the membrane. Multiple approaches to generate and purify the complexes of TAs with their targeting factors have been attempted. Co-expression of model TAs with SGTA (the mammalian homolog of Sgt2) or with Get3 in *E. coli* provides a facile method to generate recombinant SGTA•TA or Get3•TA complexes (Mateja et al., 2015; Mock et al., 2015; Rome, Chio, Rao, Gristick, & Shan, 2014; Rome, Rao, Clemons, & Shan, 2013). Nevertheless, the stoichiometry of the Get3•TA complex appears to be highly dependent on the level of protein expression (Chio, Chung, et al., 2017; Mateja et al., 2015; Suloway, Rome, & Clemons, 2012), raising questions as to whether the composition and activity of Get3•TA are perturbed by recombinant overexpression. The recombinant SGTA•TA complex also exhibited a low efficiency (5–10%) of TA transfer to Get3 (Mock et al., 2015). Alternatively, the *E. coli* PURE-IVT system, which contains the minimal components required for protein synthesis, was used to generate the TA substrate (Mateja et al., 2015; Shao, Rodrigo-Brenni, Kivlen, & Hegde, 2017). Supplementing the PURE-IVT

reaction with purified Sgt2 (or SGTA) and/or Get3 provides an excellent means to reconstitute functional Sgt2•TA or Get3•TA complexes (Mateja et al., 2015; Shao et al., 2017). Super-physiological concentrations of Sgt2 or Get3 (12 or 25 μ M) were necessary in the PURE-IVT based reconstitutions, likely to compensate for the absence of additional factors in this pathway (Mateja et al., 2015; Shao et al., 2017).

In vitro translation based on the *E. coli* S30 extract supplemented with near physiological concentrations of Sgt2 or Get3 provides a facile alternative platform to reconstitute targeting complexes with high activity (Chio, Chung, et al., 2017; Rao et al., 2016). Coupling this platform to the amber suppression system (J. Wang et al., 2006) further allows the site-specific incorporation of fluorescent probes into the TA substrate during translation. The method described here allows completion of all three reactions, translation, site-specific fluorescence labeling, and targeting complex formation, in a single procedure. Subsequent affinity purification enables the generation of targeting complex with high purity, whose activity and interaction can be further interrogated. Although the procedures described here are specific for studying substrate loading, targeting, and insertion in the GET pathway, this platform is generally applicable to other membrane protein targeting pathways.

Critical Parameters and Troubleshooting

In Basic Protocol 1, it is important to optimize both the translation yield and amber suppression efficiency in small-scale trial translation reactions prior to a large-scale translation reaction for preparation of the targeting complexes. The parameters to be optimized include the position of the amber codon, and the concentrations of T7 RNA polymerase, the plasmid DNA template, CmRS, and RF-1.

The FRET-based TA transfer assay in Basic Protocol 2 demands a high quality of sample preparation. Fluorescence measurements are extremely sensitive to the presence of scattering particles such as protein aggregates, which give rise to Raman scatter that overlaps with the fluorescence emission peak of the donor dye. The use of Basic Protocol 1 is critical for generating soluble Sgt2•TA^{Cm} complexes that give good quality fluorescence spectra. The solubility of TA•Sgt2 is further maintained by having a modest excess of Sgt2 over TA^{Cm} during sample storage and measurement, as specified in Basic Protocol 2. For substrates with weaker affinities for Sgt2, a higher Sgt2 concentration is recommended.

Ultracentrifugation of proteins and complexes immediately prior to fluorescence measurements is also critical for generating consistent and high quality fluorescence data. Finally, as with any modifications introduced into a protein, control experiments are needed to ensure that fluorescence labeling does not perturb the interaction and activity of the protein of interest using established assays.

Statistical Analyses

In general, we recommend 2–3 technical replicates each for at least two independently prepared samples to determine the reproducibility of the measured parameters and the potential sources of error. Results are reported as mean $\pm$ S.D. for all the replicates.

Anticipated Results

In Basic Protocol 1, the amber suppression efficiency at the specified residue on the model TA (SUMO-Bos1) is over 90% (Fig. 2B) (Rao et al., 2016). In Basic Protocol 2, the typical efficiency of Sfp-mediated BODIPY-FL-CoA conjugation onto ybbR-Get3 is close to 100% (Fig. 3B). In the FRET-based TA transfer assay, the typical FRET efficiency observed between TA^{Cm} and Get3^{BDP} is 60 – 80% upon completion of TA transfer (Rao et al., 2016). In addition, transfer of TA^{Cm} to unlabeled Get3 does not give rise to any donor fluorescence change; this indicates that the TA^{Cm} fluorescence is not sensitive to the changes in local environment upon transfer to Get3 (Fig. 3C). The concentration of Get3 required for 50% TA transfer from Sgt2 is ~5 nM at an Sgt2 concentration of 200 nM (Fig. 4C). In Basic Protocol 3, the insertion efficiency of purified Get3•TA complex into *get3*yRM is approximately 80%, and the half-time for TA insertion is ~ 5 min (Fig. 4B).

Time Considerations

The optimization for Basic Protocol 1 requires 2–3 days. Budget an extra week if the position of the amber codon needs to be changed to improve suppression efficiency. The preparation of the Sgt2•TA^{Cm} complex takes two additional days. The labeling and purification of Get3^{BDP} in Basic Protocol 2 takes 1 day. The FRET-based TA transfer assay requires 1–2 days. If different TA substrates or additional chaperones are tested, the experiments require extra time. Basic Protocol 3 takes 2 days if optimization is not required.

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Significance Statement

Membrane proteins play essential roles in cellular function. These proteins are initially synthesized in the cytosol and require dedicated targeting machineries for delivery to and insertion into the appropriate cellular membrane. *In vitro* reconstitution is indispensable for understanding the molecular basis of membrane protein biogenesis. This unit describes recently developed quantitative and semi-quantitative assays to reconstitute and dissect the individual molecular steps in the Guided Entry of Tail-anchored protein (GET) pathway, which has emerged as an excellent system to interrogate the molecular principles of membrane protein targeting. The platform and tools we developed are generalizable to studies of other membrane protein targeting pathways.

Figure 1.

Summary of the current model of the GET pathway. Sgt2 is the most upstream factor identified thus far that captures nascent TAs after completion of protein synthesis (step 1). Sgt2 transfers TA to Get3, and this step is stimulated by the Get4/5 complex that bridges between Sgt2 and Get3 (step 2). Get3 further delivers TA to the Get1/Get2 receptor complex, which mediates the insertion of TA into the ER membrane (step 3).

Figure 2.
In vitro reconstitution of Sgt2•TA^{Cm} complex. (A) Schematic depiction of the generation and purification of Sgt2•TA^{Cm}. In the presence of His₆-Sgt2, TA is synthesized and labeled with the fluorescent amino acid Cm using IVT in *E. coli* S30 extract coupled to amber suppression technology. Subsequent affinity purification generates the purified Sgt2•TA^{Cm} complex. (B) Sample SDS-PAGE analysis of amber suppression efficiency in a trial IVT reaction for a model TA substrate, Bos1. Reaction 1 contains a control plasmid that does not have the amber codon. Reaction 2 and 3 were carried out using the amber codon-containing

plasmid in the absence or presence of Cm, CmRS and RF-1, respectively. This figure was adapted from Figure 5B in (Rao et al., 2016).

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Figure 3.

A FRET assay to measure TA transfer from Sgt2 to Get3. (A) Scheme of the FRET assay to monitor TA loading onto Get3. Green and red stars denote the donor and acceptor dyes, respectively. (B) Sample SDS-PAGE of the Get3 labeling reaction stained with Coomassie blue. Lane 1 is Get3-ybbR labeled with BODIPY-FL-CoA via Sfp-mediated conjugation. Lane 2 is unlabeled Get3-ybbR. (C) Representative donor fluorescence emission spectra for the purified Sgt2•TA^{Cm} complex (purple; donor only), Sgt2•TA^{Cm} incubated with unlabeled Get3, Get4/5 and ATP (blue; donor + unlabeled acceptor), Get3^{BDP} and Get4/5 (magenta; acceptor only), and Sgt2•TA^{Cm} complex incubated with Get3^{BDP}, Get4/5 and ATP (green; donor + acceptor). Adapted from Figure 6B in (Rao et al., 2016). (D) Representative data for equilibrium titrations of TA transfer from Sgt2 to Get3. Adapted from the data with the 2AG substrate in Figure 6E in (Rao et al., 2016).

Figure 4.

Assay for *in vitro* TA insertion into the ER. (A) Scheme of the TA insertion assay. The purified Get3•TA complex was presented into *get3* rough ER microsomes. Proper insertion of the TA results in the glycosylation of its C-terminal opsin tag in the ER lumen. (B) Top, representative autoradiogram of the TA insertion reaction. Bottom, quantification and analysis of the insertion time course. The line is a fit of the data to Eq 3. Glyc-Bos1 denotes glycosylated Bos1. Adapted from Figures 7B and 7C in (Rao et al., 2016).