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# A biochemical analysis of the constraints of tail-anchored protein biogenesis

*Pawel Leznicki, Jim Warwicker and Stephen High\**

Faculty of Life Sciences, University of Manchester, Oxford Road,  
Manchester, M13 9PT, UK.

\*To whom correspondence should be addressed: [stephen.high@manchester.ac.uk](mailto:stephen.high@manchester.ac.uk)

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## SYNOPSIS

Tail-anchored (TA) proteins utilise distinct biosynthetic pathways, including TRC40-mediated, chaperone-dependent and/or unassisted routes to the endoplasmic reticulum (ER) membrane. We have addressed the flexibility of cytosolic components participating in these pathways, and explored the thermodynamic constraints of their membrane insertion, by exploiting recombinant forms of Sec61 $\beta$  and Cytb5 bearing covalent modifications within their TA-region. In both cases efficient membrane insertion relied on cytosolic factors capable of accommodating a surprising range of covalent modifications to the TA-region. For Sec61 $\beta$  we find that both SGTA and TRC40 can bind this substrate with a singly PEGylated TA-region. However, by introducing two PEG moieties, TRC40 binding can be prevented resulting in a block of subsequent membrane integration. Whilst TRC40 can bind Sec61 $\beta$  polypeptides singly PEGylated at different locations, membrane insertion is more sensitive to the precise location of PEG attachment. Modelling and experimentation indicate that this post TRC40 effect results from an increased energetic cost of inserting different PEGylated TA-regions into the lipid bilayer. We therefore propose that the membrane integration of TA proteins delivered via TRC40 is strongly dependent upon underlying thermodynamics, and speculate that their insertion is via a phospholipid-mediated process.

**ABBREVIATIONS**

Cytb5 – cytochrome b5  
DDM – dodecyl- $\beta$ -D-maltopyranoside  
EndoH – endoglycosidase H  
ER – endoplasmic reticulum  
Get - guided entry of tail-anchored proteins pathway  
HisTrx – Histidine Thioredoxin tag  
Hsp – heat-shock protein  
LDAO – lauryldimethylamine-N-oxide  
OG – octyl- $\beta$ -D-glucopyranoside  
OPG – opsin glycosylation tag  
PEG – polyethylene glycol  
SGTA – small glutamine-rich tetratricopeptide repeat-containing protein alpha  
SRP – signal recognition particle  
TA – tail-anchored  
TMS – transmembrane segment  
TRC40 – transmembrane domain recognition complex of 40 kDa  
TX-100 – Triton X-100

## INTRODUCTION

Tail anchored (TA) proteins constitute a group of integral membrane proteins characterised by the presence of a single, C-terminally localised, stretch of hydrophobic amino acids that acts as both the sub-cellular targeting signal and membrane anchor [1]. Whilst TA-proteins are found in most, if not all, intracellular membranes, the endoplasmic reticulum (ER) acts as the entry site for TA-proteins destined for the various compartments of the secretory pathway [2-5]. Importantly, the C-terminal location of the membrane-spanning region precludes its co-translational recognition by the signal recognition particle (SRP), ensuring that TA-protein targeting to, and insertion into, the ER membrane are post-translational [4].

The precise route of TA-protein delivery to the ER membrane is precursor-dependent, and correlates to the relative hydrophobicity of the transmembrane segment (TMS) of the tail-anchor region [6-8]. Thus, comparatively hydrophilic tail-anchors, such as those of cytochrome b5 (Cytb5) and protein tyrosine phosphatase 1B, define a pathway(s) that is either mediated by Hsp70/Hsp40 chaperones [8, 9] and/or does not utilise any cytosolic components [10]. This pathway(s) does not rely on a specific receptor at the ER membrane, hence, protease-treated microsomes are still fully capable of accepting the TA-protein substrate [6, 11].

The majority of TA-proteins, such as RAMP4 and Sec61 $\beta$ , have a TA-region predicted to be more hydrophobic than that of Cytb5 [12]. These precursors use a specialised targeting factor, known as TRC40 (Asna1) in mammalian cells [13, 14] and Get3 in *Saccharomyces cerevisiae* [15, 16], for their delivery to the ER membrane. The TRC40/Get3 pathway requires additional cytosolic factors such as Get4/Get5 and Sgt2 in yeast [17-20], and a membrane-bound receptor that has been defined as the Get1/2 complex in *S. cerevisiae* [16]. Recent structural analyses of the Get3 protein [21-25], and associated components [26, 27], have provided new insights into the relationship between its ATPase cycle and TA-protein delivery to the ER membrane and identified the likely substrate-binding site. However, since none of the current Get3 structures were obtained as a complex with a TA-protein substrate, several aspects of substrate recognition by Get3 remain to be elucidated. Moreover, the precise details of TA-protein recognition by upstream components such as Sgt2 and its mammalian equivalent SGTA, are also currently unclear [see 20, 28].

To date many biochemical studies addressing the biogenesis of TA-proteins at the ER membrane have relied upon substrates generated by *in vitro* translation using cell lysates that contain essential cytosolic factors (see [7]). However, a bacterial system has recently been exploited to co-express model TA-proteins with TRC40/Get3 [21, 29] and the resulting complexes shown to be sufficient for facilitating membrane insertion, indicating that TRC40/Get3 acts at a very late stage of TA-protein delivery to the ER. Studies using purified recombinant TA-proteins expressed in the absence of known cytosolic interacting partners are limited, and have largely focused on Cytb5 [10, 30]. The use of recombinant TA-proteins allows polypeptide synthesis to be physically and temporally separated from the ER delivery and membrane insertion steps, facilitating studies of the

components involved in these processes. Indeed, we have recently taken this approach to study Sec61 $\beta$  biogenesis, and identified a novel role for Bat3 during TRC40-dependent ER delivery [28]. Notwithstanding the potential advantages of using recombinant forms of TA-proteins [31], this approach has not been exploited extensively to date.

In the current study, we describe the successful production of recombinant TA-protein models for each of the two major biosynthetic pathways, namely Sec61 $\beta$  and Cytb5. By chemically modifying these recombinant TA-protein substrates we show that both routes are surprisingly unconstrained, and can accommodate substrates with significantly modified transmembrane domains. Remarkably, PEGylated forms of both Sec61 $\beta$  and Cytb5 can also be efficiently integrated into the ER membrane, albeit via different routes. We find that the cytosolic factors SGTA and TRC40 can both accommodate substrates with a PEGylated TA region, and provide a model for the ER delivery and membrane integration of PEGylated Sec61 $\beta$ . By analysing additional variants of Sec61 $\beta$ , we show that the location of the PEG moiety within the TA region strongly influences membrane integration in a manner that correlates with the predicted thermodynamic cost of this process. On this basis, we speculate that the membrane insertion step of the TRC40 dependent pathway for TA-protein biogenesis may be a purely lipid mediated event that does not require a dedicated integrase.

## EXPERIMENTAL

*Materials.* Bacterial expression vector pHisTrx was a gift from Richard Kammerer (Paul Scherrer Institute, Villigen, Switzerland). Rabbit polyclonal antiserum recognising TRC40 was a gift from Bernhard Dobberstein (ZMBH, Heidelberg, Germany). All detergents were from Anatrace, except Triton X-100 (Sigma). Cross-linking reagents and EZ-link-biotin were from Pierce, whilst BODIPY and IASD were from Molecular Probes. PEG-5000 and PEG-20000 maleimides were from Nektar. Nuclease-treated rabbit reticulocyte lysate was supplied by Promega.

*Chemical modification of recombinant TA-proteins.* Recombinant TA-proteins were expressed and purified as previously described [28], with the various cysteine mutants generated by site directed mutagenesis and confirmed by DNA sequencing. Thiol-reactive probes as indicated were mixed with solutions of single cysteine mutants of Sec61 $\beta$ OPG<sup>S77C</sup> (77  $\mu$ M) in buffer B (50 mM Tris-Cl, pH 7.4; 300 mM NaCl; 10 mM imidazole; 10 % (v/v) glycerol, 0.75 % (w/v) octyl- $\beta$ -D-glucopyranoside (OG)) and Cytb5OPG<sup>S119C</sup> (25  $\mu$ M) in buffer Q (50 mM HEPES-KOH, pH 7.9, 50 mM KOAc, 200 mM NaCl, 10% (v/v) glycerol, 0.1 % (w/v) lauryldimethylamine-N-oxide (LDAO)) (cf. Supplementary Table S1) using the reagents at a final concentration of 1-3 mM and incubating for 2 h at room temperature in the dark. Reactions were quenched by addition of 20 mM 2-mercaptoethanol and further incubation for 10 min. Modified proteins were resolved by SDS-PAGE, and then stained with Coomassie Brilliant Blue or immunoblotted with the anti-opsin tag antibody, or they were used for membrane integration reactions. All other Sec61 $\beta$ OPG cysteine mutants (see Supplementary Table S1) were purified and labelled as described using 1.5 mM mPEG-5000, pre-quenched with glycine, for 1 h at 25°C. After quenching any unreacted maleimides, labelled proteins were analysed as before or used for integration assays. Membrane integration assays of recombinant TA-proteins were performed using sheep pancreatic microsomes as previously described [28].

*Binding of PEGylated Sec61 $\beta$ OPG to recombinant SGTA and endogenous TRC40.*

Human SGTA was expressed in *E. coli* BL21 Gold (DE3) pLysS cells and purified essentially as described above for TA-proteins (see "Purification of TA-proteins"). HisTrxSGTA, or BSA control, were then immobilised on UltraLink Biosupport beads (ThermoScientific) according to manufacturer's instruction. Recombinant PEGylated Sec61 $\beta$ OPG variants were mixed with beads in buffer S (50 mM HEPES-KOH, pH 7.5, 40 mM KOAc, 5 mM MgCl<sub>2</sub>, 0.05 % (w/v) pMAL-C12 amphiphile (from Anatrace)), to give to a final concentration of 3.3  $\mu$ M and incubated for 1 h at 4°C. Beads were washed with buffer S plus 0.5 M NaCl and any bound material eluted first with buffer S supplemented with 0.5 % (v/v) TX-100, and then Laemmli buffer.

Association of PEGylated Sec61 $\beta$ OPG variants with endogenous TRC40 was tested by incubating 1 – 1.9  $\mu$ M of recombinant modified Sec61 $\beta$ OPG with 140  $\mu$ l of rabbit reticulocyte lysate for 30 min at 30°C. Reactions were then split in two and each half added to 20  $\mu$ l (50 % (v/v) suspension) of Protein A Sepharose coated with 7.5  $\mu$ l of rabbit anti-TRC40 or anti-PDI antisera. The lysate was incubated with Protein A

Sepharose-antibody beads for 1 h at 4°C, the resin washed extensively with buffer R (50 mM HEPES-KOH, pH 7.5, 40 mM KOAc, 5 mM MgCl<sub>2</sub>) supplemented with 0.5 M NaCl and bound proteins eluted with buffer R containing 0.5 % (v/v) TX-100 (cf. [13]). The eluate was mixed with the Laemmli sample buffer, samples resolved on SDS-PAGE and immunoblotted with the anti-opsin tag antibody. Quantitative analysis was carried out using a LiCor Biosciences system (see below). The non-specific binding of the indicated Sec61βOPG variants to anti-PDI antibody-coated Protein A Sepharose beads was subtracted from the results obtained using anti-TRC40 antibody-coated Protein A Sepharose, and the resulting values were normalised to the amount of each Sec61βOPG species present in the starting material (see Supplementary Fig. S5 for a full worked example).

*Quantitative immunoblotting.* Following transfer of the samples onto a low-fluor PVDF membrane and incubation with a primary anti-opsin epitope tag antibody, a secondary anti-mouse fluorescent-dye conjugated antibody was added at 1:10000 dilution. The membrane was incubated for 1 h at room temperature, scanned using a LiCor Biosciences system and the results quantified with Odyssey 2.1 software.

*Protease protection of membrane integrated Sec61βOPG variants.* Two membrane integration reactions were combined, microsomes isolated as described [28] and resuspended in 68 µl of low-salt sucrose (LSS) buffer (250 mM sucrose; 100 mM KOAc; 5 mM Mg(OAc)<sub>2</sub>; 50 mM HEPES-KOH, pH 7.9). The suspension was then split in three and each 20 µl aliquot received 2 µl of water, 2 µl of proteinase K (2.5 mg/ml) or 2 µl of proteinase K (2.5 mg/ml) and 2.2 µl of 10 % (v/v) Triton X-100. Digestion was carried out on ice for 30 min and proteinase K was inhibited by the addition of 2.5 mM PMSF and further incubating on ice for 10 min. Laemmli buffer was then added and the samples immediately heated to 95°C for 10 min. Samples were resolved on a 16 % (v/v) Tris-Tricine gel and immunoblotted with the anti-opsin epitope tag antibody.



## RESULTS

### ***Recombinant TA-proteins are inserted into the ER membrane.***

Preliminary analysis indicated that a HisTrx-based fusion was superior to a glutathione S-transferase tag for the high-level expression and purification of the recombinant TA-proteins studied here (data not shown). We used this approach to purify and characterize two model recombinant TA-proteins, Sec61 $\beta$  and Cytb5, each of which favours a distinct pathway for their biogenesis at the ER membrane [see Introduction and Refs. 7, 8, 13, 14, 32]. Cells expressing recombinant TA-proteins were lysed in the presence of detergent, soluble fractions incubated with NiNTA agarose and, after washing the beads with buffer containing a low concentration of detergent, the various proteins were released from the tag by thrombin-mediated cleavage [10, 28, 30, see also Experimental and Supplementary Table S1]. Coomassie blue staining showed an acceptable level of protein purity, with only a fraction of Sec61 $\beta$ OPG and Cytb5OPG present in truncated forms (see Supplementary Fig. S1A).

The membrane integration capacity of the recombinant proteins was investigated using a well-established N-glycosylation assay that relies on a short C-terminal opsin-derived epitope tag [4, 8, 11, 13, 33]. Crucially, this modification can only take place when the protein spans the membrane and the tag has entered the ER lumen consistent with authentic membrane integration. Substantial levels of endoglycosidase H (EndoH)-sensitive protein species for both Sec61 $\beta$ OPG and Cytb5OPG were observed (Supplementary Fig. S1B, cf. lanes 1 and 2, 3 and 4), clearly indicating that these two precursors are efficiently integrated into the ER membrane in the presence of cytosol.

### ***Chemically modified TA-proteins are efficiently inserted into the ER membrane.***

Purified Sec61 $\beta$ OPG and Cytb5OPG, both bearing single cysteines within their TA-regions (Sec61 $\beta$ OPG<sup>S77C</sup> and Cytb5OPG<sup>S119C</sup>, see Supplementary Table S1), were treated with a variety of thiol-reactive probes differing in size and physicochemical properties (Figs. 1A and 1C). In the case of PEGylation, the modification was confirmed by a shift in electrophoretic mobility (see Figs. 1A and 1C), whilst for BODIPY the fluorescently tagged protein could be visualised after membrane integration (Supplementary Fig. S2). In the case of IASD and biotinylation, we have not directly confirmed labelling, and cannot rule out the possibility that little if any protein was modified. However, the biotinylation reagent also generated covalently linked dimers of Sec61 $\beta$ OPG, most likely resulting from trace amounts of bifunctional cross-linkers present in the commercial reagent (Fig. 1A, lane 2, see “•”). This conclusion is strongly supported by the fact that we can generate apparently identical species upon treatment with a bifunctional maleimide (Fig. 1A, lane 7, see “•”). Treatment with mPEG-5000 and BMH also generated even larger species (Fig. 1A, lanes 5 and 7, see “?” and “o”, respectively), but since these derivatives were not recovered in the membrane fraction after further analysis (cf. Fig. 1B, lanes 9 and 10, 13 and 14) they were not further characterised. In the case of Cytb5OPG, a small amount of SDS-stable dimer was observed in the absence of any additional treatment (Fig. 1C, lane 1). A similar species became more prominent after incubation with both mPEG-5000 and BMH, presumably due to covalent cross-linking of the dimer (Fig. 1C). Both the monomeric and dimeric forms of Cytb5OPG appeared to be

modified with mPEG-5000 (Fig. 1C, lane 2, see “◇” and “□”, respectively). Additional high molecular weight species were also observed with BMH (Fig. 1C, lane 3, see “○”), but as for Sec61βOPG, little if any of these unassigned species were recovered in association with ER-derived membranes (cf. Fig. 1D). Hence, our analysis showed that cysteine residues introduced into the TA regions of both recombinant proteins can be covalently modified by PEGylation and cross-linking reagents, whilst Sec61βOPG can also accept a number of other thiol-reactive probes.

A role for cytosolic components during the membrane integration of Cytb5 is controversial [7, 8, 10]. Our previous analysis of recombinant Cytb5OPG found that immunodepletion of TRC40 or Bat3 from cytosol had no effect on its insertion, clearly distinguishing its integration from that of Sec61βOPG [28]. This difference is further underlined when the kinetics of membrane insertion for recombinant Cytb5OPG and Sec61βOPG are compared (see Supplementary Fig. S1, panels C and D), with Cytb5OPG integration reaching completion substantially more rapidly [cf. 34]. We therefore established whether the membrane integration of recombinant Cytb5OPG is cytosol dependent in our assay. We can only detect the N-glycosylation of Cytb5OPG in the presence of both cytosol and ER derived membranes (Fig. 1D, lanes 1 to 4), using experimental conditions where the efficient N-glycosylation of recombinant TA proteins has been observed in the presence of buffer alone [21, 29]. We therefore conclude that the efficient membrane integration of the recombinant Cytb5OPG used in our experimental system is cytosol dependent.

To examine the constraints on the cellular components involved in the biogenesis of Sec61βOPG and Cytb5OPG, we tested the cytosol dependent integration of the two modified recombinant polypeptides into ER-derived membranes. Remarkably, none of the treatments with thiol-reactive reagents tested resulted in a complete block of membrane integration, despite the location of the target cysteine residue within the TA region of both proteins (Fig. 1B, lanes 1-12 and Fig. 1D, cf. lanes 3 and 4, 7 and 8). In fact, even homodimers of both Sec61βOPG and Cytb5OPG were still measurably inserted into the lipid bilayer (Fig. 1B, cf. lanes 3, 4, 13 and 14 and Fig. 1D, cf. lanes 3, 4, 11 and 12). Membrane integration of a Cytb5OPG dimer occurred both with and without prior BMH treatment, suggesting that the close proximity of the two TA-regions of the protein monomers does not preclude membrane integration of a Cytb5OPG dimer (Fig. 1D, cf. lanes 3 and 4, 11 and 12). A more detailed analysis of Sec61βOPG dimers, generated by using cross-linking reagents of different length spacers, revealed that membrane integration was not qualitatively affected by the distance between individual subunits (Supplementary Fig. S3A). Moreover, partial EndoH digestion of the membrane-associated material showed that both subunits of Sec61βOPG and Cytb5OPG dimers are N-glycosylated indicating that both polypeptides are fully inserted into the ER membrane (Supplementary Figs. S3B and S3C). Remarkably, even the addition of large hydrophilic ER membrane-impermeable probes [35], including PEG-5000 (Fig. 1B, cf. lanes 9 and 10, see “\*”), and Fig. 1D, lanes 7 and 8, see “∞”) and, for Sec61βOPG, PEG-20000 (Fig. 1B, cf. lanes 11 and 12), to the TA-regions did not prevent authentic membrane insertion of the recombinant TA-proteins. Perhaps most strikingly, a Cytb5OPG dimer modified with mPEG-5000 was still capable of being efficiently

inserted into the ER membrane (Fig. 1C, lane 2, and Fig. 1D, cf. lanes 7 and 8, marked as “Δ”). It is conceivable that the introduction of a PEG moiety into the TA region of a protein might alter its choice of biosynthetic route. In particular, we wished to confirm that the requirements for PEGylated Sec61βOPG were the same as those previously defined for the unmodified protein. Sec61β is known to exploit the TRC40 dependent pathway for ER integration, a route that is characterised by a protease sensitive membrane component [11, 14]. We therefore investigated the effect of trypsin treatment upon the membrane integration of Sec61βOPG and found that the membrane association and N-glycosylation of both PEGylated and non-PEGylated forms of the protein were identically sensitive to protease treatment (Supplementary Fig. S4A). In contrast, the TRC40 independent membrane integration of Cytb5 was unaffected by trypsin treatment, confirming both the resilience of the N-glycosylation machinery and the specificity of the assay (Supplementary Fig. S4A, see also Refs. 11, 14). We also discovered that prequenching of mPEG-5000 with glycine prevented the formation of aberrant products (cf. Fig. 1A, lane 5 and Supplementary Fig. S4B), and used this approach for all subsequent experiments.

### ***PEGylation of Sec61βOPG affects distinct stages of its membrane integration.***

Delivery of Sec61β to the ER membrane is mediated by the TRC40 protein [14], and if this cytosolic factor is removed the TA-protein fails to reach its target destination efficiently [10, 28]. Several studies also suggest a role for yeast Sgt2p [18, 26], and its mammalian equivalent SGTA [28, 36], acting on the same pathway but upstream of Get3/TRC40 [17, 20]. To address the potential role of mammalian SGTA in TA-protein biogenesis we analysed the interaction of our recombinant TRC40 substrate, Sec61βOPG, with immobilised recombinant SGTA. We find that Sec61βOPG binds efficiently to SGTA, but not to a BSA control, in a Triton X-100 sensitive manner (Fig. 2, lanes 2 and 3). PEGylated Sec61βOPG appears to bind at least as efficiently as the unmodified protein (Fig. 2A, lanes 2 and 3), and is also released by Triton X-100 consistent with a hydrophobic interaction via the TA region as previously observed for TRC40 binding [cf. 13]. In contrast, an alternative version of Sec61β that lacks the hydrophobic TA region but also bears a single PEG residue shows no association with immobilised SGTA, confirming that the association of a PEG moiety alone is not capable of mediating a Triton sensitive interaction with SGTA (Fig. 2B, lanes 2 and 3).

Crystal structures of Get3 [21, 23, 24], the yeast homologue of TRC40, reveal that it has a deep groove at the dimer interface that most likely acts as a TA-protein recognition site. On the basis of our results (cf. Fig. 1B), like SGTA, TRC40 must presumably accommodate a Sec61β polypeptide modified with a single mPEG-5000 moiety. To further investigate the apparent flexibility of substrate binding by TRC40, we mutated both Ser77 and Leu79 of the Sec61βOPG TA-region into cysteines and modified this polypeptide with mPEG-5000. Assuming that the transmembrane region adopts an α-helix-like conformation, this double modification would yield a TA-protein with two PEG-5000s extending from opposite sides of the polypeptide chain (Fig. 3A). Immunoblotting analysis confirmed the efficient labelling of Sec61βOPG<sup>S77C,L79C</sup> yielding populations bearing both one and two mPEG-5000 molecules (Fig. 3B, cf. lanes 1 and 2). When membrane insertion was tested both unmodified and singly PEGylated

forms of Sec61 $\beta$ OPG<sup>S77C,L79C</sup> were visibly N-glycosylated in agreement with our previous results (Fig. 3C, cf. lanes 3 and 4; cf. Fig. 1B, lanes 1 and 2, 9 and 10). However, no N-glycosylation of Sec61 $\beta$ OPG<sup>S77C,L79C</sup> labelled with two mPEG-5000 molecules was seen even after prolonged exposure of the immunoblot (Fig. 3C, cf. lanes 3 and 4; and data not shown).

The lack of any membrane-inserted Sec61 $\beta$ OPG<sup>S77C,L79C</sup> that had been modified with two PEG-5000 species could result from either its inefficient delivery to the ER membrane or an inability to be integrated into the phospholipid bilayer. To address this issue we tested the association of the PEG-labelled Sec61 $\beta$ OPG<sup>S77C,L79C</sup> with TRC40 by immunoisolation and quantitative immunoblotting. We found that the addition of two PEG probes to a single transmembrane segment of Sec61 $\beta$ OPG<sup>S77C,L79C</sup> almost completely abolished TA-protein binding to TRC40 (Fig. 3D, cf. lanes 3 and 4, see also Supplementary Fig. S5). This study also revealed that the attachment of even a single PEG probe to Sec61 $\beta$ OPG<sup>S77C,L79C</sup> reduced its binding to TRC40 to about half the level observed with the unmodified protein (Fig. 3E). This is consistent with a qualitative reduction in the membrane integration of the singly modified chain when compared to the unmodified version of Sec61 $\beta$ OPG (Fig. 3C, cf. lanes 3 and 4, “0xPEG” and “1xPEG”).

We next established whether a protein with a TA-region modified with two PEG-5000 molecules predicted to be located on the same side of an  $\alpha$ -helix-like conformation can be bound by TRC40 and delivered to the ER membrane. To this end, a third derivative of Sec61 $\beta$ OPG with both Ser77 and Val84 mutated to cysteines (Fig. 4A) was purified and modified with mPEG-5000 (Fig. 4B, cf. lanes 1 and 2). When the membrane integration of the two double cysteine variants of Sec61 $\beta$ OPG was compared, surprisingly little if any N-glycosylation of even singly PEGylated Sec61 $\beta$ OPG<sup>S77C,V84C</sup> could be detected under conditions where the singly modified Sec61 $\beta$ OPG<sup>S77C,L79C</sup> was reproducibly integrated (Fig. 4C). When the binding of Sec61 $\beta$ OPG<sup>S77C,V84C</sup> to TRC40 was analysed, the interaction of the singly PEGylated-Sec61 $\beta$ OPG<sup>S77C,V84C</sup> species was unperturbed, although association of the doubly PEGylated species was clearly diminished (Figs. 4D and 4E). Hence, although TRC40 binding is relatively tolerant of the addition of a single PEG-5000 moiety to the Sec61 $\beta$  TA-region, a subsequent step of the membrane insertion process appears much more sensitive to the precise location of such a probe and PEGylation of Cys84 appears not to be tolerated.

In order to test this hypothesis further, we generated a single cysteine mutant, Sec61 $\beta$ OPG<sup>V84C</sup>, and studied its membrane integration compared to the Sec61 $\beta$ OPG<sup>S77C</sup> mutant. The cysteine at residue 84 was more efficiently PEGylated than residue 77 (Fig. 5A and Supplementary Fig. S5A), but the resulting Sec61 $\beta$  derivative showed little if any membrane dependent N-glycosylation whilst Sec61 $\beta$ OPG<sup>S77C</sup> was again efficiently modified (Fig. 5A, lanes 3 to 6), as previously observed (Fig. 1B, lanes 9 and 10). Furthermore, the effect of PEGylation of residue 84 could not be correlated to a reduction in TRC40 binding (Supplementary Fig. S5). Hence, the PEGylation of a single cysteine located at residue 84 of Sec61 $\beta$ OPG effectively prevents membrane integration as judged by N-glycosylation. One potential explanation for this location specific effect of

PEGylation is the thermodynamic cost of integrating the modified TA-region into a phospholipid bilayer (see also Fig. 6B, Supplementary Data and Discussion). We therefore explored the consistency of this positional effect by analysing the membrane integration of PEGylated forms of three further single cysteine mutants, L74C, L87C and a novel cysteine, added to the C-terminus of Sec61 $\beta$ OPG, and denoted 110C. We observe that the membrane integration of PEGylated Sec61 $\beta$ OPG<sup>L74C</sup> and Sec61 $\beta$ OPG<sup>S77C</sup> is clearly more efficient than that of PEGylated Sec61 $\beta$ OPG<sup>V84C</sup>, Sec61 $\beta$ OPG<sup>L87C</sup> and Sec61 $\beta$ OPG<sup>110C</sup> (Figs. 5A, B and C). To confirm that the location specific effect of PEGylation was due to a perturbation of membrane integration, and not N-glycosylation *per se*, we also carried out a protease protection analysis [cf. 10]. We could identify a discrete, membrane dependent, protease protected fragment of PEGylated Sec61 $\beta$ OPG<sup>S77C</sup> (Fig. 5D, lane 5, filled triangle). In contrast, no such product was observed with Sec61 $\beta$ OPG<sup>V84C</sup> or Sec61 $\beta$ OPG<sup>110C</sup> (Figs. 5D, lane 8 and 5E, lane 2, predicted location indicated by filled triangle). On this basis, we conclude that the PEGylation of amino acid residues located towards the C-terminus of Sec61 $\beta$ OPG preferentially inhibits membrane integration.

## DISCUSSION

We have exploited recombinant TA-proteins as a tool for better understanding their biogenesis at the endoplasmic reticulum. Both Sec61 $\beta$ OPG [28] and Cytb5OPG (this study) are efficiently integrated into ER derived membranes, and in both cases this process is dependent upon the presence of cytosol. The role of cytosolic factors in the membrane integration of Cytb5 remains controversial [7, 8, 10]. Nevertheless, in this study we can only detect an N-glycosylated membrane integrated form of the protein in the presence of lysate [cf. 7]. In the case of Sec61 $\beta$  the requirement for cytosol correlates to a need for TRC40/Asna-1 [13, 14], and other cytosolic factors including Bat3 [28] and the mammalian homologues of Get4 and Get5 [37]. We now provide the first evidence for a direct interaction between Sec61 $\beta$  and SGTA, the mammalian equivalent of yeast Sgt2. Sgt2 has recently been implicated in the transfer of TA-protein substrates to Get3 [20]. These data support our previous observation of a specific association between SGTA and TA-regions [28], and we now conclude that this represents a direct interaction of SGTA with a hydrophobic substrate [38], and speculate that SGTA normally plays a role in TA protein transfer to TRC40.

Sec61 $\beta$  can tolerate the covalent attachment of a variety of reagents to a single cysteine residue located within its TA-region, whilst retaining the ability to be integrated into the ER membrane. Likewise, the membrane integration of Cytb5 is not blocked by the addition of a PEG-5000 moiety close to the middle of its TA-region or by the formation of a protein dimer. In the case of Sec61 $\beta$ , polypeptides with various attachments including PEG-5000, and even PEG-20000, can all be post-translationally integrated. This suggests that the cellular components that mediate its delivery to, and integration into, the ER membrane can accommodate a range of non-physiological modifications without a substantial perturbation of their function. Our analysis of the binding of Sec61 $\beta$ OPG to cytosolic factors implicated in its delivery to the ER showed that the direct binding of recombinant Sec61 $\beta$ OPG<sup>S77C</sup> to SGTA was unaffected by PEGylation. Since the PEG moiety is attached to the hydrophobic TA region, this would suggest that SGTA has a hydrophobic groove-like binding site capable of accommodating this unnatural substrate. Interestingly, both TRC40 and the 54 kDa subunit of the signal recognition particle each have flexible hydrophobic clefts capable of accommodating a range of hydrophobic polypeptide regions [21, 23, 39], further supporting this notion [see also Ref. 20].

To analyse the effects of PEGylation upon TRC40 binding, we engineered double cysteine mutants of Sec61 $\beta$ OPG that generated a mixture of singly and doubly modified species upon PEGylation and determined their association by co-immunoprecipitation. This analysis showed that the singly PEGylated form of Sec61 $\beta$ OPG<sup>S77C,L79C</sup> can still bind to TRC40 with substantial efficiency, whilst a doubly modified form cannot. When this PEGylated TA region is modelled into the substrate-binding site of TRC40, our data are in good agreement with the proposed deep pocket [21-24] that could accommodate the addition of one centrally localised PEG-5000 to the substrate, but not two (see Fig. 6A). Hence, we conclude that the doubly PEGylated form of Sec61 $\beta$ OPG<sup>S77C,L79C</sup> is

incapable of membrane integration due to its inability to bind TRC40. When an alternative cysteine was used to generate a doubly PEGylated variant, Sec61 $\beta$ OPG<sup>S77C,V84C</sup>, although the singly modified protein could bind TRC40, it was no longer membrane-integrated. Subsequent analysis of the single cysteine mutant, Sec61 $\beta$ OPG<sup>V84C</sup>, showed that PEGylation of cysteine 84 specifically perturbs biosynthesis at a step occurring after TRC40 binding; for example substrate release by TRC40 or the subsequent membrane integration step (Fig. 6C). Our calculations of the free energy changes resulting from the insertion of a PEG polymer into a lipid bilayer favour the introduction of a constraint at the membrane insertion step. Hence, the free energy associated with the transfer of the unmodified Sec61 $\beta$ OPG transmembrane segment into a lipid bilayer can be estimated as -11.5 kcal/mol (see Supplementary Data), assuming a non-Sec61 translocon mediated pathway for insertion [7, 40, 41]. By the same criteria, the integration of Sec61 $\beta$ OPG PEGylated at cysteine 77 has an estimated energetic cost of -4 kcal/mol, and hence remains favourable (Fig. 6B, S77C). In contrast, the energetic cost of integrating Sec61 $\beta$ OPG PEGylated at cysteine 84 is +1 kcal/mol (Fig. 6B, V84C, see Supplementary Data for detailed calculations). Thus, our modelling supports the idea that the membrane integration efficiency of a PEGylated Sec61 $\beta$  TA-region is dependent on the precise location of the probe relative to the lipid bilayer (Fig. 6B).

To further challenge this model, we created three further single cysteine mutants of Sec61 $\beta$ OPG, Sec61 $\beta$ OPG<sup>L74C</sup>, Sec61 $\beta$ OPG<sup>L87C</sup> and Sec61 $\beta$ OPG<sup>I10C</sup> and analysed the membrane integration of their PEGylated species. As predicted the modification of residue 74 towards the N-terminal side of the TA region did not prevent membrane integration, whilst the alteration of residues 87 towards the C-terminal end, and the addition of a novel cysteine at the extreme C-terminus, both did. Taken together, these observations suggest that thermodynamic constraints are a major factor in the membrane integration of TRC40 dependent TA proteins. Whilst we cannot exclude the possibility that the membrane insertion of Sec61 $\beta$  requires a specialised integrase, we believe that the distinct behaviour of the differently PEGylated Sec61 $\beta$  TA regions is more consistent with a simple partitioning model for membrane insertion. In this scenario, the recently identified membrane-bound receptor for TRC40 [42] would act to deliver TA-proteins to the surface of the ER membrane, but subsequent integration would rely solely on the favourable thermodynamics of TA-protein partitioning into the lipid bilayer (Fig. 6C, cf. Ref. 7).

In the case of Cytb5, the precise identity of any cellular components that mediate its membrane delivery and integration are uncertain [10, 13, 14, 28, 29]. Nevertheless, we see an equally flexible pathway for Cytb5 biogenesis that can tolerate both PEGylation and enforced dimerisation. We conclude that any cytosolic factors that facilitate Cytb5 integration can tolerate these species without substantial perturbation (Fig. 1). Once at the membrane, the rate at which Cytb5OPG can be N-glycosylated appears to be substantially faster than that of Sec61 $\beta$ OPG (Supplementary Fig. S1), and Cytb5OPG integration is also insensitive to trypsin treatment of the membranes (Supplementary Fig. S4), further supporting the use of different pathways for integration into the ER [7, 33, 34]. Whether the flexibility of the post-translational pathways that underlie TA-protein

biogenesis is of physiological significance remains to be determined. However, our results are in good agreement with the finding that certain viral proteins are palmitoylated within their transmembrane segments prior to membrane integration [43]. They are also consistent with the proposal that soluble SNAP25 is initially targeted to the ER membrane in a complex with the TA-protein syntaxin [44], presumably via the binding of the complex to TRC40 prior to ER delivery [14]. It may even be the case that SGTA and TRC40 can bind to covalently modified TA-proteins that are generated *in vivo* such as ubiquitinated species, consistent with reports of the co-translational ubiquitination of membrane proteins [45] and known links between the yeast homologue of TRC40, Get3, and the ubiquitin-proteasome system [46].

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## FIGURE LEGENDS

### Figure 1. Chemically modified TA-proteins are integrated into ER-derived membranes.

Sec61 $\beta$ OPG<sup>S77C</sup> (A) and Cytb5OPG (C) with single cysteines located in their transmembrane segments (see Supplementary Table S1) were modified with thiol-reactive probes or the homobifunctional crosslinking reagent, BMH, as indicated and the products analysed by immunoblotting. Full length Sec61 $\beta$ OPG (>), a truncated form (■) and cross-linked dimers (●) are indicated, as are the monomer and dimer of Cytb5OPG (1xb5 and 2xb5, respectively). PEG-5000 and PEG-20000 modified Sec61 $\beta$ OPG (▲ and ◆ respectively), and PEGylated Cytb5OPG monomer (◇) and dimer (□) are shown. Other products assumed to represent aberrant cross-linked species (? and ○) were not competent for membrane integration and their precise identity was not pursued (see panels B and D). N-glycosylated species are indicated with an asterisk (\*) except the N-glycosylated, PEG-modified Cytb5OPG dimer (Δ) (see Panel D, lane 7). A product corresponding to the PEGylated Cytb5OPG dimer, and an N-glycosylated, non-PEGylated dimer that comigrate is also indicated (∞).

### Figure 2. Recombinant Sec61 $\beta$ OPG binds to immobilised SGTA.

Sec61 $\beta$ OPG<sup>S77C</sup> (A) or Sec61 $\beta$ OPG-TA<sup>110Ceq</sup> (B) were PEGylated as previously described and mixed with immobilised recombinant SGTA or a BSA control. The Triton-X100 (TX) or Laemmli buffer (SDS) eluted fractions were resolved by SDS-PAGE, and Sec61 $\beta$ OPG variants then detected by immunoblotting as before. Each panel shows samples resolved on a single blot and exposed for the same time with irrelevant lanes removed. An uncharacterised, but PEGylation dependent, product of Sec61 $\beta$ OPG-TA<sup>110Ceq</sup> is indicated (Y).

### Figure 3. PEGylation of Sec61 $\beta$ OPG<sup>S77C,L79C</sup> prevents TRC40 binding.

Sec61 $\beta$ OPG<sup>S77C,L79C</sup> was generated (see Supplementary Table S1) and the purified protein modified with mPEG-5000. A schematic representation of the doubly labelled polypeptide is presented assuming an  $\alpha$ -helix-like conformation of the transmembrane region (A). Covalent attachment of PEG-5000 was confirmed by immunoblotting (B) and membrane integration by EndoH sensitivity (C). Small amounts of Sec61 $\beta$ OPG species most likely corresponding to a dimer formed during protein purification were observed (B, ●). Binding of PEG-modified Sec61 $\beta$ OPG<sup>S77C,L79C</sup> to TRC40 was addressed by pre-incubating the protein with rabbit reticulocyte lysate followed by co-immunoprecipitation with anti-TRC40 or control anti-PDI antibodies. Bound Sec61 $\beta$ OPG was eluted and detected by quantitative immunoblotting (D) allowing relative binding to TRC40 to be compared (E, see Supplementary Fig. S5 for a full worked example). The values shown are the average from two independent experiments.

**Figure 4. PEGylation of Sec61 $\beta$ OPG<sup>S77C,V84C</sup> inhibits membrane integration.**

Purified recombinant Sec61 $\beta$ OPG<sup>S77C,V84C</sup> (see Supplementary Table S1) was modified with mPEG-5000 and is shown schematically as described for Fig. 3 (A). PEGylation was confirmed by immunoblotting (B) with a presumptive Sec61 $\beta$ OPG dimer indicated (●). Membrane integration was determined by EndoH sensitivity (C), and TRC40 binding (D and E) was analysed as detailed in the legend to Fig. 3 (see also Supplementary Fig. S5).

**Figure 5. Distinct effects of PEGylation at different locations within the Sec61 $\beta$  TA-region.**

Additional single cysteine Sec61 $\beta$ OPG variants were generated (see Supplementary Table S1) and PEGylated as before. The membrane integration of PEGylated Sec61 $\beta$ OPG<sup>S77C</sup> and Sec61 $\beta$ OPG<sup>V84C</sup> (A), Sec61 $\beta$ OPG<sup>L74C</sup> and Sec61 $\beta$ OPG<sup>L87C</sup> (B) and Sec61 $\beta$ OPG<sup>S77C</sup> and Sec61 $\beta$ OPG<sup>110C</sup> (C) were determined by EndoH sensitivity. In addition, the membrane integration status of Sec61 $\beta$ OPG<sup>S77C</sup> and Sec61 $\beta$ OPG<sup>V84C</sup> (D) and Sec61 $\beta$ OPG<sup>110C</sup> (E) was determined by protease protection (see Experimental). The filled triangle indicates the actual (panel D, lane 5) or predicted (panel D, lane 8 and panel E, lane 2) location of a protease protected, PEGylated, fragment of Sec61 $\beta$ OPG. In panel C, the 110C samples were “overexposed” in order to best visualise any N-glycosylated PEGylated products.

**Figure 6. Membrane delivery and integration of PEG-modified Sec61 $\beta$ .**

A) Human TRC40 was modelled using the *S. cerevisiae* Get3 dimer structure as a template (PDB identifier 2woj) (left panel) and with a 23 residue  $\alpha$ -helical transmembrane segment of Sec61 $\beta$  (green) shown in the proposed binding groove. The surface of the TRC40 dimer is colour-coded by amino acid polarity: grey/non-polar, red/negatively-charged, blue/positively-charged, yellow/polar-uncharged. A schematic representation for the association of PEGylated Sec61 $\beta$  with TRC40 (right panel) showing binding of polypeptides PEGylated at residue 77 or 84, but not of a doubly modified Sec61 $\beta$ <sup>S77C,L79C</sup> variant. B) Estimated energy changes (see Supplementary Data) for the incorporation of different singly PEG-modified TA-regions of Sec61 $\beta$ OPG into a lipid bilayer (left panel). The relative  $\Delta G$  values for PEGylation at residues 74, 77, 84 and 87 are indicated. Representation of PEGylated Sec61 $\beta$  TA-regions within a model lipid bilayer using membrane coordinates taken from [47] (right panel). Experiments confirm the efficient membrane integration of a Sec61 $\beta$ OPG PEGylated on Cys 74 and 77 but not Cys 84, 87 or 110. C) Working model for the delivery of PEGylated Sec61 $\beta$  to the ER membrane. A variety of upstream components including Bat3, SGTA and mammalian Get4 and Get5 [27, 28, 37] facilitate TRC40-mediated delivery to the mammalian ER membrane via its receptor [42]. Any requirement for a membrane integrase remains hypothetical [cf. 7]. Covalent modification of the TA-region of Sec61 $\beta$  could in principle perturb any one of the steps illustrated.

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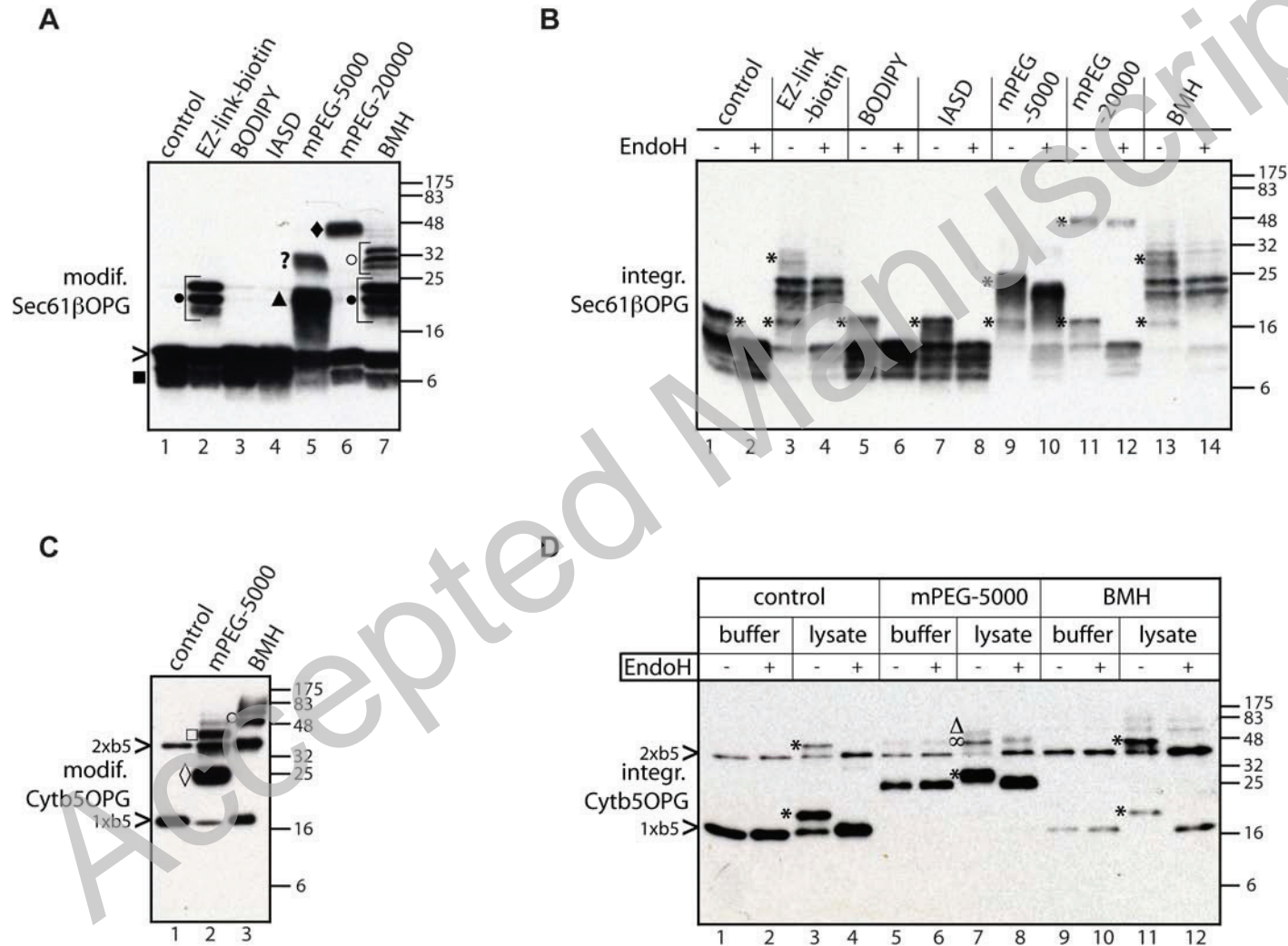
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Figure 1



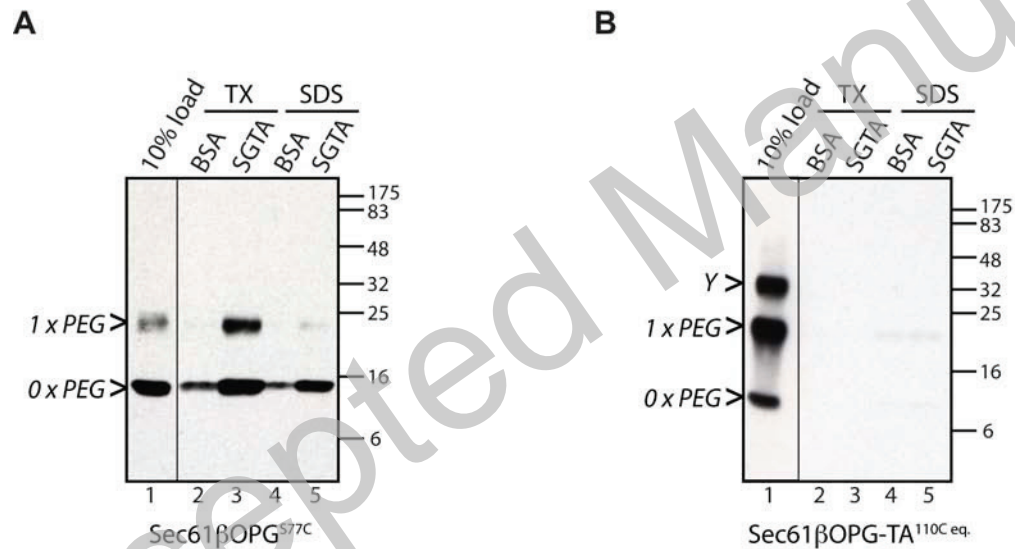
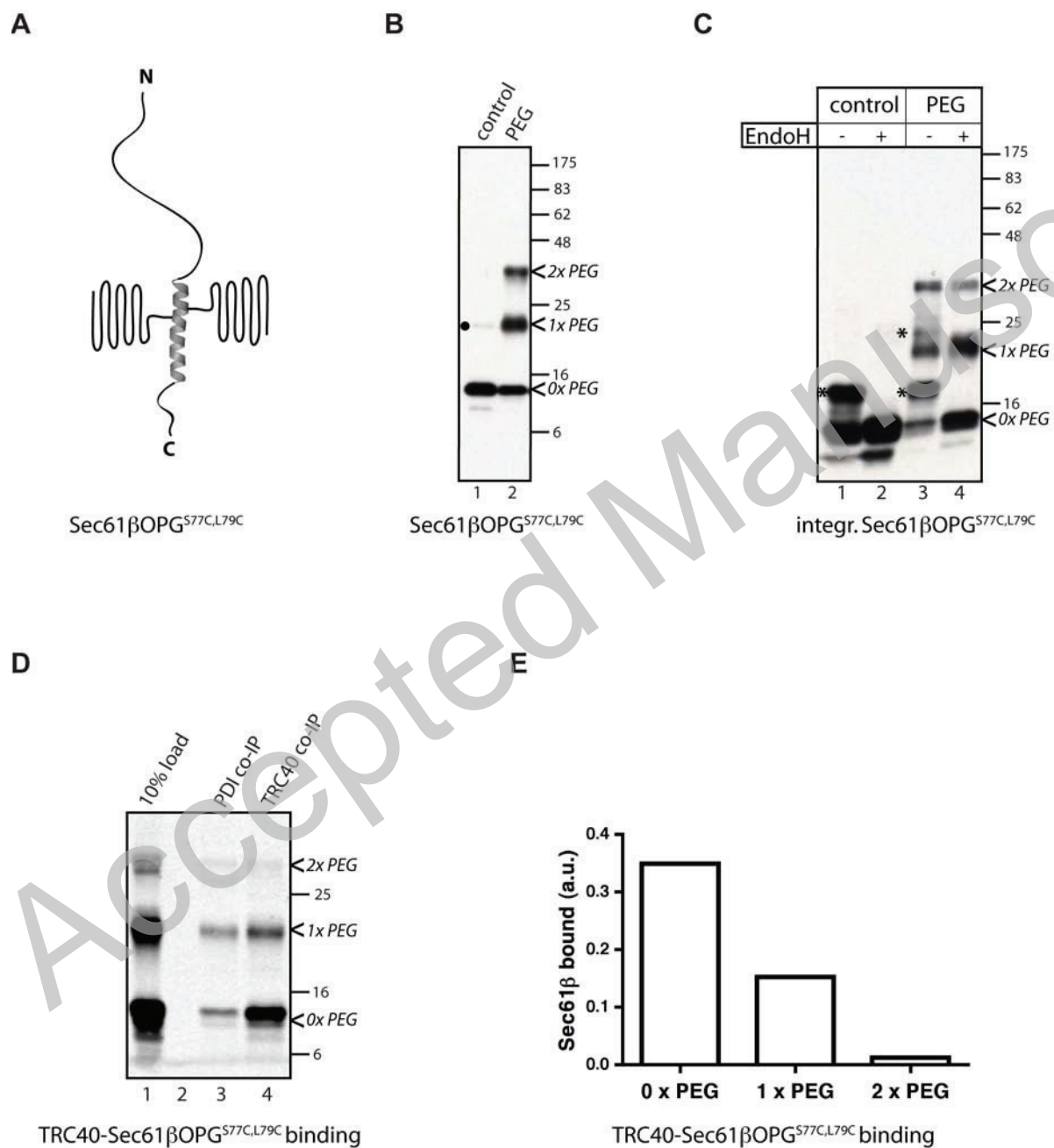


Figure 2



Figure 3



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Figure 4

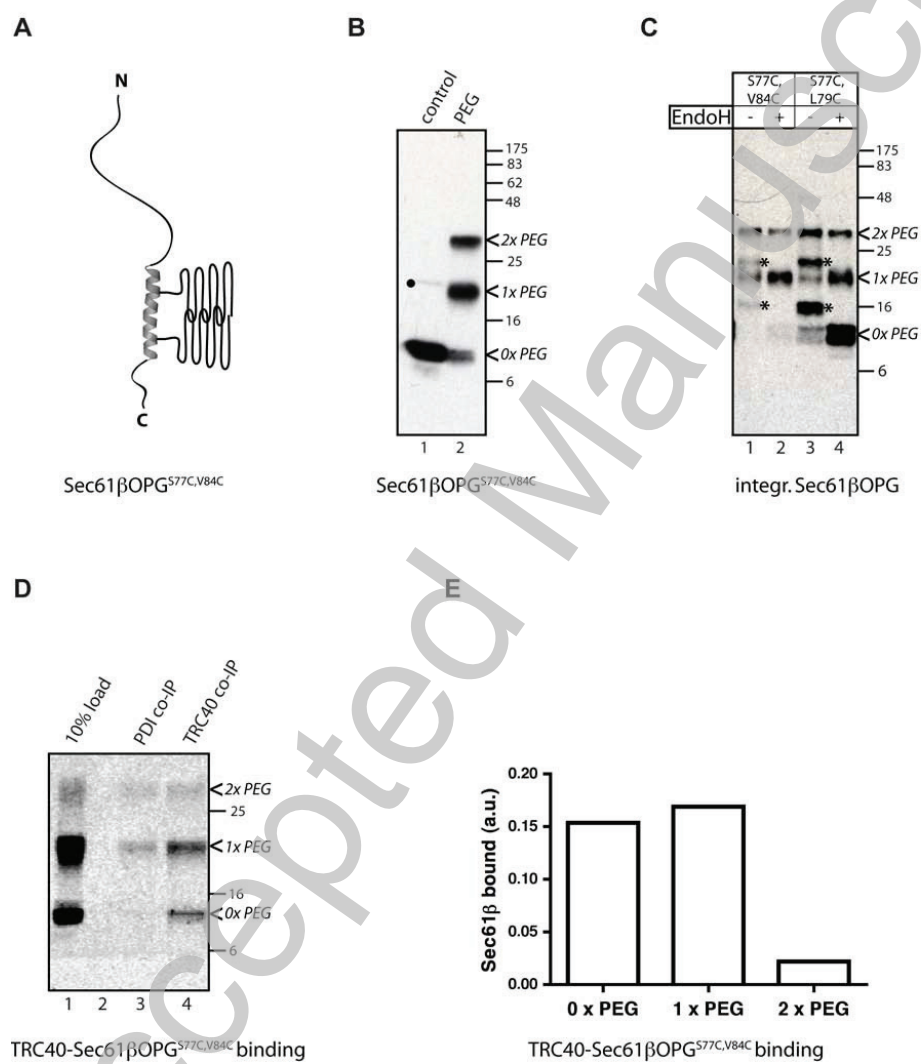
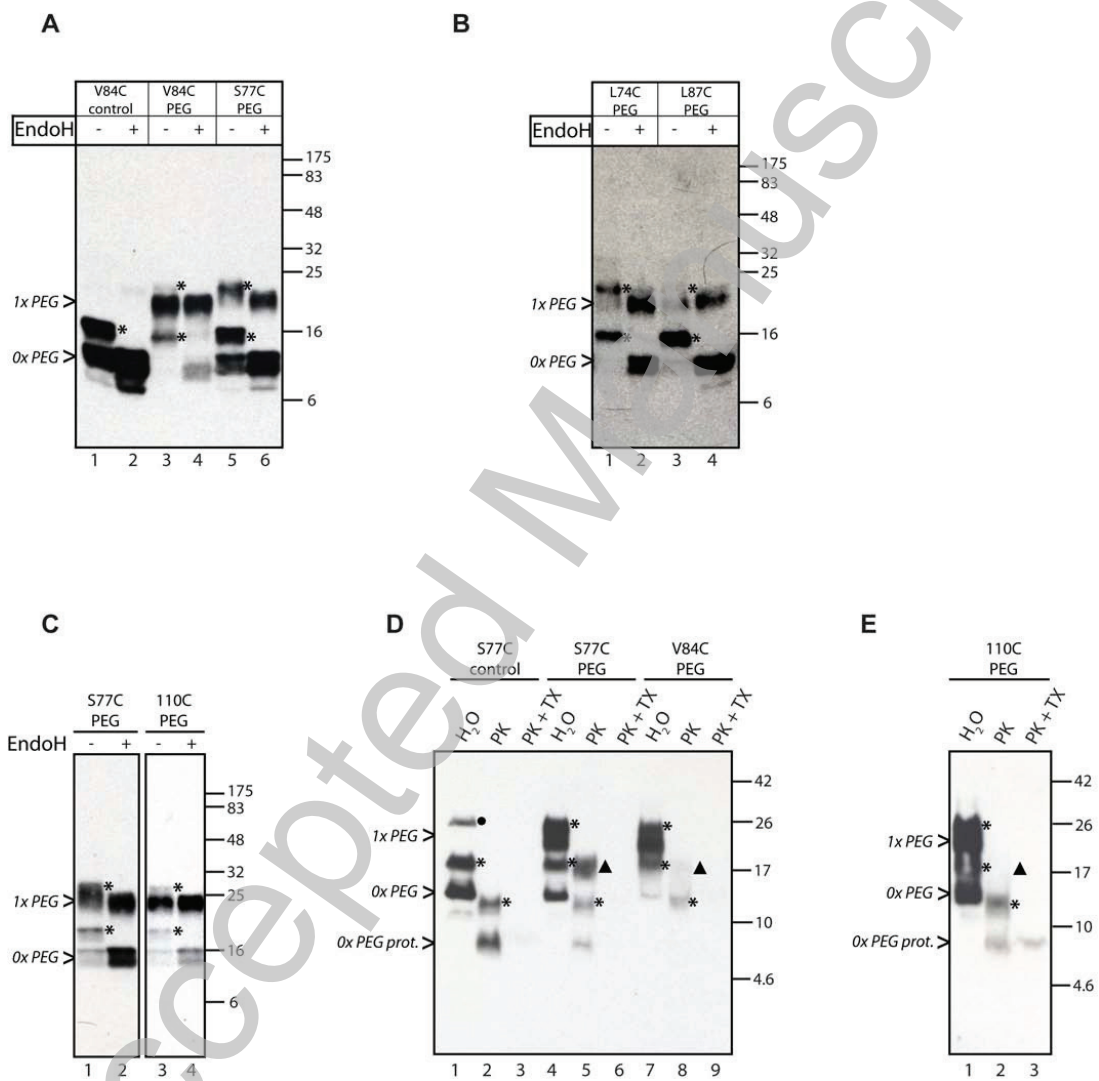
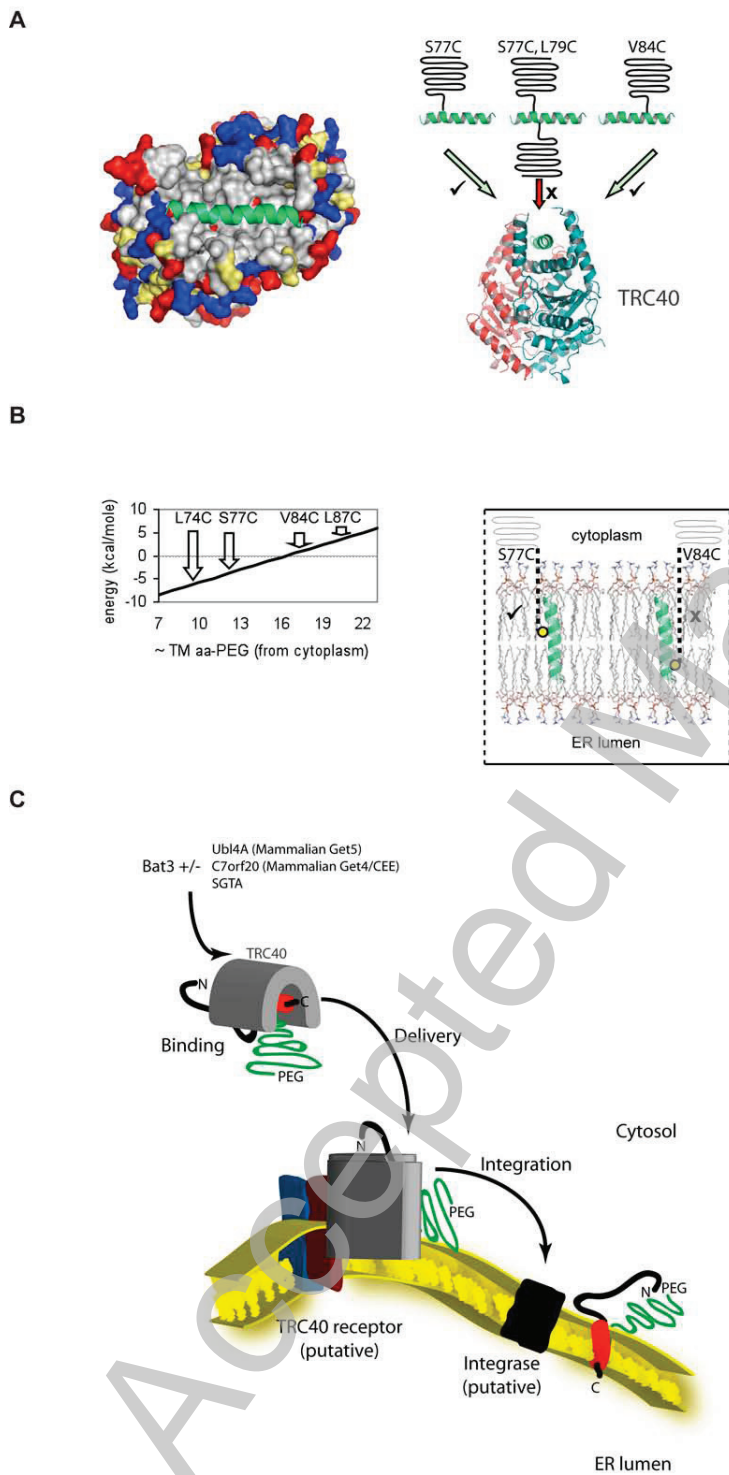


Figure 5



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Figure 6



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