Refined topology model of the STT3/Stt3 protein subunit of the oligosaccharyl transferase complex

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Running title: Membrane insertion of STT3/Sttp3

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Keywords: STT3/Stt3p protein, oligosaccharyltransferase, protein synthesis, transmembrane domain, membrane protein, membrane topology, membrane biogenesis

ABSTRACT

The oligosaccharyl transferase (OST) complex, localized in the endoplasmic reticulum (ER) of eukaryotic cells, is responsible for the N-linked glycosylation of numerous protein substrates. The membrane protein STT3 is a highly conserved part of the OST, and likely contains the active site of the complex. However, understanding the catalytic determinants of this system has been challenging, in part because of a discrepancy in the structural topology of the bacterial vs eukaryotic proteins and incomplete information about the mechanism of membrane integration. Here, we use a glycosylation mapping approach to investigate these questions. We measured the membrane integration efficiency of the mouse STT3-A and yeast Stt3p transmembrane domains (TMDs) and report a refined topology of the N-terminal half of the mouse STT3-A. Our results show that most of the STT3 TMDs are well inserted into the ER membrane on their own or in the presence of the natural flanking residues. However, for the mouse STT3-A hydrophobic domains 4 and 6, and yeast Stt3p domains 2, 3a, 3c and 6 we measured reduced insertion efficiency into the ER membrane. Furthermore, we mapped the first half of the STT3-A protein, finding two extra hydrophobic domains between the third and the fourth TMD. This result indicates that the eukaryotic STT3 has 13 transmembrane domains, consistent with the structure of the bacterial homolog of STT3 and setting the stage for future combined efforts to interrogate this fascinating system.

INTRODUCTION

N-linked glycosylation is one of the most common types of eukaryotic protein modifications. The attachment of carbohydrates to asparagine residues in proteins takes place during protein translocation across the endoplasmic reticulum (ER) membrane and is catalysed by the OST-complex, referred to OT in yeast. The OST/OT catalytic site is located on the lumenal side of the ER membrane and the whole complex is located near the translocon, the protein translocation machinery that is composed of Sec61α, β and γ. The translocon facilitates the translocation of proteins across or into the ER membrane (1,2). Proteins of the translocon that come into contact with the nascent polypeptide chains during their translocation across the ER membrane have been identified by different crosslinking methods (3,4). The mechanism of membrane protein integration in these studies is based upon in vitro biochemical and biophysical assays (reviewed in (5-7)).

The central subunit of the OST complex is the highly conserved STT3, found in eukaryotes
and archaea, and in some eubacteria. Both the mammalian OST and the yeast OT are composed of eight to nine identified subunits (8,9). Of these STT3 (Stt3p in yeast) is believed to be the catalytic subunit that contains the active site (10,11). It was first discovered as a staurosporine and temperature sensitive mutant in Saccharomyces cerevisiae (12) and was later found to be a subunit of the heteroligomeric OT complex (11). The 3.4 Å resolution X-ray structure of the bacterial OST, the single-subunit PglB protein from Campylobacter lari, reveals the fold of the STT3 proteins. It also indicates the locations of the transmembrane (TM) helices, the connecting loops and conserved residues forming the active site of PglB (13). The sequence alignment between PglB and other STT3 proteins show conserved motifs in loops between helices and in the C-terminal part of the proteins (13-15). Furthermore, the similarity in membrane topology indicates that PglB and eukaryotic STT3s share a common reaction mechanism for N-linked glycosylation (8,10,16). Mammalian cells express two homologues of the yeast Stt3p (STT3-A and STT3-B) that form tissue specific complexes with other OST subunits (ribophorins I and II, OST48, DAD1 and Ost4) (9,17,18). Stt3p from S. cerevisiae has 50% sequence identity to the human homologues of STT3, and highly conserved stretches of residues are found throughout the protein (19). Both STT3 and Stt3p are multispanning membrane proteins, with 10-13 predicted TM segments by hydrophobicity plot, while the X-ray structure of the single-subunit membrane protein, PglB indicated 13 TM segments (13). STT3 is predicted not to have a cleavable signal sequence. It has a glycosylated C-terminal domain containing the WWD motif in the lumen of the ER, which is suggested to define the polypeptide substrate specificity (13). We have earlier shown, that the topologies for both yeast Stt3p and mouse STT3A are similar, with the N-terminus being located in the cytoplasm, the C-terminus in the lumen, and the number of TM segments to be 11 with possible alterations of first half of the protein (20).

Although, the crystal structure of a bacterial single-subunit STT3 protein is available (13), the molecular mechanism underlying the STT3 membrane integration is still not fully understood. We therefore, performed a membrane integration study of yeast Stt3p and mouse STT3-A in the ER membrane. Initially, we defined the STT3 TM domains (TMDs) by the ΔG-predictor and then we analyzed the insertion efficiency of the predicted TMDs using an in vitro assay based on asparagine-linked glycosylation. Our results of the membrane insertion efficiency show that most of the STT3/Stt3p TMDs, when including the natural flanking residues surrounding the TMD, integrate effectively into the membrane. Exceptions are TMD4 and TMD6 of mouse STT3A and TMD2, 3a, 3c, and 6 of yeast Stt3p. Here, we also present experimental data for a refinement of the first half of the mouse STT3-A protein, including two extra TMDs between the third and the fourth TMD. We therefore conclude that eukaryotic STT3s have 13 TM segments that are consistent with the structural model of PglB (Figure 1).

**RESULTS**

**Prediction of the position of the TMDs of mouse STT3-A and yeast Stt3p** - Our study started by defining the positions of the mouse STT3-A and yeast Stt3p TMDs using the ΔG predictor server (http://dgpred.cbr.su.se/). The server calculates the theoretical ΔG value of all predicted TMDs (Table 1 and 2), which is a measure of the tendency of any sequence to insert into a biological membrane (21). Adding TMD flanking residues allows the program to find the most optimal TMD sequence with the lowest ΔG value. We therefore, calculated and measured the ΔG value for each TMD sequence alone and when, the flanking loops were included. Loop here is defined as the protein sequence between two adjacent TMDs. For the long loop sequences, we extended the TMD flanks with a maximum of 20 residues. We then compared the predicted TMD positions with the experimentally determined eleven-TM topology model of STT3 (20) and the recently published crystal structure of PglB (13) and found them to agree well (Figure 1).

**Membrane integration efficiency of individual TMDs of mouse STT3-A** - To measure the efficiency of the translocon-mediated membrane integration of the mouse STT3-A TMDs we used a well-established experimental approach based on N-linked glycosylation of a
modified version of the Escherichia coli inner membrane protein leader peptidase (Lep) (22-25). We used two engineered versions of Lep (LepH2 and LepH3) that allow experimentally based quantitative measurements of the insertion efficiency of STT3-A TMD segments in their natural orientation combined without and with adjacent loops. The LepH2 version is engineered such that Lep TM helix 2 (N_{in}-C_{out}) can be replaced by any sequence of interest (23). Here, we replaced it with all STT3-A TMDs with uneven numbers without (TMD1, 3, 5, etc.) or with flanking residues (TMD1F, 3F, 5F, etc.). Likewise, we introduce the even numbered TMDs of STT3-A as well as their extended versions into the P2 domain of LepH3 (N_{out}-C_{in}) (21,22) (Table 1, Figure 2). The efficiency of the translocon-mediated membrane integration of the TMD inserts can be monitored, by the extent of glycan modification of the second of two engineered acceptor sites for N-linked glycosylation in LepH2 (G1, G2) or LepH3 (G2, G3) (Figure 2A). We expressed each cloned construct in vitro in presence of ER microsomal membranes and separated subsequently the singly and doubly glycosylated Lep model proteins containing TMDs of STT3-A by SDS-PAGE. Then, we quantified and calculated the insertion efficiency as described under experimental procedures (Figure 2B, C) (21-23).

When LepH2 constructs are co-translationally integrated into microsomal membranes, a fraction of the molecules with membrane integrated TMD is often cleaved by signal peptidase (SPase) into two protein fragments with lower molecular mass (Figure 2B). The extent of cleavage is dependent on the nature of the TMD sequence and can be prevented by addition of a SPase inhibitor during translation (23,26). Thus the fraction of cleaved protein can be added to the fraction of proteins with integrated STT3 TMD.

Interestingly, when expressing TMD2 without flanking loops in LepH3 we observed an extra band on the SDS-gel that corresponds to a triply glycosylated LepH3 protein. We verified the triple glycosylation by EndoH treatment (Figure 2B and 3). An unusual glycosylation site N_{114}VC (Asn-Val-Cys) was discovered in TMD2 of STT3 (Table 1) (27-31) and we confirmed the site by substituting it to Q_{114}VC (Gln-Val-Cys), which completely abolished the glycosylation. We used this modified construct to avoid complications when quantifying the insertion efficiency of TMD2. The acceptor site was found in the beginning of TMD2 and can only be modified when TMD2 is not inserted into the membrane. It has a lower extent of glycosylation efficiency than the more common Asn-X-Thr/Ser site (about 40%).

Our results for ten of the eleven previously annotated STT3-A TMDs (20) (TMD3 is discussed later) show that most TMDs integrate efficiently into the ER membrane, when taken out of their natural context together with the flanking regions and introduced into the LepH2 or LepH3 model proteins. The exceptions are the following: TMD2 integrates to only 6% into the membrane and by including the flanking loops the insertion increases to 60%. TMD4 integrates poorly, even in the presence of its natural flanks (29% as “isolated TMD”, 38% including flanking loops) (Table 1, Figure 2B and C). Finally, TMD6 integrates to 8% in the membrane and by adding its adjacent regions the integration efficiency increases barely to 22%. The results for TMD2 and TMD6 correlate well with their highly positive, predicted ΔG values for the TMDs by themselves (2.7 respectively 2.5 kcal/mol) and together with their loop-TMD-loop sequence (1.9 respectively 2.2 kcal/mol). For TMD4 on the other hand, the values do not correlate so well with its predicted ones (0.1 without and 0.1 kcal/mol with flanking residues).

Refining the topology of the N-terminal part of the STT3 protein - The unresolved question, of the experimentally determined eleven-TMD topology model of STT3 and Stt3p, is how two hydrophobic stretches could serve as TMD3 (TMD3A, TMD3B) (20). This together with the recently published thirteen-TMD crystal structure of the bacterial homologue PglB (13) motivated us to experimentally take a closer look at the first half of the STT3 protein. We noted a marginally hydrophobic sequence directly upstream adjacent to TMD4 of STT3-A with a theoretical ΔG value of 2.1 kcal/mol. This encouraged us to hypothesize a thirteen-TMD topology model also for the mouse STT3 homologues. TMD3A, TMD3B and the marginally hydrophobic sequence, named here TMD3C, would in this case all be STT3 TMDs. Indeed, TMD3A, B and C, cloned into the
appropriate Lep variant, inserted efficiently into microsomal membranes in presence of their flanking loops (A: 81%, B: 70%, C: 83%, Figure 2B and C).

To strengthen our results, we decided to map the location of the C-terminus of STT3-A truncations by fusing them to the Lep-P2 domain, which include an engineered glycosylation site. For this we generated mouse STT3-A truncations that ended directly after the C-terminal loop of the TMDs of interest, i.e. right before the next TMD. We included one to seven TMDs from STT3 generating STT3-A-Lep-P2 fusions. (Figure 4A). For a sequence with more than two TMDs, multiple topologies are possible. By adding strategically glycosylation sites in the N-terminus and in one loop in the STT3-A-Lep fusion series, it was possible to determine the most populated topologies more precisely (Figure 4A). Therefore, one extra site except from the one in the P2 domain of LepH2 at position 120 was engineered at position N55TT (Asn-Thr-Thr) in the large luminal domain connecting TMD1 and 2 in STT3-A. The other was introduced in the N-terminal loop of the TMD1-2-P2 construct at position N3ST (Asn-Ser-Thr). We reason this would allow us to monitor the location of the P2 domain of every protein fusion construct (Figure 4A). The results of the two series of constructs without and with the glycosylation site in the loop correlate well with each other. This indicates no or little disturbance of the natural protein topology due to creation of additional glycosylation sites.

The glycosylation pattern of STT3 truncated versions reflects to some extent the measured insertion efficiency of the individual TMDs, as for TMD1-2-P2 and TMD2F in LepH3 (56% and 60%). The same was observed for TMD1-3C-P2 compared to TMD3CF in LepH3 (79% respectively 83%). Adding a glycosylation site (replacing K3LG for N3ST) in the N-terminus made it possible to determine the most populated topology of TMD1-2-P2 Lep fusion more precisely (Figure 4B). The data confirm that the N-terminus of the TMD1-2-P2 is located in the cytosol and the singly glycosylated in construct N55TT suggests a cytosolic orientation of the P2 domain (around 56%). TMD1-P2 inserts with about the same efficiency as for TMD1F (93% respectively 85%) (Figure 2B, C and 4B), when calculated with both glycosylated species in N55TT construct. Although, both TMD1-3A-P2 and TMD1-5-P2 insert around 20% less than the individual TMD3A and TMD5 they insert strongly into the membrane (66% respectively 72%). The case of TMD1-3A-P2 two faster migrated bands could be seen that correspond to a SPase cleavage of the glycosylated fusion construct on the luminal side of the membrane after TMD3A Figure 2B and 4C). The two fragments were confirmed by calculating the size of the fragments and by expression of a P2 construct (Figure 4C).

The TMD1-3B-P2 and TMD1-4-P2 protein pattern reveals a single band of monoglycosylated protein that corresponds to a protein population with exclusively the expected topology. That is in contrary to the low insertion efficiency of the individual TMD4F (38%), and in agreement with the high insertion of TMD3BF (70%). In similar manner, TMD1-6-P2 possibly adopts its putative topology to 82% in contrast from the individual TMD6F (22%) (Figure 2B, C and 4B). The TMD1-7-P2 includes the long C-terminal loop containing a possible translational arrest or premature ribosome release resulting in truncated protein forms that have been previously observed (20). By deleting the last 19 amino acid residues of the truncated construct prevents this phenomenon and simplifying the quantification of glycosylated protein (Figure 5A).

The very short cytosolic loops connecting TMDs of the first half of STT3-A make it possible to use a protease protection assay to access if TMDs are certainly integrated into the microsomal membranes and not retained on the cytosolic side of the membrane. We utilized this to verify our results of the STT3-A P2 fusion series (Figure 5B). In this case the second glycosylation site in the large luminal domain connecting TMD1 and 2 was not present. We could observe that for nearly all the proteins that had the C-terminus on the luminal side of the membrane were protected and only unglycosylated, untargeted proteins were degraded (TMD1-P2, TMD1-3A-P2, TMD1-3C-P2, and TMD1-5-P2). In contrast, for the rest of the truncated proteins (TMD1-2-P2, TMD1-3B-P2, TMD1-4-P2 and TMD1-6-P2) the P2 domain was completely degraded because of its location in the cytosol (Figure 5B). In addition, for longer truncations (from TMD1-3B-P2) we could observe a lower molecular weight band that corresponds to the protected N-terminal part of the protein, which
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were long enough to be detectable in the SDS-gel (Figure 5B).

Insertion efficiency of individual TMDs and integration of the N-terminal part of yeast Stt3p - To determine the insertion efficiency of individual TMDs of the yeast Stt3p, we utilized the SP-Lep model protein (32). It consists of the cleavable signal peptide (SP) of the yeast secretory protein invertase and the C-terminal domain of Lep. The Stt3p TMDs were individually introduced into the SP-Lep construct, flanked by insulating GGPG/GPGG tetra-peptides, as well as two potential glycosylation sites, up and down stream of the TMDs (Figure 6A). The N-terminal SP targets the model protein efficiently to the ER membrane and is subsequently cleaved by the membrane resident enzyme SPase. Membrane insertion of the test TMDs was assessed by a glycosylation assay. If the TMD is inserted into the membrane, only the first glycosylation site becomes modified, while translocation of the TMD into the ER lumen leads to glycosylation of both glycan acceptor sites (Figure 6A).

Here, should be mentioned that even TMD3 of Stt3p was less defined in our earlier model (20) and hence, the three most hydrophobic stretches between TMD2 and 4 were tested in the SP-Lep model protein. SP-Lep model proteins containing TMDs 1, 2, 3a, 3b, 3c, 4, 6, 7, 8, or 11 were expressed in yeast and the glycosylation state of the model proteins was assayed by Western blotting (Figure 6B). The insertion efficiency of the individual Stt3p TMDs varied greatly reaching from 11% (TMD2) to 85% (TMD1) (Figure 6B, Table 2). The ΔG_{app} values, calculated from the experimentally obtained insertion efficiencies, were similar to the predicted ΔG_{app}, using the ΔG predictor software (21). Comparing the experimentally ΔG_{app} between mouse and yeast however, we found some differences between some of the TMDs, such as TMDs 3a (-0.5 vs 1.2; mouse vs yeast), 3c (-0.2 vs 0.8) and 11 (-1.2 vs -0.4) (Table 1 and 2).

To test whether the addition of the natural flanking regions would also increase the insertion efficiency, as observed for the mouse TMDs, SP-Lep model proteins containing TMD3b or TMD8 including their natural flanks were constructed. We found that the insertion efficiency of TMD3b was altered by the presence of the natural flanks, increasing the membrane insertion from 53% to 95% (Figure 6C). The insertion efficiency of TMD8 however was unaffected by the addition of its natural flanks and remained at ~45%.

To further test the membrane integration of Stt3p, two Stt3p truncations were expressed in yeast with the length of 1-173 and 1-221 amino acid residues. The sequence length 173 contains TMD1-3a and the length 221 includes TMD1-3c both with two C-terminal NST sites. The idea was to show that both 3a and 3c are real TM segments. When expressed, both constructs become glycosylated, and hence, the C-terminal part is located in the ER, being an indicator that 3a and 3c serve as TMDs. From this we can also deduce that when expressed in context of the first TMDs of Stt3p their flanking regions, 3a and 3c, integrate better than when expressed individually in the Lep model. The glycosylation rate was about 55% for the length of 173 and 75% for amino acid sequence length 221. For Stt3p 1-221 construct, more of singly glycosylated form appeared (Fig. 6D) due to that the first N-linked glycosylation site was located too close to TMD3c (11 residues away from the end of TMD3c). If the N-linked glycosylation site is located at a distance of less than 12 residues from the end of the TMD, it is not efficiently glycosylated by the oligosaccharyl transferase (33). The possible topologies of the truncated constructs 173 and 221 are shown in Figure 6D.

DISCUSSION

In this report, we present a detailed study on the membrane integration of two homologous proteins from two different organisms, the mouse STT3A and the yeast Stt3p, which have been reported to be the catalytic subunit of the OST/OT enzyme complex (11,34). The different eukaryotic STT3 proteins are highly conserved and they are believed to share a common reaction mechanism for N-linked glycosylation, even the bacterial single-subunit STT3s share the same mechanism (10,16).

Most eukaryotic membrane proteins insert and fold in the ER membrane. The insertion process is mediated by the Sec-translocon, and both the hydrophobicity of the TMDs and the charged residues flanking the TMD play important roles during integration (6). Many multi-spanning membrane proteins are predicted to contain TMDs, that are not hydrophobic enough to insert
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into the ER membrane by themselves (35,36), and will therefore need assistance from their neighboring TMDs to integrate into the membrane. Here, we have taken advantage of a well-established in vitro translation system combined with ER-vesicles to study the targeting, translocation and integration process in detail (37-39). The individual ΔG predicted TMDs of mouse STT3-A without and with flanking residues were introduced into the model protein LepH2 or LepH3 depending on their natural orientation (21-23). Remarkably, six isolated TMDs (2, 3B, 4, 6, 8 and 10) inserted poorly into the membrane from 6 to 34%. Four of them (TMD2, 3B, 8 and 10) improved their insertion efficiency by adding their natural flanking loops from 60 to 88%. On the other hand, TMD4 and TMD6 retained their low integration efficiency even after including their flanking residues (38% respectively 22%). TMD6 has a very high, predicted ΔG value, which reflects the low integration, in contrary to TMD4 that has a rather low ΔG value. However, it is important to keep in mind that these results display how individual TMDs behave in the lipid bilayer. The N-terminal truncated results (shown later) reflect the importance of the adjacent TMDs in assisting their marginally hydrophobic neighbors for integration into the membrane (35).

Previously, we reported an 11 TMDs topology model of STT3 protein, with the uncertainty of where TMD3 was located (20). In this paper we attempt to respond to that question by studying the TMD3 region and also the whole N-terminal part (TMD1-TMD7) of the STT3 protein. We made 7 truncations starting from the N-terminal of STT3 and ending at the beginning of each TMD sequence (TMD2-TMD8) and fused it to the P2 domain of LepH2 that contains a glycosylation site. Furthermore, we introduced an extra glycosylation site in the long loop between TMD1 and 2 of the STT3 protein in order to determine the correct topology of each truncation. Most of them adopted their correct topology to a high degree (more than 60%), TMD1-P2 inserted with the same efficiency (nearly 100%) compared to the individual TMD1F, which inserted to 85%, including both singly and doubly glycosylation. Both singly and doubly glycosylation was observed for the N$_{55}$TT TMD1-P2-construct. One possible explanation for this could be the position of TMD1 in the membrane hindering access to the OST so that the N$_{55}$TT (Asn-Thr-Thr) site is less efficiently glycosylated generating the singly glycosylation species (33). Another explanation could be if the very weak hydrophobic domain between TMD1 and TMD2 (Figure 1) influence the orientation of the very short construct like TMD1-P2 to some extent and thereby also the glycosylation status. The small amounts of unglycosylated species tell us that targeting seems not to be a problem. In two cases the insertion for the truncations were almost the same as for the individual TMDs (TMD1-2-P2 and TMD1-3C-P2). Furthermore, although TMD1-3A-P2, TMD1-5-P2 and TMD1-7-P2 inserted worse than their individual counterparts their insertion efficiency was higher than 60%. Importantly, our results show that the poorly inserted TMD4 and 6 could fully integrate into the membrane in conjunction with their more hydrophobic TMD neighbors. It is known that marginally hydrophobic TMDs are common in multispanning membrane proteins (35,36,39,40) and helices that do not insert into the membrane by themselves have been identified in both P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR) (36,39), in aquaporin-1 (AQP1)(41), and in the plant K$_c$ channel KAT1(42). When working with more than one TMD the possibilities of acquiring more than one topology arise, we can confidently exclude the less dominant topology and assign the most dominant one by ingeniously placing glycosylation sites in the gene of STT3. Nevertheless, this can be more difficult the more TMDs that are added. Therefore, we confirmed these results by using a proteinase protection assay. If the TMDs are correctly inserted into the membrane, the connecting loops are too short to be digested by the proteinase K (PK) in the cytosol (35). The long loop connecting TMD1 and 2 is on the luminal side and therefore, unreachable for digestion. However, the position of the soluble Lep-P2 domain is determined by the last TMD that is studied. Depending on the location of the Lep-P2 domain, we could observe fully protected truncated proteins or protease sensitive ones. Moreover, we only kept the glycosylation site in the P2 domain, and did not add any additional in the STT3 gene. Clearly, unglycosylated species are completely degraded, while the inserted glycosylated proteins are protected. TMD1-4-P2 has its C-terminal in the cytosol, so in this case the
The unglycosylated specie is the fully correctly inserted protein that has been protected in agreement with the previous result (Figure 4B and 5B).

For yeast Stt3p, TMD3 was poorly defined and we tested membrane insertion efficiencies of three possible TMDs (3a, 3b, and 3c). While TMD3a and 3c were not membrane inserted by themselves, TMD3b showed ~50% membrane insertion and much enhanced membrane efficiency when the natural flanking residues were included. With 173 and 221 the truncated constructs however, the C-terminal glycosylation sites are glycosylated in the lumen of the ER, suggesting that both 3a and 3c TMDs are located in the membrane.

In summary, we have made a thorough analysis of STT3A/Stt3p with regard to topological and structural aspect. We have found that flanking residues and neighboring TMDs have an impact on membrane integration in more than one way and to various extents. Most importantly our data suggests that eukaryotic STT3s have the same number of TM segments as the bacterial homolog and that our data are consistent with the bacterial structural model. Future studies are warranted to obtain a structure of the eukaryotic STT3.

EXPERIMENTAL PROCEDURES

**Mouse plasmid construction** - Mouse STT3-A constructs were made as previously described (20,25). For the membrane integration assay, double-stranded oligonucleotides encoding the mouse STT3 TMDs of the Itm1 gene encoding STT3-A (Genbank accession no. L34260) (20,43). The gene sequence for TMDs alone or together with their connecting loops, were introduced as amplified and purified PCR fragments (QIAquick PCR Purification kit (Hilden, FRG)) with an N-terminal Spe1 and a C-terminal Kpn1 restriction site into the previously described modified lepB gene in pGEM1 vector (Promega, Madison, WI, US) (22,23). The PCR fragments were obtained using Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, FI) or Expand Long Template PCR System (Roche Diagnostics GmbH, FRG) with primers complementary to the 5' and 3' ends of the selected part of the gene. For the study of the first half of the STT3-A protein the gene sequence for the truncations were introduced with an N-terminal Xba1 and a C-terminal Kpn1 restriction site into the lepB gene. Glycosylation acceptor sites (NX[S/T/C] found in both genes (lepB and Itm1) were mutated to QX[S/T/C] and extra glycosylation acceptor sites were introduced using the QuikChange™ Site-Directed Mutagenesis protocol (Stratagene, CA) by replacing one, two or three appropriately positioned codons with codons for the acceptor tripeptide NXT (Asn-X-Thr). All constructs were confirmed by sequencing of plasmid DNA at Eurofins MWG Operon (Ebersberg, FRG) or BM labbet AB (Furulund, SE). All cloning steps were done according to standard procedures using restriction enzymes from Promega (Madison, WI, US) or Fermentas (Burlington, Ontario, CA).

In vitro expression of mouse STT3-A - STT3-A constructs cloned in pGEM1 vector were transcribed and translated in the TNT® SP6 Quick Coupled System (Promega) in the presence and absence of column-washed rough microsomes (CRMs) of pancreas from dog (tRNA Probes, TX, US) (44). 10 µl of reticulocyte lysate, 150-200 ng DNA template, 1 µl of [35S]Met (5 µCi) and 0.5 µl CRMs were mixed and incubated at 30 °C for 90 min.

For endoglycosidase H (EndoH) treatment 9 µl of the TNT reaction was mixed with 1 µl of 10X glycoprotein denaturing buffer. Following addition of 1 µl of EndoH (500,000 units/ml; NEB, MA, US), 7 µl of dH2O and 2 µl of 10X G3 reaction buffer, the sample was incubated at 37 °C for 1 h (23).

Proteinase K (PK) treatment was performed by adding 1 µl CaCl2 (200 mM) and 0.2 µl Proteinase K (4.5 U/µl) to the translation reaction. After incubating on ice for 30 min, 1 µl PMSF (20 mM ethanolic solution) was added to inactivate PK and further incubated on ice for 5 min (35).

Translation products were analyzed by SDS-PAGE gels and visualized in a Fuji FLA-3000 phosphoimager (Fujifilm) using the Image Reader V1.8J/Image Gauge V 3.45 software. The MultiGauge (Fujifilm) software was used to generate a one-dimensional intensity profile of each gel lane and the multi-Gaussian fit program (Qtiplot, www.qtiplot.ro) was used to calculate the peak areas of the glycosylated protein bands. The
membrane insertion of each studied construct was calculated as the quotient between the peak area of the singly or doubly glycosylated band and the peak area of doubly respectively singly glycosylated protein band (depending on which Lep construct was used). On average, the glycosylation levels vary by no more than ±5% between repeated experiments.

**Yeast plasmid construction and transformation** - All yeast plasmids were constructed from p424GPD-SP-Lep (23,32). To substitute the sequence coding for the hydrophobic (H) segment of the SP-Lep model protein with various yeast Stt3p TMDs, with or without adjacent loops, a Smal site was inserted within the H-segment by site-directed mutagenesis. Stt3p TMDs were amplified from genetic DNA, using primers that include sequences, which complement the sequences upstream and downstream of the H-segment. The PCR products were inserted into the Smal-linearized p424GPD-SP-Lep by homologous recombination. Yeast transformants were selected on -Trp plates, plasmids were isolated and the correct sequence was confirmed by sequencing. Stt3p 1-173 and Stt3p 1-221 constructs were prepared by amplifying the indicated fragments by PCR and subcloning into p424GPDHA vector (23) carrying the engineered two N-linked glycosylation sites (Fig. 6D). For both cloning and expression the yeast strain W303-1a (MATa, ade2, can1, his3, leu2, trp1, ura3) (45) was used.

**Western blotting analysis of yeast Stt3p** - Protein preparation and Western blotting was carried out as described in (46). In brief, model protein expressing yeast cells were grown over night in appropriate selective media, harvested by centrifugation, washed with dH2O, and resuspended in SDS-PAGE sample buffer. After incubation at 60°C for 15 min and centrifugation, the supernatant was loaded onto SDS-gels and subjected to Western blotting, using mouse anti-HA antiserum (Covance). To remove glycans, samples of the whole-cell lysates were treated with EndoH (Roche Diagnostics) prior to SDS-PAGE, as previously described (46).

**Acknowledgments:** We gratefully thank Prof. William Lennarz for providing yeast strains and plasmids, Prof. Arthur E. Johnson for providing rough microsomes, and Prof. Joseph Merregaert for providing a mouse STT3 plasmid (Itm1 cDNA).

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions:** Planned experiments: (PL, KÖ, JR, HK, IN), performed experiments: (PL, KÖ, JR, AHo, YM, AHa, DM), analyzed data: (PL, KÖ, JR, HK, IN), wrote the paper: (PL, KÖ, JR, HK, IN).
REFERENCES


Membrane insertion of STT3/Stt3p


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FOOTNOTES
This work was supported by grants from the Swedish Cancer Foundation (130624) to IN and GvH, from the Swedish Foundation for International Cooperation in Research and Higher Education (STINT) (210/083(12) and KU 2003-4674) to IN, from the Swedish Strategic Foundation (A302:200 and SSF-Infection Biology 2012(SB12-0026)) to IN, from National Research Foundation of Korea (NRF-2016R1A2B2013459) and Promising-Pioneering Researcher Program through Seoul National University to HK.

The abbreviations used are: CRM, column-washed rough microsomes from dog pancreas; OST, oligosaccharyl transferase; ER, endoplasmic reticulum; TM, transmembrane; TMD, transmembrane domain; EndoH, endoglycosidase H; PK, proteinase K
FIGURE LEGENDS

FIGURE 1. Alignment of the TMD region of STT3 and PglB. The alignment when using hydrophobicity plot suggests that mouse STT3A (PDB code: P46978) (1-474) and Campylobacter lari PglB (PDB code: B9KDD4) (1-433) have a similar membrane topology.

FIGURE 2. Model protein Lep and membrane insertion efficiency of mouse STT3-A. A) The leader peptidase model proteins, LepH2 (left) and LepH3 (right). B) In vitro translation in the presence and absence of column-washed rough microsomes (CRMs) of pancreas from dog of constructs containing all STT3-A TMDs without and with flanking residues (F). Odd numbered TMDs to the left were inserted into LepH2 and even numbered to the right in LepH3. The NVC glycosylated product is indicated with a *, SPase cleaved fragments are indicated with C, not fully translated full-length protein is indicated with Fr, and unglycosylated, singly, doubly and triply glycosylated products are indicated with unfilled circle respectively one, two or three filled circles. The percent insertion is calculated from at least three independent experiments. C) Insertion efficiency of all mouse TMDs without and with natural flanking residues. TMDs without and with flanking residues are indicated with black respectively gray color. The error bars are calculated from at least three independent experiments.

FIGURE 3. Verification of mouse TMD2 NVC glycosylation site. To verify the glycosylation of the NVC site in mouse TMD2, the sample was EndoH treated and as a control a sample with the same buffer conditions but without EndoH (Mo) was made. To specifically verify the NVC glycosylation, the site was destroyed by a replacement of the N (Asn) to a Q (Gln). Unglycosylated, singly, doubly and triply glycosylated products are indicated with unfilled circle respectively one, two or three filled circles. The percent insertion is calculated from at least three independent experiments.

FIGURE 4. A detailed study of the N-terminal topology of mouse STT3. A) Truncations of mouse TMD1-TMD7 including loop residues between the TMDs and the P2 domain of Lep containing an engineered glycosylation site. The residues at the end of the different truncations are indicated. B) In vitro translation both without and with CRMs of truncation constructs TMD1-P2 to TMD1-6-P2. The constructs with an extra glycosylation site located in the N-terminal loop or in the first lumenal loop of STT3A are indicated with N3ST and N55TT. C) For the construct TMD1-3A-P2 two SPase cleaved fragments (C) are indicated dependent on size with P2 and TMD1-3A. The extra glycosylation site located in the first lumenal loop of STT3A is indicated with N55TT. Unglycosylated, singly, and doubly glycosylated products are indicated with unfilled circle respectively one, or two filled circles. The percent insertion is calculated from at least three independent experiments.

FIGURE 5. Verifying the N-terminal topology of mouse STT3 using EndoH and protease protection. A) In vitro translation without and with CRMs of the TMD1-7-P2 fusion construct. The appearance of the shorter fragment of TMD1-7-P2 is dependent of the last 19 residues of the truncated TMD1-7, and it was confirmed by deletion of the residues, N341-S359. To verify the glycosylation the samples were EndoH treated and as a control the same buffer conditions but without EndoH (Mo) was made. B) Proteinase K (PK) treatment after translation of all constructs, TMD1-P2 to TMD1-7-P2. The protease protection assay shows a protected lumenal and glycosylated band for TMD1-P2, TMD, TMD1-3A-P2, TMD1-3C-P2, TMD1-5-P2 and only unglycosylated band is completely degraded. In the case of TMD1-3A-P2 the SPase cleaved P2 domain is also protected. The other constructs are more or less completely degraded except for a protected N-terminal fragment (P). Not fully translated full-length protein is indicated with Fr, protected fragment is indicated with P, and unglycosylated, singly, and doubly glycosylated products are indicated with unfilled circle respectively one, or two filled circles. The percent insertion is calculated from at least three independent experiments.
FIGURE 6. SP-Lep model protein and membrane insertion efficiency of yeast Stt3p TMDs. A) The model protein containing a cleavable signal peptide. B) Insertion efficiency of yeast TMDs. C) Insertion efficiency of TMD3b and TMD8 without and with flanking residues. D) Membrane integration of two Stt3p truncations with the length of 1-173 and 1-221 amino acid residues. Sequences of the end of TMD 3a (last residue 158) and 3c (last residue 221) are shown with the N-linked glycosylation sites in the linker preceding the HA epitope. EndoH treatment confirmed the glycosylation of both constructs. A cartoon shows the membrane topologies of the two truncations. Unglycosylated, singly, and doubly glycosylated products are indicated with unfilled, one, and two filled circles, respectively. The percent insertion is calculated from at least three independent experiments.
TABLE 1. Position of the 13 mouse TMD helices of STT3-A determined by the ΔG predictor - Predicted vs. measured ΔG values for each of the selected hydrophobic domains in mouse STT3-A. For the predicted values the ΔG prediction server was used (http://www.cbr.su.se/DGpred/). All TMDs were predicted from the full-length mouse STT3-A protein and selected to be 19-residues long. These 19-residues long TMD segments without and with natural flanking residues (F) were predicted by the ΔG predictor using length corrections and sub-sequences with lowest ΔG (red). The number in parenthesis correlates with the position of the TMDs. All TMDs without natural flanking residues and with flanks composed of less than 10 residues were insulated by a tetra-peptide, GGPG...GPGG. Two potential glycosylation sites are underlined. The integration (%) is calculated from results based on at least 3 independent experiments.

<table>
<thead>
<tr>
<th>Region (position)</th>
<th>Flank</th>
<th>TMD-sequence</th>
<th>Flank</th>
<th>Integration (%)</th>
<th>Predicted ΔG (kcal/mol)</th>
<th>Measured ΔG (kcal/mol)</th>
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<td>TLLILLSSMAAVLSFSTRLF AVLRF</td>
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<td>1.6</td>
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<td>ITIDIRNVCVFAPLFFSSFTTIV T YHITL</td>
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<td>1.6</td>
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<tr>
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<tr>
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<td>IYWAACALAYFYMVSSWGGYV GYV</td>
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<tr>
<td>TMD5F (233-264)</td>
<td>…SH</td>
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<tr>
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<td>1.4</td>
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<td>SKLN…</td>
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<td>2.2</td>
<td>0.7</td>
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<td>TMD7 (302-320)</td>
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<td>84</td>
<td>-0.8</td>
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<tr>
<td>TMD7F (289-340)</td>
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<tr>
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<td>YYFDLQLLVFMFPVGLYCF</td>
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<td>1.8</td>
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<td>------</td>
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<td>-----</td>
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<tr>
<td>TMD9F</td>
<td>(381-403)</td>
<td>…SN</td>
<td>LSDARIFIIMYGVTSMYFSAVM</td>
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<td>83</td>
<td>1.5</td>
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<tr>
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<td>TMD10F</td>
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<tr>
<td>TMD11F</td>
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<td>…IKNE</td>
<td>VASGMILVMAFFLITYTFH</td>
<td>HSTW…</td>
<td>87</td>
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TABLE 2. Yeast Stt3p TMDs and their predicted and measured ΔG values - The positions of the TMD segments were predicted from full-length yeast Stt3p using the ΔG predictor (21). The TMD length was set to be 19 residues. ΔG values in kcal/mol were also predicted with the ΔG predictor using length corrections and sub-sequences with lowest ΔG (red). The TMDs are either flanked by insulating GGPG/GPGG tetra-peptides or their natural flanks (F), as indicated. The integration efficiency of the TMDs is based on results from at least 3 independent experiments and was used to calculate the ‘measured’ ΔG values in kcal/mol.

<table>
<thead>
<tr>
<th>Region (position)</th>
<th>Flank</th>
<th>TMD-sequence</th>
<th>Flank</th>
<th>Integration (%)</th>
<th>Predicted ΔG (kcal/mol)</th>
<th>Measured ΔG (kcal/mol)</th>
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<td>TMD1 (16-34)</td>
<td>GGPG</td>
<td>LKLVIFVAIFGAAISSRLF</td>
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<td>VCVLFAPLFSVGVTAWATYE</td>
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<td>TMD3c (195-212)</td>
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</table>
Membrane insertion of STT3/Stt3p

Figure 3

[Diagram showing membrane insertion of STT3/Stt3p with protein regions labeled and an inset with Mr and protein bands under CRM and EndoH conditions.]

Insertion (%): 6 (±1)
Figure 4

A

B

C

Membrane insertion of STT3/Stt3p

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<tr>
<th>Mr</th>
<th>TMD1-P2</th>
<th>TMD1-2-P2</th>
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<td>WT</td>
<td>WT</td>
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<td>35</td>
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<tr>
<td>Insertion (%)</td>
<td>93 (±1)</td>
<td>56 (±2)</td>
<td>100 (±0)</td>
<td>79 (±2)</td>
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<table>
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<td>WT</td>
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<td>35</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Insertion (%)</td>
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<td>72 (±7)</td>
<td>82 (±2)</td>
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<table>
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<tr>
<td>Insertion (%)</td>
<td>66 (±4)</td>
<td>66 (±4)</td>
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</table>
Membrane insertion of STT3/Stt3p

Figure 6

A

B

C

D

---PGYISRSVAGSYDNEAIATGGNSTAANSTSYDPGYA---

---WGGYYPITNLILHGSTAANSTSYDPGYA---

---PGYISRSVAGSYDNEAIATGGNSTAANSTSYDPGYA---

---WGGYYPITNLILHGSTAANSTSYDPGYA---
Refined topology model of the STT3/Stt3 protein subunit of the oligosaccharyl transferase complex
Patricia Lara, Karin Öjemalm, Johannes Reithinger, Aurora Holgado, You Maojun, Abdesselam Hammed, Daniel Mattle, Hyun Kim and IngMarie Nilsson
J. Biol. Chem. published online May 16, 2017 originally published online May 16, 2017

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