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Biochemical and biological assays of mycolactone-mediated inhibition of Sec61

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Abstract

Mycobacterium ulcerans, the causative agent of Buruli ulcer disease, is unique among human pathogens in its capacity to produce mycolactone, a diffusible macrolide with immunosuppressive and cytotoxic properties. Recent studies have shown that mycolactone targets the host membrane translocation complex (Sec61), with an unprecedented potency compared to previously identified Sec61 blockers. Mycolactone binding to the pore-forming subunit of Sec61 inhibits its capacity to transport nascent secretory and membrane proteins into the endoplasmic reticulum, leading to their cytosolic degradation by the ubiquitin:proteasome system. In T lymphocytes, Sec61 blockade by mycolactone manifests as a sharp decrease in the cell's ability to express homing receptors and release cytokines following activation. Sustained exposure of cells to mycolactone typically generates proteotoxic stress responses in their cytosol and ER, ultimately inducing apoptosis. Here we describe cell-free systems for studying Sec61-mediated protein translocation that allow the impact of mycolactone on the biogenesis of secretory and membrane proteins to be probed. We also describe biological assays of mycolactone-driven inhibition of Sec61 providing rapid and sensitive means to quantitatively assess the presence of the toxin in biological samples.

Keywords

Co-translational translocation, endoplasmic reticulum (ER), enzyme-linked immunosorbent assay (ELISA), flow cytometry, *in vitro* transcription and translation, microsomes, Sec61 translocon, secreted protein, transmembrane protein (TMP).

1 Introduction

In mammalian cells, roughly one third of mRNAs code for secretory and membrane proteins. Of these proteins, the majority are targeted to and then translocated across (secretory proteins), or laterally inserted into (transmembrane proteins, TMPs), the ER membrane in a concerted process termed co-translational translocation [1]. Initiated by the binding of the signal recognition particle (SRP) to a hydrophobic region of nascent polypeptides as it emerges from the ribosome, ribosome-nascent chain complexes are targeted to the ER membrane, via SRP engagement with its cognate receptor, and then transferred to a membrane embedded translocon, whose central component is the Sec61 complex. The Sec61 complex forms a proteinaceous channel through which polypeptides are either translocated across or inserted into the ER membrane bilayer, concomitant with their ribosomal synthesis [1, 2].

In order to faithfully recapitulate these tightly coupled translational and translocational events *in vitro*, the synthesis of proteins of interest must be performed in the presence of cytosolic factors, such as SRP, and a source of ER membranes. Efficient cell-free systems were first devised in the 1970s [3] and typically involve the translation of radiolabelled proteins in either a wheat germ or rabbit reticulocyte lysate (cytosolic extract) that is supplemented with ER-derived microsomes isolated from canine pancreas (ER membranes) [3, 4]. Studies utilising this system, and variations thereof [5], have since provided definitive insights into the mechanisms of co-translational translocation, enabled the discovery of post-translational ER-targeting pathways [2, 6, 7] and permitted the study of subsequent events such as N-glycosylation [8, 9] and ER-associated degradation (ERAD) [10]. Manipulations of this cell-free, *in vitro*, system have also permitted the characterisation of small molecule inhibitors that target these processes [11, 12], including mycolactone which selectively inhibits Sec61-mediated co-translational protein translocation [13–17]. Herein we provide a protocol for the

evaluation of mycolactone as an inhibitor of co-translational translocation using a cell-free system which utilises sequential *in vitro* transcription and translation reactions to synthesise radiolabelled secretory or membrane protein precursors of interest. Assessment of a mycolactone-induced blockade of protein translocation is determined by translation of *in vitro* transcribed mRNA templates, isolation of ER membranes and analysis of the levels of processed secretory and membrane proteins in the presence and absence of mycolactone. By quantifying changes in the ER lumen specific processing events of signal sequence cleavage and/or protein N-glycosylation, the impact of Sec61 inhibition can be determined. This protocol is widely applicable to the study of any small molecules inhibiting either co-translational translocation [18–20] or other ER-dependent processes [21].

In vitro assays of protein translocation and proteomic analyses of mycolactone-treated cells have shown that secretory proteins (with a signal peptide but no transmembrane domain), TMPs with a signal peptide (Type I) and TMPs without signal peptide that have a cytosolic N terminus (Type II) are normally highly susceptible to mycolactone inhibition [17]. In contrast, the rarer subset of TMPs that lack a signal peptide but adopt a topology with an exoplasmic N terminus (Type III) are resistant to mycolactone inhibition both *in vitro* and *in vivo* [17]. At a cellular level, the effectiveness with which mycolactone decreases the steady-state level of susceptible proteins depends on the protein's turnover rate, and hence proteome alterations vary extensively across cell types [16, 22, 23]. However, CD62L (also known as SELL, L-selectin) is a single-pass Type I TMP expressed by quiescent lymphocytes that is acutely suppressed by mycolactone treatment [16, 24]. Likewise, Interleukin (IL)-2 and Interferon (IFN)-γ are both secreted cytokines whose production by activated T lymphocytes is efficiently prevented when cells are incubated with mycolactone during activation [16, 25]. Here we describe assays for CD62L expression and cytokine production using the Jurkat T cell line, as they provide rapid and reproducible methods for detecting nanomolar

concentrations of mycolactone by flow cytometry and ELISA, respectively. Because a mycolactone-mediated Sec61 blockade is cytotoxic in most cell types after 48 hours, assays of cell viability including thiazolyl blue tetrazolium bromide MTT reduction have also been widely used to detect or quantitate mycolactone in biological samples [17]. However, in addition to the necessity for a minimum 48 hours of treatment, such cytotoxicity assays are typically less sensitive and less reliable than those we describe here, and we do not recommend them for an accurate determination of mycolactone concentration.

2 Materials

2.1 Mycolactone solutions

Millimolar solutions of mycolactone made in ethanol or dimethyl sulfoxide (DMSO) and protected from light are suitable for long term storage at -20°C or -80°C. Working solutions should be prepared as and when they are required, by dilution of mycolactone stocks in organic solvents or cell culture medium, and discarded after use as exposure to light causes mycolactone degradation [26].

2.2 Biochemical assays

Prepare all solutions using Milli-Q water and analytical grade reagents at room temperature. All reagents are suitable for long term storage at 4°C, -20°C or -80°C as indicated and may be freeze thawed multiple times unless otherwise specified.

2.2.1 In Vitro Transcription

1. RNase free water.
2. Eppendorf tubes.

3. Transcription buffer 5X: 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl and 200 mM Tris-HCl (pH 7.9). Dissolve 243 mg Tris-base, 61 mg $MgCl_2$, 14.5 mg spermidine and 29.2 mg NaCl in 9 mL water, mix and adjust to pH 7.9 using HCl. Make up to 10 mL with water. Alternatively, use pre-made transcription buffer (eg. Promega) (*see* **Note 1**).
4. 100 mM DTT (1,4-dithiothreitol): Dissolve 154 mg in 10 mL water. Alternatively, use pre-made solution (eg. Promega) (*see* **Note 1**).
5. Ribonucleoside Triphosphates (rNTPs): 25 mM of each rCTP, rATP, rUTP and rGTP in water (*see* **Note 2**).
6. Purified linear DNA template (Fig. 1A) (*see* **Note 3**).
7. RNase inhibitor solution (*see* **Note 1**).
8. RNA polymerase (*see* **Note 1**).
9. Any appropriate column-based kit for the isolation and purification of mRNA (eg. ISOLATE II RNA Mini Kit, Bioline) (*see* **Note 4**).

2.2.2 *In Vitro* Translation

1. Eppendorf tubes.
2. Rabbit reticulocyte lysate. Store at -80°C (*see* **Note 5**).
3. Amino acid mixture minus methionine: 1 mM of each of the 20 amino acids without methionine in aqueous solution. Prepare 20 mM stock solutions of each amino acid in either 0.01 M HCl (Asn, Asp, Glu, Ile, Lys, Phe, Trp, Val), 0.1 M HCl (Tyr) or water (Ala, Arg, Cys, Gln, Gly, His, Leu, Met, Pro, Ser, Thr) then mix 0.5 mL of each stock together and add 0.5 mL of water. Alternatively, a pre-made stock may be purchased (eg. Promega).

4. 1000 Ci/mmol [^{35}S] methionine in aqueous solution (eg. EasyTag EXPRESS ^{35}S Protein Labelling Mix containing [^{35}S] methionine, PerkinElmer). Store in aliquots of 100 µL at 4°C and take appropriate precautions when using radioisotopes.
5. Mycolactone (*see* **Note 6**).
6. DMSO.
7. ER-derived microsomes isolated from canine pancreas. Store at -80°C. Loss of activity may result from multiple freeze-thaw cycles (*see* **Note 7**).
8. Transcription-generated mRNA template (sections 2.2.1 and 3.1.1 and Fig. 1).
9. 2.1 mM Puromycin (Optional) (*see* **Note 8**). Prepare a 21 mM master stock by dissolving 9.9 mg in 1 mL water. Dilute 1:10 to prepare a working 2.1 mM stock for inclusion in translation reactions.
10. High salt cushion: 0.75 M sucrose, 0.5 M KOAc, 5 mM $Mg(OAc)_2$ and 50 mM HEPES-KOH (pH 7.9). Dissolve 25.67 g sucrose, 4.91 g KOAc, 71 mg $Mg(OAc)_2$, 1.19 g HEPES in 90 mL water, mix and adjust to pH 7.9 using KOH. Make up to 100 mL with water. Suitable for long term storage at -20°C.
11. 2X SDS sample buffer: 100 mM Tris-HCl, pH 6.8, 100 mM DTT, 4% (w/v) SDS, 20% (w/v) glycerol, 1% (v/v) L-methionine, 10 mM EDTA, 0.02% bromophenol blue.
12. Endoglycosidase H (Endo H) (*see* **Note 9**).
13. Equipment and buffers for SDS-PAGE according to standard protocols and using an appropriate molecular weight marker [27].
14. Fix mix: 20% methanol, 10% glacial acetic acid. Add 200 mL methanol, 100 mL glacial acetic acid and 700 mL water. This solution may be stored long term at room temperature.
15. Ultracentrifuge, ultracentrifuge tubes and compatible rotor.

16. Filter/blotting paper.
17. Cling film.
18. Heated polyacrylamide gel vacuum dryer (eg. BioRad Gel Dryer) [28].
19. Exposure cassette with phosphor screen (eg. FujiFilm) (*see* **Note 10**).
20. Phosphorimager (eg. Typhoon FLA-7000, GE Healthcare) equipped with appropriate software (eg. AIDA, Raytest Isotopenmeßgeräte).

2.3 Biological assays

2.3.1 Cell culture and activation

1. Jurkat, Clone E6.1 (ATCC® TIB152™).

2. Complete culture medium: RPMI-1640 medium (with L-glutamine eg. GlutaMAX™, and sodium bicarbonate) supplemented with 10 % fetal calf serum (FCS).

3. 96-well cell culture treated plates.

4. Phorbol 12-myristate 13-acetate (PMA), 2 mg/mL stock solution in DMSO stored at -20°C in the dark.

5. Calimycin (A23187), 50 mg/mL stock solution in DMSO stored at -20°C in the dark.

2.3.2 Flow cytometry analysis (FACS)

1. Anti-human CD62L antibody (Clone DREG-56), conjugated to a fluorochrome such as FITC, PE or APC.

2. Fluorochrome compatible cell impermeable dye, such as propidium iodide (PI), 10 μg/mL stock solution in PBS stored at +4 °C in the dark.

3. Dulbecco's Phosphate Buffered Saline (PBS), no calcium no magnesium: 2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCL, 8.06 mM Na_2HPO_4-7H2O in distilled water. PBS can also be prepared from commercially available tablets or 10X solutions (note that such

concentrated stock solutions may precipitate when cooled and precipitates should be completely dissolved before dilution). The pH of PBS is ~7.4. If necessary, it can be adjusted using hydrochloric acid or sodium hydroxide. PBS solutions should be sterilized by autoclaving or filtration for long term storage at room temperature or +4°C.

4. FACS buffer: PBS + 2% FCS.

5. Two-laser Flow cytometer (eg. CytoFLEX B2R2, Beckman Coulter).

2.3.3 ELISA

1. Matched antibody pair for the capture and detection of human IL-2 (or IFN-γ).
2. Recombinant human IL-2 (or IFN-γ).
3. Streptavidin-HRP.
4. 96-well ELISA plates.
5. Assay diluent: PBS + 1% bovine serum albumin (BSA).
6. Wash buffer: PBS + 0.05% Tween-20.
7. TMB substrate solution.
8. Stop solution: 2N H_2SO_4.
9. Microplate reader capable of measuring absorbance at 450 nm.
10. Wash bottle or automated microplate washer.

3 Methods

3.1 Biochemical assays

The following *in vitro* transcription protocol (section 3.1.1) is sufficient for synthesis of one mRNA template from one DNA template (Fig. 1) and all procedures may be performed at room temperature unless indicated otherwise. The *in vitro* translation protocol (section 3.1.2, Figs. 2 and 3) is sufficient for 12 separate translation reactions obtained from a translation

master mix and may be scaled up or down depending on the number of experiments required (*see* **Note 11**). Unless otherwise specified, all reagents must be precooled on ice prior to use and all procedures must also be performed on ice in order to preserve the activity of ER-derived microsomal membranes.

3.1.1 *In Vitro* Transcription

1. To an Eppendorf tube add in the following order: 40 µL RNase free water, 20 µL of Transcription buffer 5X, 10 µL DTT solution, 5 µL rNTPs solution, 6 µL linear DNA template (*see* **Note 5**), 2.5 µL RNase inhibitor solution and 4 µL of the appropriate RNA polymerase (circa 80 units, *see* **Note 1**).
2. Mix thoroughly by vortexing.
3. Incubate at 37°C with agitation for 2-24 h (*see* **Note 12**).
4. Isolate and purify mRNA using a commercial column-based purification kit according to the particular manufacturer's instructions (*see* **Note 4**).

3.1.2 *In Vitro* Translation

1. To generate the translation master mix add in the following order to an Eppendorf tube: 200 µL rabbit reticulocyte lysate, 6.25 µL amino acids minus methionine, 18.75 µL [^{35}S] methionine and 18.75 µL nuclease-treated ER-derived microsomes (*see* **Note 7**).
2. Thoroughly mix the translation master mix by vortexing and then split into 17.5 µL aliquots in 12 new Eppendorf tubes.
3. Add 1 µL of mycolactone or DMSO to each individual aliquot (*see* **Note 6**).
4. Next, add 2 µL of the RNA template of interest to each individual aliquot (*see* **Note 13**). Immediately mix thoroughly by vortexing and incubate samples at 30°C for 20 min to perform the translation reaction (*see* **Note 14**).

5. During this translation time, pre-cool the ultracentrifuge to 4°C, label 12 centrifuge tubes with reaction identifiers (*see* **Note 15**) and add 80 μL of high-salt cushion (*see* **Note 16**) to the labelled centrifuge tubes.

6. Following translation, add 1 μL of puromycin, mix thoroughly by vortexing and incubate at 30°C for a further 5 min (*see* **Note 8**).

7. To isolate the membrane-associated material (*see* **Note 17**), mix samples by vortexing once more, remove 18 μL from each translation reaction (*see* **Note 15**) and layer on top of the high-salt cushion so that the centrifuge tube contains two discrete layers, with the high-salt cushion (colourless) on the bottom and the translation reaction (red) on the top (*see* **Note 18**).

8. Centrifuge the samples at 4°C for 10 min at 100,000 ***g***.

9. Being careful not to disturb the pellet, remove and discard the supernatant (*see* **Notes 15 and 19**).

10. Re-suspend the pellet in 30 μL 2X SDS sample buffer (*see* **Notes 15 and 20**) and transfer each re-suspended sample to a new labelled Eppendorf tube. Once all pellets have been re-suspended, add 1 μL Endo H to the appropriate samples (*see* **Note 9**) and incubate all samples at 37°C for 30 min.

11. Denature samples by incubating samples at 70°C for 5 min and then resolve proteins by SDS-PAGE according to standard protocols [27].

12. Following protein resolution by SDS-PAGE, transfer the gel to a plastic container containing a large enough volume of fix mix such that the gel is completely submerged. Incubate, with rocking, at room temperature for 5-10 min.

13. Next, gently transfer the gel to a polyacrylamide gel vacuum dryer according to standard protocols [28]. Briefly, layer two fix mix-soaked pieces of blotting paper (slightly larger than the gel to be dried) on top of the dryer, followed by the fix mix incubated gel and a layer of

cling film to form a sandwich before sealing the gel dryer, removing any air bubbles and drying at 65°C for 2 h (*see* **Note 21**).

14. Once dry and cool, tape the gel to a gel cassette and carefully place a spot of 1:400 diluted [^{35}S] methionine onto the bands of the molecular weight markers so their positions can be visualised by phosphorimaging and leave these radioactive marks to dry for 5 minutes at room temperature. Next, place a phosphorimaging screen face down on top of the gel and close the cassette [29] (*see* **Note 10**). After exposure for a minimum of 24 h, radiolabelled translation products may be viewed using a phosphorimager (Fig.3).

3.2 Biological assays

The following protocols were optimized for assessment of the Sec61 inhibitory activity of pure (synthetic or bacteria-derived) mycolactone. Assay conditions also proved well suited to the characterization of Ipomoeassin F, a newly identified, plant-derived, Sec61 blocker [20] (*see* **Note 22**). Both approaches are applicable to the study of synthetic derivatives of mycolactone [25] or mycolactone-containing biological samples. They use the Jurkat T cell line, which can be easily maintained at 37°C in a 95% air/5% CO_2 atmosphere in complete culture medium. Jurkat T cell concentration should be kept within10^5 and 10^6 viable cells/mL, by addition of fresh medium every 2 to 3 days (depending on cell density), or by centrifugation with subsequent resuspension at 10^5 viable cells/mL.

3.2.1 Downregulation of CD62L surface expression

1. Harvest exponentially growing cells, and adjust cell suspension to a concentration of 10^6 viable cells/mL in complete culture medium.

2. Seed a 96-well cell culture treated plate with 50 µl of this cell suspension per well.

3. Prepare a 1 µM working solution of mycolactone by dilution of your stock solution in complete culture medium (section 2.1), and serial two-fold dilutions spanning the 1-500 nM concentration range (*see* **Note 22**).

4. Add 50 µl of mycolactone dilutions to cell containing wells (total volume/well is 100 µl). Set up duplicate wells for each mycolactone dilution and 2 x duplicate wells in 100 µL complete culture medium to serve as untreated/unstained controls and untreated/stained controls, respectively.

5. Incubate for 16 to 24 h at 37°C, in a 95% air/5% CO_2 atmosphere.

6. Harvest the cells by centrifugation of the plates at 125 ***g*** for 5 min at +4°C.

7. Wash the cells by adding 200 µl ice-cold PBS, centrifuge at 125 ***g*** for 5 min, remove the supernatant from the wells, repeat with 200 µl ice-cold FACS buffer.

8. Resuspend the cells after the second wash in 50 µl conjugated antibody (0.1-10 µg/mL in ice-cold FACS buffer). Resuspend unstained controls in 50 µl ice-cold FACS buffer. Mix all cells thoroughly by pipetting up and down 2 times.

9. Incubate for 30 min in dark at +4°C.

10. Add 200 µl ice-cold PBS containing 25 µl PI staining solution. Mix gently and incubate for 1 min. Centrifuge at 125 ***g*** for 5 min, remove the supernatant from the wells, resuspend in 200 µl ice-cold FACS buffer, repeat.

11. Resuspend the cells after the second wash in 200 µl ice-cold FACS buffer. Keep the cells in the dark on ice, or at +4°C in a fridge, until analysis. For best results, analyse the cells on the flow cytometer as soon as possible.

12. Acquire mean fluorescence intensities (MFI) in the chosen CD62L fluorophore channel, after exclusion of dead cells and doublets. Subtract the MFI of unstained controls. Plot MFI of stained samples as a function of mycolactone concentration. A typical dose-response curve is shown in Fig. 4A.

3.2.2 Inhibition of cytokine production

This assay can be fully completed in two days: *Day 0* for cell activation in the presence or absence of mycolactone, and *Day 1* for quantitative determination of IL-2 (or IFN-γ) in cell culture supernatants by ELISA. Alternatively, supernatants may be harvested on *Day 1* and transferred into microtiter plates for storage at -20°C or -80°C until cytokine analysis.

Day 0

1. Harvest exponentially growing cells, and adjust cell suspension to a concentration of 10^6 viable cells/mL in complete culture medium.

2. Seed a 96-well cell culture treated plate with 50 µl of this cell suspension per well.

3. Prepare a 1 µM working solution of mycolactone by dilution of the stock in complete culture medium (section 2.1), and serial two-fold dilutions of this solution (*see* **Note 22**).

4. Add 50 µl of mycolactone dilutions to cell containing wells (total volume/well is 100 µl). Set up duplicate wells for each mycolactone dilution and 2 x duplicate wells in 100 µL complete culture medium to serve as untreated/unactivated controls and untreated/activated controls, respectively.

5. Incubate for 1h at 37°C, in a 95% air/5% CO_2 atmosphere.

6. Prepare a 10 X solution of activation containing 500 ng/mL PMA and 10 µg/mL A23187 in complete culture medium.

7. Add 10 µl of activation mix to the wells containing treated cells and untreated/activated controls (total volume/well is 110 µl). Add 10 µL of complete culture medium to untreated/unactivated controls.

8. Incubate for 16 to 24 h at 37°C, in a 95% air/5% CO_2 atmosphere.

Day 1

8. Pellet the cells by centrifugation of the plates at 125 ***g*** for 5 min at +4°C. Cell culture supernatants may be harvested and transferred into a fresh 96-well plate for storage at -20°C, or immediately processed for cytokine quantitation (*see* **Note 23**).

9. A wide range of ELISA kits and reagents are commercially available that provide pairs of anti-IL-2 (or IFN-γ) antibodies and an HRP colorimetric detection system to quantitate cytokines in samples, with detailed protocols. Assay cytokines on neat supernatants, following the manufacturer's recommended procedure (*see* **Note 23**).

10. Acquire absorbance levels of samples and standards (*see* **Note 24**). Cytokine concentrations are best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If not available, plot the standard curve on log-log axis graph paper with analyte concentration on the x-axis and absorbance on the y-axis. Draw a best fit line through the standard points and determine analyte concentrations graphically. If the samples were diluted, multiply by the appropriate dilution factor. A typical dose-response curve is shown in Fig. 4B.

4 Notes

1. These buffers/reagents may all be prepared in-house, however, we use pre-made solutions of Transcription buffer 5X, 100 mM DTT, 40 U/μL recombinant RNAsin® Ribonuclease inhibitor (Promega) and either 20 U/μL T7 RNA polymerase or 19 U/μL SP6 RNA polymerase depending on the transcription promoter present in the linear DNA template (Fig. 1A). T7/SP6 RNA polymerase are supplied in their respective buffers (20 mM HEPES-KOH (pH 7.6), 50 mM KCl, 8 mM DTT and 20 mM potassium phosphate buffer (pH 7.7), 1 mM EDTA, 10 mM DTT, 100 mM NaCl, 0.1% (v/v) Triton® X-100) and as 50% (v/v) glycerol solutions.

2. rNTPs purchased as 100 mM stock solutions in water (400 µL of rCTP, rATP, rUTP and rGTP, Promega) are combined, mixed and then diluted by the addition of 4.8 mL water to yield a solution containing 25 mM of each rNTP. Aliquots of 50 µL are suitable for long term storage at -20°C may be freeze thawed multiple times.

3. Linear DNA templates must have an appropriate transcription promoter (usually T7 or SP6, although other promoters may be used) typically located ~50 to 150 base pairs upstream of the coding region of interest which must contain a start and stop codon (Fig 1A). Linear DNA templates are prepared by PCR amplification [30] from a plasmid containing the cDNA of interest using a forward primer which anneals upstream of the T7/SP6 promotor (5'-3' direction) and a reverse primer which anneals after the in-frame stop codon (3'-5' direction). For longer proteins, or proteins with few methionine residues, a reverse primer designed with an overhang of three methionine residues and an artificial stop codon may be used in order to incorporate more [^{35}S] methionine into the resulting translation products and improve the visualisation of radiolabelled proteins. Any thermostable DNA polymerase may be used according to the manufacturer's protocol (eg. KOD Hot Start DNA Polymerase, Sigma-Aldrich). Isolation and purification of the PCR-generated linear DNA templates may be performed using a commercial column-based PCR isolation and purification kit (eg. ISOLATE II PCR and Gel Kit, Bioline). Following purification, linear DNA templates, as stock solutions in water (~75-150 ng/µL), may be stored long term at -20°C until use for *in vitro* transcription reactions.

4. Typically, each transcription reaction from a single PCR-generated linear DNA template yields ~200-1000 ng/µL of purified mRNA. An indication of mRNA purity may be determined by running parallel samples of the DNA template and mRNA on an agarose gel [31] in which, upon a successful purification, the band corresponding to the DNA template will give rise to a lower molecular weight band of mRNA. Alternatively, the ratio of

absorbance measurements (A_{280}/A_{260}) acquired at wavelengths of 280 nm and 260 nm using a spectrophotometer or plate reader may be used. We find that, using a Synergy H1 Hybrid multi-mode plate reader (BioTek), purified PCR-generated DNA templates have a A_{280}/A_{260} ratio of ~1.8 whereas transcription-generated mRNA templates have a A_{280}/A_{260} ratio of ~2.1.

5. Rabbit reticulocyte lysate may be prepared in-house [32] and prior to inclusion in translation reactions must be: i) supplemented with hemin, an inhibitor of the translational inhibitor eIF-2 kinase [33] and ii) treated with micrococcal nuclease in order to digest endogenous mRNAs [5]. Alternatively, nuclease treated and hemin supplemented rabbit reticulocyte may be purchased commercially (eg. Promega) and stored long term at -80°C. Do not freeze-thaw the commercial lysate more than twice.

6. Synthetic mycolactone A/B (a mixture of *E*- and *Z*-isomers) [34] was first solubilised in DMSO containing 10% bovine serum albumin (BSA) to a concentration of 33 μM. This stock was then diluted in DMSO to give a working stock of 20 μM such that, in translation reactions (typically 20 μL volume), the final concentration of mycolactone when added at 1 in 20 (v/v) is 1 μM and the level of remaining BSA negligible. A 1 μM final concentration of mycolactone, and other substrate selective inhibitors of co-translational translocation such as Ipomoeassin-F [20], will effectively block the insertion and/or translocation of secretory and certain classes of transmembrane proteins without the need for prior incubation with microsomes [11].

7. ER-derived microsomes capable of efficiently translocating precursor proteins synthesised in cell-free translation reactions can be made from the pancreas of a variety of species including dog, pig, sheep and cattle [3, 35]. Microsomes from different sources, and indeed different batch preparations from the same tissue, though all ER-derived, may differ in their activity [35]. Microsomal membranes prepared from dog pancreas following a well-established protocol [3] are usually very effective and we typically use them in each

translation reaction at a final concentration of 6.5% (v/v) (from a stock with OD_{280} = 44/mL). Canine pancreatic microsomes may also be purchased commercially (eg. Promega) although typically in small amounts. Microsomes may be stored long term at -80°C. Multiple freeze-thaw cycles can result in a loss of activity. Failing access to such pancreatic microsomes, semi-permabilised mammalian cells may be used as an alternative source of ER membrane [36]. In this case, the semi-permeabilised cells must be freshly made before each experiment [37].

8. Puromycin induces premature chain termination during translation [38] ensuring the complete ribosomal release of all polypeptide chains. Performing this step is optional and it may be omitted.

9. Endo H cleaves the bond between the two *N*-acetylglucosamine (GlcNAc) residues in the core of N-linked glycans such that high-mannose N-glycans are cleaved to leave one GlcNAc residue attached to the radiolabelled polypeptides. Following Endo H treatment and resolution by SDS-PAGE, loss of a higher molecular weight band (the N-glycosylated form) permits identification of the N-glycosylated versus non-N-glycosylated species (Fig 2B). Recombinant Endo H is commercially available (New England Biolabs) and can be stored as 50% glycerol solutions at -20°C. We find that Endo H (30 kDa) and Endo Hf (70 kDa) (New England Biolabs) function well when added directly to SDS sample buffer at high concentration (1000 U), despite previous reports of SDS-mediated enzyme inactivation [39]. Ideally, an N-glycan cleaving enzyme should be used that has a different molecular weight/migration to your radiolabelled protein of interest so that the bands are not distorted when analysed by SDS- PAGE.

10. Traditional autoradiography may be used, however, phosphor screens are often more sensitive than X-ray film, reusable and typically require approximately one-tenth of the exposure time. Furthermore, the resulting digital signals are easy to quantify. For alternative

techniques for the visualisation of radiolabelled proteins following SDS-PAGE and gel drying, standard procedures of autoradiography and densitometry may be followed [29].

11. A typical experimental design consists of three reactions performed in triplicate: i) a control translation reaction performed in the absence of mycolactone, ii) a second control translational reaction performed in the absence of mycolactone but subsequently Endo H treated to permit identification of N-glycosylated species, and iii) a translation reaction performed in the presence of mycolactone (Fig. 2C). Use of a 'master mix' ensures that the ratio of reagents across all translation reactions are consistent. For proteins of a longer length, such as polytopic membrane proteins or those with more than ~ 300 amino acids, or proteins with few methionine residues, we perform a two-fold scale up of the translation reactions.

12. A good yield of mRNA can be obtained in 2 h and higher yields can be obtained from longer reaction times, particularly for DNA templates containing longer coding regions. Reactions have always been purified after a maximum of 24 h.

13. Translation reactions will only proceed once the mRNA template has been added. Keeping all samples on ice, adding the mRNA last and transferring all samples to 37°C at the same time synchronises translation initiation across the samples.

14. Typically, 20 min translation time is sufficient to generate radiolabelled proteins which can be visualised by phosphorimaging. For longer proteins, in addition to a two-fold scale up of translation reactions the translation time can be extended to 1 h in order to increase the quantity of radiolabelled products synthesised and, thus, increase their detection by phosphorimaging.

15. When labelling centrifuge tubes, marking a line along the top outer edge of the tube is particularly useful in identifying the location of the ER membrane pellet, which can often be difficult to see. By placing centrifuge tubes in a fixed angle rotor with the marked line on the outside of the tube, ie. where the microsome pellet will form during centrifugation, the

location of the pellet may be more easily identified after removal from the rotor. Furthermore, by angling the pipette tip towards the opposite side of the centrifuge tube, removal of the high-salt cushion may now be performed without disturbing the pellet. Alternatively, angling the pipette tip on the same side allows more efficient resuspension of the pellet.

16. For reactions performed on a 2X translation scale, 120 µL of high-salt cushion is sufficient for loading of 38 µL of translation reaction material.

17. Resolution of radiolabelled proteins at this stage may be performed, however, the large amount of background [^{35}S] methionine often masks small polypeptides of interest. Isolation of membrane-associated material (ie. the microsome fraction), enriches the signal of the radiolabelled protein of interest by removal of background [^{35}S] together with any non-targeted/integrated proteins in the lysate. This enhances the visualisation of those proteins which have been efficiently membrane translocated/integrated. If total translation products are analysed, then the fix-mix should be changed twice to remove unincorporated [^{35}S] methionine from the gel.

18. Slowly dispensing the translation material through a pipette tip positioned at a 45° angle relative to the centrifuge tube and at the top of, but still inside, the high-salt cushion is a good technique to avoid mixing of the two layers during addition of the translation material to the cushion.

19. In order to maximise removal of background [^{35}S] methionine, the centrifugation step may be performed twice, re-suspending the microsome pellet in another 80 µL of high-salt cushion and centrifuging again at 4°C for 10 min at 100,000 ***g***. Due to the viscosity of the high-salt cushion, dislodgement and re-suspension of the pellet requires several up and down motions with the pipette plunger. Performing this step, whilst recommended, may, however, be omitted.

20. The microsome pellet can be re-suspended by dispensing 2X SDS sample buffer into the bottom of the centrifuge tube on the side previously marked with a line, pipetting up and down several times, accompanied by scraping of the tube with the pipette tip. Perform the scraping and re-suspension slowly and gently as 2X SDS sample buffer has a propensity to foam which makes transfer of the samples into a new Eppendorf tube difficult and time-consuming.

21. During gel drying, the underside of the blotting paper can stick to the gel dryer itself, making removal of the dried and blotting paper mounted gel difficult to do without tearing. By using two sheets of the blotting paper, the upper piece of blotting paper to which the gel has dried may be lifted easily off the gel dryer and the lower piece of blotting paper removed without damaging the gel. The cling film placed on top of the gel allows for easy removal of the silicone cover from the gel after drying. However, great care should be taken to ensure the cling film is flat and devoid of any ripples before the vacuum seal is applied, otherwise the gel can crack or fragment during drying and distort the radiolabelled protein products of interest. After sealing, removal of any air bubbles under the cling film by gently rolling a cylindrical surface across the gel (eg. a 5 mL pipette) will also help to prevent 'cracking' of the gel.

22. No toxic effects to cells was observed with ethanol or DMSO at 0.1% final concentration (v/v). IC_{50} for mycolactone (A/B form) and Ipomoeassin F were both in the 5-10 nM range, for downregulation of CD62L expression and for inhibition of IL-2 production by Jurkat T cells in the conditions described [20].

23. A typical ELISA experiment includes 12 wells for standard curve corresponding to 5 recombinant human cytokine dilutions and blank in duplicates, and 1-2 wells for each cell culture supernatant. The intra-assay precision of ELISAs is typically <10%, and in our experience variability among biological replicates was below 20%.

24. If a sample's absorbance value falls outside the standard curve range, that test sample needs to be reanalysed at a higher or lower dilution as appropriate.

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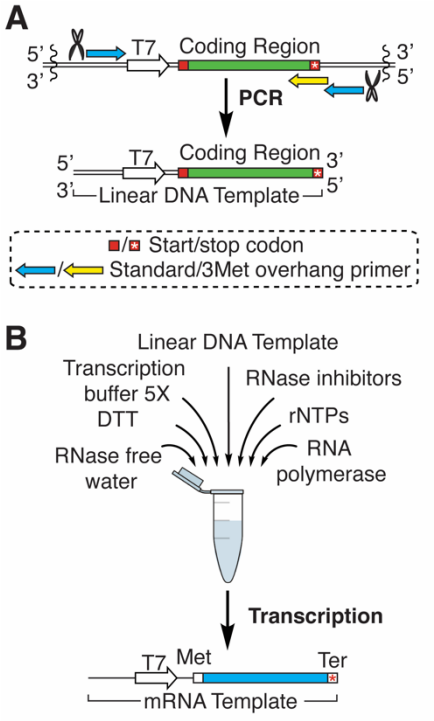


Fig 1. size: 2.25 x 3.75 in

Fig. 1. Schematic depicting (**a**) the preparation of linear DNA templates suitable for use in (**b**) *in vitro* transcription reactions. See main text for details.

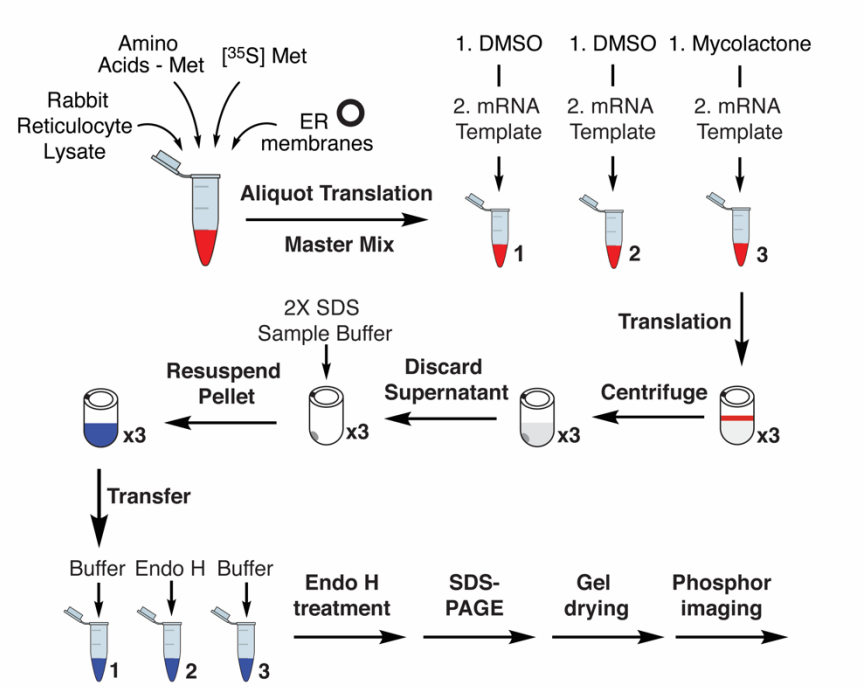


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Fig. 2. A schematic of the typical workflow for *in vitro* translation reactions involving a control, Endo H treated and mycolactone samples. See main text for details.

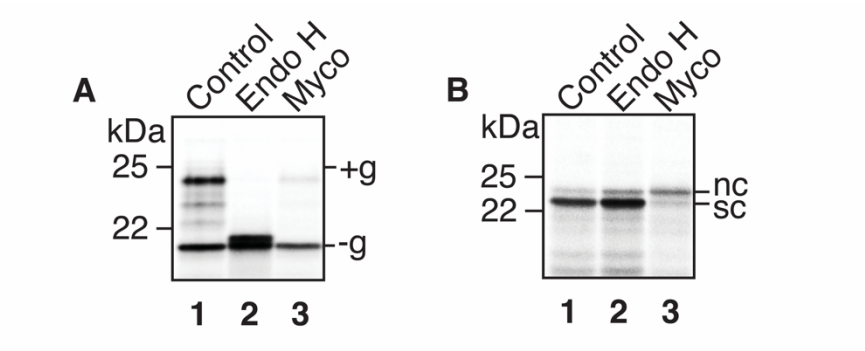


Fig 3. size: 4.5 x 1.83 in

Fig. 3. Phosphorimages obtained following the *in vitro* transcription and translation protocols as described in the main text. Translation reactions were performed in the absence of mycolactone and analysed directly (lane 1) or treated with Endo H (lane 2) or performed in the presence of mycolactone and analysed directly (lane 3). (**A**) N-glycosylation and/or (**B**) signal sequence cleavage may be used as read-outs to assess levels of ER membrane translocation by comparison of the ER processed species in lanes 1 and 3. -g, non-N-glycosylated species, +g, N-glycosylated species, nc, non-signal sequence cleaved, sc, signal cleaved.

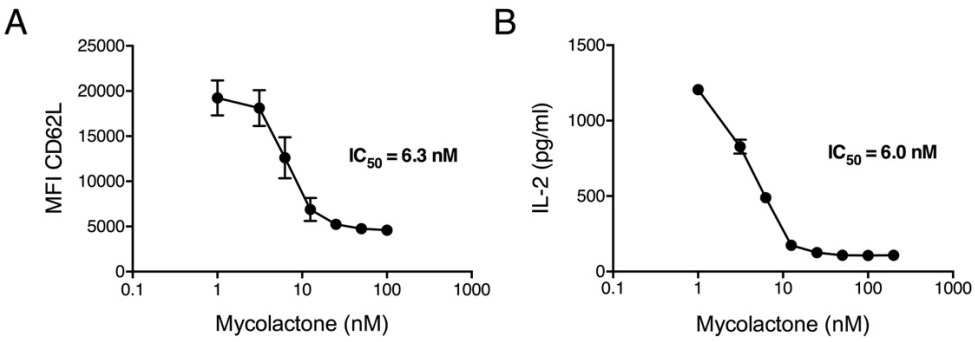


Fig 4. size: 5 x 1.83 in

Fig. 4. Mycolactone-mediated inhibition of Sec61 in Jurkat T cells, as reflected by (**A**) dose-dependent decrease in basal CD62L expression and (**B**) dose-dependent decreased ability to produce IL-2 upon activation.