SUPPLEMENTARY ONLINE DATA
A biochemical analysis of the constraints of tail-anchored protein biogenesis

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Calculations of the energetic cost for membrane integration of PEGylated Sec61βOPG

Boundaries of the membrane-spanning region of Sec61βOPG were determined using a prediction algorithm for changes in free energy, ΔG, upon Sec61 translocon-mediated protein integration into a lipid bilayer [1]. The transfer energy for the predicted 23-amino-acid segment (see Table S1) was calculated on the basis of the water/octanol partition scale [2] and a value of −11.5 kcal/mol was obtained.

LogP values of atom fragments of the mPEG-5000 maleimide moiety were computed by best fit to a training set of 2473 compounds [3], and the sum obtained (−5.0 to −5.3) was in good agreement with the data available for the logP of PEG-400 derived from water/hexane partitioning (−4.824), assuming that PEG-400 is composed of, on average, eight or nine CH2CH2O repeats, and taking into account any potential discrepancy between water/hexane and water/octanol systems. Making equivalent calculations for a 35 Å (1 Å = 0.1 nm) stretch of PEG, corresponding to the length of the predicted tail-anchor region, and converting the results to ΔG, the cost of transferring the PEG polymer into a lipid bilayer in a conformation that spans both leaflets was estimated as approximately +3.6 kcal/mol.

A rotamer entropy loss for an extended PEG chain was estimated on the basis of a comparison with studies of aliphatic side chain conformers in proteins [4] and the role of ligand rigidification in modulating binding affinities [5]. Hence, the entropy was calculated from the equation $S = -R \sum (P \ln P)$, where $S$ is the universal gas constant, $R$ is the probability for each rotamer, and the sum is taken over all rotamers. For a torsional system with three stable rotamers, assuming a transition between equal probabilities for each, and just one (extended chain) rotamer,

Figure S1  TRC40 binding to PEGylated Sec61βOPG

(A) PEGylated Sec61βOPG77C or Sec61βOPG84C were incubated in the presence of rabbit reticulocyte lysate followed by co-immunoprecipitation with anti-TRC40 or control anti-PDI antibodies as indicated. Bound Sec61βOPG variants were then eluted and detected by quantitative immunoblotting using a LiCor system (see the Experimental section of the main text) to estimate the association of the unmodified and PEG-modified forms with TRC40. Molecular-mass markers are shown in kDa on the right-hand side. (B) The actual values obtained from the LiCor system for each of the products analysed (see boxes in (A) for two separate experiments are shown, together with the method for calculating Sec61βOPG binding. Non-specific binding to Protein A-Sepharose coated with anti-PDI antibody was subtracted from the binding to anti-TRC40 antibody-coated Protein A-Sepharose, and the resulting values were normalized to the amount of each Sec61βOPG species. These results are expressed graphically for Sec61βOPG77C (C) and Sec61βOPG84C (D).

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a free energy contribution of $+0.6 \text{ kcal/mol}$ at $T = 300 \text{ K}$ for each torsion was obtained. Over the 35 Å TMS length, this amounts to $+17.0 \text{ kcal/mol}$ for a fully extended chain.

A contribution of the mPEG-5000 maleimide linker region to the energetic cost of inserting PEGylated Sec61/βOPG into a lipid bilayer was estimated making an assumption, on the basis of the manufacturer's data, that in the experimental conditions used the maleimide-linker ring remains intact. Taking into account the nature of the maleimide-linker components and their bond rotamer status, the following parameters were calculated: length in the extended conformation, 10 Å; $\Delta G$ for octanol partitioning of $-0.5 \text{ kcal/mol}$ (calculated from $\log P$); rotamer entropy loss in extended conformation contributing $+3.6 \text{ kcal/mol}$, i.e. a net value of $+3.1 \text{ kcal/mol}$.

The estimated contribution of each component to the overall energetic cost for the membrane integration of the PEGylated forms of Sec61/βL74C, Sec61/βS77C, Sec61/βV84C and Sec61/βL87C is presented in Table S2. Although the $\Delta G$ values shown for the lipid partitioning of PEG attached to these distinct sites are estimates and absolute values may differ, our calculations clearly indicate that the integration of PEGylated Sec61/β is finely balanced, such that it changes from favourable to unfavourable as the site of PEG modification is moved deeper into the membrane from the cytosolic face. It should be noted that, in all calculations presented above, an extended conformation of the maleimide-PEG moiety, parallel to the TA of Sec61/β, was assumed. This conformation minimizes occlusion of the PEG chain from the solvent and provides a defined conformer for calculations. Although other conformations are theoretically possible, these would most likely require higher energy for their membrane integration, due to the increased number of the PEG repeats that would have to be accommodated by the lipid bilayer. In addition, it is assumed that the bulk of the PEG molecule does not translocate across the membrane, consistent with experimental data, so that PEG traversal of the membrane is always modelled from the cysteine/maleimide linker to the cytosolic side.

Figure S2 Characterization of recombinant Sec61/βOPG and Cytb5OPG

(A) Coomassie Blue-stained gel of purified recombinant Sec61/βOPG$^{\text{STTC}}$ and Cytb5OPG$^{\text{STTC}}$ both prepared using buffers supplemented with 0.75 % (w/v) octyl-β-D-glucopyranoside (see also [6]). (B) Membrane integration reactions of recombinant Sec61/βOPG$^{\text{STTC}}$ and Cytb5OPG$^{\text{STTC}}$ carried out in the presence of reticulocyte lysate (see [6]), and using immunoblotting for detection and EndoH sensitivity of N-glycosylated products (*) to confirm membrane integration. (C and D) Time course for the membrane integration (integr.) of Sec61/βOPG$^{\text{STTC}}$ (C) and Cytb5OPG$^{\text{STTC}}$ (D) performed as described for (B). Truncated versions of Cytb5OPG (C); Sec61/βOPG (B) and N-glycosylated Sec61/βOPG (D) are indicated. Molecular-mass markers in kDa are also indicated.
Figure S3  BODIPY® labelling of Sec61βOPG

Purified recombinant Sec61βOPG\textsuperscript{S77C} was modified with BODIPY® maleimide or not (unmodif.) as indicated, and membrane integration (integr.) was analysed as described in the text (see also Figures 1A and 1B of the main text). Fluorescently labelled products recovered with the membrane fraction were visualized directly using a FUJIFILM LAS-3000. N-glycosylated products are indicated (*). Molecular-mass markers in kDa are also indicated on the right-hand side.

Figure S4 Both monomers of TA protein dimers are authentically integrated into the ER membrane

(A) Dimers of Sec61βOPG were generated by cross-linking the single cysteine mutant S77C (see Supplementary Table S1) with 1 mM of the indicated thiol-reactive reagents with a spacer length of 8–13 Å. The reaction mixtures were incubated for 10 min at 25 °C, quenched with 15 mM 2-mercaptoethanol and the modified proteins were used in a membrane integration assay (see [6]). The membrane-associated fraction was isolated by centrifugation, treated with Endo H where indicated and products were analysed by immunoblotting. N-glycosylated monomers and dimers are indicated (1x glyc. and 2x glyc. respectively). BMB, 1,4-bis(maleimido)butane; BMOE, bis(maleimido)ethane. (B and C) BMH cross-linked single cysteine mutants of Sec61βOPG (B) and Cytb5OPG (C) were used in a membrane integration reaction [6], and the isolated membrane fraction subjected to partial digestion with Endo H (0.21 units/μl). At the indicated times, aliquots were mixed directly with Laemmli sample buffer, and products were subsequently analysed by immunoblottting. Migration of Sec61βOPG and Cytb5OPG dimers is indicated, and various N-glycosylated (glycosyl.) species of both proteins are shown (*). The doublet corresponding to the Sec61βOPG dimer results from a truncated form of the recombinant protein (see Figure S2), leading to a population of mixed dimers. These species are also responsible for the additional N-glycosylated protein species identified by EndoH treatment (B). Molecular-mass markers in kDa are indicated on the right-hand side.
Table S1  Sequence of TA proteins used in the present study

Sequences of TA proteins used in the present study are shown, with the predicted transmembrane segments in bold and the opsin-derived, C-terminal tag underlined. Mutations introduced into the amino acid sequences are indicated by asterisks (*). Amino acid numbering reflects that of the wild-type proteins; however, the recombinant versions lack an initiator methionine residue and have instead a GlySer dipeptide (italics) resulting from the thrombin-mediated cleavage of the N-terminal HisTrx tag.

| Protein (species) | Sequence |
|------------------|----------|
| Sec61βOPG77C (human) | GSPGPTPS6TNVGGSRSPSKAVAARAAGSTVRQRKNASS*GTRSAGRTTSAGTGGMWRFYTEDPSGLKVGPVPVLVMC*LLFIASVFMLHIWGGY-TRSGPNFYVPFSNKTG |
| Sec61βOPG84C (human) | GSPGPTPS6TNVGGSRSPSKAVAARAAGSTVRQRKNASS*GTRSAGRTTSAGTGGMWRFYTEDPSGLKVGPVPVLVMC*FMNLHIWGGY-TRSGPNFYVPFSNKTG |
| Sec61βOPG124C (human) | GSPGPTPS6TNVGGSRSPSKAVAARAAGSTVRQRKNASS*GTRSAGRTTSAGTGGMWRFYTEDPSGLKVGPVPVC*VMSLFIASVFMLHIWGGY-TRSGPNFYVPFSNKTG |
| Sec61βOPG87C (human) | GSPGPTPS6TNVGGSRSPSKAVAARAAGSTVRQRKNASS*GTRSAGRTTSAGTGGMWRFYTEDPSGLKVGPVPVLVMC*FMNLHIWGGY-TRSGPNFYVPFSNKTG |
| Sec61βOPG77C,L79C (human) | GSPGPTPS6TNVGGSRSPSKAVAARAAGSTVRQRKNASS*GTRSAGRTTSAGTGGMWRFYTEDPSGLKVGPVPVLVMC*LLFIASVFMLHIWGGY-TRSGPNFYVPFSNKTG |
| Sec61βOPG77C,V84C (human) | GSPGPTPS6TNVGGSRSPSKAVAARAAGSTVRQRKNASS*GTRSAGRTTSAGTGGMWRFYTEDPSGLKVGPVPVLVMC*FMNLHIWGGY-TRSGPNFYVPFSNKTG |
| Sec61βOPG110C (human) | GSPGPTPS6TNVGGSRSPSKAVAARAAGSTVRQRKNASS*GTRSAGRTTSAGTGGMWRFYTEDPSGLKVGPVPVLVMC*LHFIASVFMLHIWGGY-TRSGPNFYVPFSNKTG |
| Sec61βOPG-TA110C eq. (human) | GSPGPTPS6TNVGGSRSPSKAVAARAAGSTVRQRKNASS*GTRSAGRTTSAGTGGMWRFYTEDPSGLKVGPVPVLVMC*LHFIASVFMLHIWGGY-TRSGPNFYVPFSNKTG |
| Cytb5OPGS119C (human) | GSAEQSDEAVKYYTLEEIQKHNHSKSTWLILHHKVYDLTKFLEEHPGGEEVLREDAGDATENFEDVGHESTDAREMSTKTPFLGELHPDNPKLNPETLITTIDSSSSWTTNWVPAIC*AVAVALMYRLYMAEDGPNFYVPFSNKTG |

Figure S5  Characterization of PEGylated Sec61βOPG

(A) PEGylated Sec61βOPG77C or unmodified (unmodif) Cytb5OPG119C were incubated in the presence of reticulocyte lysate and sheep microsomes that had been pretreated with increasing amounts of trypsin (tryp) as indicated. The membrane fraction was recovered and products were visualized via immunoblotting of the C-terminal opsin tag. N-glycosylated products are indicated (*). (B) Western blot showing purified Sec61βOPG variants that were untreated (lane 1), treated with mPEG-5000 prequenched with glycine (lanes 2 and 4) or treated with mPEG-5000 without prequenching (lane 3). PEGylated forms are indicated together with a distinct unassigned product seen only in the absence of glycine prequenching (*). S77C, Sec61βOPG77C, S77C,L79C, Sec61βOPG77C,L79C. Molecular-mass markers in kDa are indicated on the right-hand side.
Table S2  Contribution of different factors to the energetic cost of membrane integration of the PEGylated variants of Sec61β

See the text for more details. All values are in kcal/mol unless stated otherwise.

| PEG link | Tail-anchor contribution | Maleimide linker | PEG distance (Å) | PEG contribution | Sum |
|----------|--------------------------|-----------------|-----------------|-----------------|-----|
| L74C     | 11.5                     | 3.1             | 3.9             | 2.3             | −6.1|
| S77C     | 11.5                     | 3.1             | 7.5             | 4.4             | −4.0|
| V84C     | 11.5                     | 3.1             | 16              | 9.4             | 1.0 |
| L87C     | 11.5                     | 3.1             | 19.6            | 11.5            | 3.1 |

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