Membrane Topology of the STT3 Subunit of the Oligosaccharyl Transferase Complex*\[S\]

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The highly conserved membrane protein STT3 is part of the oligosaccharyl transferase complex in the endoplasmic reticulum of eukaryotic cells. Various experimental observations strongly suggest that STT3 contains the active site of the complex. Here, we report a detailed topology study of STT3 from two different organisms, Saccharomyces cerevisiae and mouse, using in vivo and in vitro topology mapping assays. Our results suggest that STT3 has 11 transmembrane helices and an overall Ncyt-Clum orientation.

STT3 is a highly conserved polytopic membrane protein found in all eukaryotic organisms. It was first discovered as a stauroporine- and temperature-sensitive mutant in Saccharomyces cerevisiae (1) and was later found to be a subunit of the hetero-oligomeric oligosaccharyl transferase (OT)\[S\] complex (2). Systematic site-directed mutagenesis in combination with photo-cross-linking using a derivatized peptide carrying an N-linked glycosylation consensus sequence suggested a direct involvement of Stt3p in substrate recognition (3).

Mammalian cells express two homologues of Stt3p (STT3-A and STT3-B) that form a complex with other OT subunits (ribophorins I and II, OST48, and DAD1) (4). STT3-A and -B are expressed in a tissue-specific manner and modulate the enzymatic activity of OT (4). A photoreactive probe incorporated into a nascent polypeptide chain carrying a cryptic glycosylation sequon (Gln-\[S\]Thr) can be cross-linked to STT3-A in dog pancreatic microsomes (5), lending further support to the idea that STT3 is the catalytic subunit of OT. An STT3 homologue has recently been discovered in the enteropathogenic bacterium Campylobacter jejuni (6), in which

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The abbreviations used are: OT, oligosaccharyltransferase; ER, endoplasmic reticulum; SFOA, 5-fluoro-orotic acid; TM, transmembrane; Endo H, endoglycosidase H; TMMHM, transmembrane hidden Markov model.

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Membrane Fractionation and Endo H Digestion—Membrane fractions were prepared as described in Ref. 11 with some modifications. Yeast transformants were grown overnight in 5 ml of auxotrophic media. Cells were harvested and washed with 5 ml of distilled H$_2$O and resuspended in 200 ml of cold lysis buffer (20 mM Tri-HCl, pH 7.6, 10 mM EDTA, 0.1 mM NaCl, 0.5 mM sorbitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, Complete protease inhibitor mixture from Roche Applied Science) and transferred to a 2-ml Eppendorf tube containing 200 ml of acid-washed glass beads (Sigma). Cell suspensions were vortexed 5 times at full speed for 45 s with 30-s intervals on ice. Cell suspension excluding glass beads was transferred to a new tube on ice, the glass beads were washed once with 200 ml of lysis buffer, and this residual cell suspension was added to the first tube. The glass bead-treated cell suspension was centrifuged for 5 s to remove unbroken cells, and the soluble fraction was transferred to a new tube and centrifuged at 14,000 rpm for 30 min at 4 °C. The membrane pellet was resuspended with 100 ml of Endo H buffer (80 mM sodium acetate, pH 5.6, 1% SDS, 1% β-mercaptoethanol) and centrifuged at 14,000 rpm for 5 min, and the pellet was discarded. 30 ml of each soluble fraction was aliquoted into two tubes; 1 ml of each soluble fraction was incubated at 30°C for 3 days. Yeast transformants carrying SUC2 insertion plasmids were streaked on -His plates supplemented with 6 mM histidinol and incubated at 30°C for 3 days. Yeast transformants carrying SUC2 insertion plasmids were streaked on 5FOA plates and incubated at 30°C for 2 days.

Plasmid Construction and in vitro Expression of Mouse STT3-A—Mouse STT3-A constructs were made as described previously (12). Briefly, in vitro transcription and translation were performed from the pGEM1 plasmid, the 5' end of the In1 gene encoding STT3-A (GenBank® accession number L34260) in the pSPORT1 plasmid (13) modified in two ways during PCR amplification: (i) by the introduction of a 5' XbaI site and (ii) by changing the context of the region immediately upstream of the initiator ATG codon to a Kozak consensus ribosome binding sequence, CCACCATGG (12, 14); both changes were encoded within the sequence, CACCACTG (12, 14). After in vitro transcription (Promega, Madison, WI), the cDNA products were purified using the QiAquick PCR purification kit from Qiagen (Valencia, CA) and developed using the ECL Advance Western blotting detection kit (Amersham Biosciences, Piscataway, NJ).

Growth Assay—Growth of yeast STT3-A constructs was determined using the growth assay described in Ref. 11 with some modifications. Yeasts were transformed with plasmids coding for wild-type or mutant STT3-A proteins as described above. Samples were analyzed by SDS-PAGE, and proteins were visualized in a Fuji FLA-3000 phosphorimaging device using the Image Reader V1.3d/Image Gauge V 3.45 software.

**RESULTS**

The membrane topology models for yeast Stt3p and mouse STT3-A predicted by the TMDMM method (18) are shown in Fig. 1. The predicted topologies differ in the number of transmembrane segments (13 for yeast, 12 for mouse) and in the location of the hydrophilic C-terminal domain (luminal in yeast, cytosolic in mouse). Guided by these predictions, we engineered glycosylation sites in most of the predicted loops and also made in-frame and C-terminal truncation reporter fusions to obtain further data on the topology of the two proteins.

S. cerevisiae Stt3p—We have taken two approaches to investigate the membrane topology of yeast Stt3p. The first is to insert a 52-residue-long segment of invertase with three N-glycosylation sites into predicted loops of full-length Stt3p, and the second is to fuse the dual Suc2/His4c topology reporter (10, 19) at the C terminus of truncated Stt3p molecules. In the first approach, glycosylation of the invertase fragment indicates a luminal location of the corresponding loop; in the second approach, glycosylation of the reporter again suggests a luminal orientation, whereas the ability of transformed his4c cells to grow on media lacking histidine but including histidinol suggests a cytosolic location of the reporter (19). In both approaches, a hemagglutinin tag was attached to Stt3p to allow detection by Western blotting.

To insert the invertase fragment into Stt3p, we amplified an internal part of the SUC2 gene encoding a 52-amino-acid segment (residues 81-133) that contains three N-glycosylation sites. It has been shown previously that insertion of this fragment into other S. cerevisiae membrane proteins does not influence the overall topology (20). The invertase fragment was inserted into predicted loops as detailed in Table I.

The constructs were transformed into an stt3 deletion strain carrying the wild-type STT3 gene on a plasmid with a URA marker (3). Since STT3 is an essential gene in S. cerevisiae, wild-type STT3 must be present on a plasmid for a haploid deletion strain to survive. To test whether the different constructs can functionally replace Stt3p, transformants were streaked onto 5FOA plates. If the Stt3p insertion construct can replace wild-type STT3, transformants will grow on 5FOA plates by releasing the UR3 plasmid carrying wild-type STT3. In contrast, if the construct does not complement the loss of wild-type Stt3p, cells cannot be cured of the wild-type STT3 plasmid and thus die on 5FOA plates due to the accumulation of toxic fluorodeoxyuridine that is formed from 5FOA and uracil in the cell.

Of the different insertion constructs, the ones with insertions at residues 105 and 204 showed a very low level of protein expression (data not shown). The constructs with insertions after residues 354 and 404 expressed detectable levels of protein, but the transformants did not grow on 5FOA (data not shown). The remaining transformants allowed growth on 5FOA (Fig. 2A), showing that these insertions are compatible with Stt3p function and thus presumably allow a significant fraction of the molecules to insert with the correct topology.

To determine the location (cytosolic or luminal) of the invertase fragment in these constructs, membrane fractions were prepared from yeast transformants expressing the different constructs and subjected to Endo H digestion. Wild-type Stt3p migrates anomalously as a smeared band with an apparent molecular mass of ~60 kDa (21). As expected, the constructs with a Suc2 insertion migrated more slowly than wild-type Stt3p (Fig. 2B). The WT (wild type), N (N-terminal), 139, 385, and 440 Suc2 insertion showed only a minor mobility shift upon

![Insert Figure 1 here]

**Fig. 1.** The predicted topologies differ in the number of transmembrane segments (13 for yeast, 12 for mouse) and in the location of the hydrophilic C-terminal domain (luminal in yeast, cytosolic in mouse). Guided by these predictions, we engineered glycosylation sites in most of the predicted loops and also made in-frame and C-terminal truncation reporter fusions to obtain further data on the topology of the two proteins.
Endo H treatment (Fig. 2B), reflecting the only endogenous glycosylation site at Asn\textsuperscript{535} in the C-terminal domain (22). Thus, the Suc2 reporter domain in these constructs is not glycosylated, suggesting that the loops where the Suc2 domain was inserted are oriented to the cytosolic side of the ER membrane.

In contrast, insertion of the Suc2 domain after amino acid residue 354 and at the C terminus resulted in a larger molecular weight difference between Endo H non-treated and treated samples (Fig. 2B), suggesting that the reporter domain was glycosylated and thus located in the lumen of the ER. Endo H treatment of the construct with an insertion after amino acid residue 113 resulted in a more diffuse band, making it hard to assess the glycosylation status of this particular construct.

To facilitate the discrimination between singly and multiply glycosylated molecules, Stt3p constructs with a Suc2 insertion at various positions were also truncated after amino acid residue 536. The site of truncation was chosen about 90 residues downstream of the last predicted TM segment to avoid any possible misinsertion of transmembrane segments into the membrane when truncated between potential TM domains. Moreover, to probe the correct translocation of the C terminus of the protein to the ER lumen, a new N-glycan site was generated at the end of the truncated Stt3p. Membrane fractions were prepared from yeast transformants carrying these constructs and treated with Endo H (Fig. 2C). The difference between one glycosylation (due to the lack of three glycosylations of Suc2 reporter if located in cytosol but one from the C terminus) and four glycosylation (three glycosylations from Suc2 domain if oriented to the luminal side of the membrane and one from the C terminus) in a truncated Stt3p was unambiguously resolved in 10% SDS-PAGE (Fig. 2C). The mobility shift upon Endo H treatment clearly showed that the Suc2 reporters inserted after amino acid residues 105, 113, and 354 in the truncated Stt3p were glycosylated, whereas the reporters inserted after amino acid residue 139, 385, and 440 were...
not (Fig. 2C). Taken together with the results obtained for the full-length Stt3p/Suc2 constructs, we conclude that the loops corresponding to amino acid residues 139, 385, and 440 are on the cytosolic side, whereas those corresponding to amino acid residues 105, 113, and 354 are on the luminal side of the ER membrane.

We finally prepared constructs in which Stt3p was truncated near the C-terminal end of predicted loops and fused to the Suc2/His4C dual reporter domain. A major advantage of using this dual reporter is that a cytosolic orientation of the fusion domain can be confirmed by growth on a selective medium (in addition to the absence of N-glycosylation) and vice versa for a luminal orientation. The C-terminal fusions at residues 78, 140, 199, 340, and 386 were all sensitive to Endo H digestion and failed to grow on media supplemented with histidinol (Fig. 3), confirming that these regions reside in the lumen of the ER. For unknown reasons, two bands were observed for the fusion at residue 140, both of which were Endo H-sensitive.

The fusions at residues 235 and 437 were insensitive to Endo H, and the one at residue 296 was partially sensitive. Cells carrying all three constructs grew on the selective media supplemented with histidinol. For the fusions at residues 235 and 437, the results clearly suggest a cytosolic location of the reporter domain, whereas for the fusion at residue 296, a cytosolic location of the reporter domain seems likely even if not absolutely certain.

In two cases, the invertase fragment insertion and the C-terminal reporter fusion approaches gave conflicting results. The Suc2 insertion at residue 139 in full-length Stt3p was not glycosylated, whereas the C-terminal reporter fusion at residue 140 was glycosylated and did not support growth on histidinol. Likewise, the Suc2 insertion at residue 385 was not glycosylated, whereas the C-terminal reporter fusion at residue 386 was glycosylated and did not grow on histidinol. These cases are discussed further below.

Mouse STT3-A—To investigate the topology of mouse STT3-A, we used glycosylation mapping (23–25) to determine the location of the N- and C-terminal ends of the protein and of the predicted loops. When expressed in vitro in the presence of microsomes, wild-type STT3-A was inserted into the microsomal membrane with the C-terminal domain in the lumen, as seen by the glycosylation of one or more of the three acceptor sites for N-linked glycosylation (Asn537-Arg-Thr, Asn544-Asn-Thr, Asn548-Asn-Thr) present in this domain (Fig. 4A). This site is situated at the end of a predicted transmembrane segment between residues 361 and 383 and is presumably not glycosylated since N-linked glycosylation in the microsome system requires a minimum distance of 10–15 residues between the glycosylation site and the nearest transmembrane segment (26–28).

When translated both in the absence and in the presence of microsomes, all constructs gave rise to a full-length (Fig. 4A, FL) protein but also to a prominent, shorter fragment (Fig. 4A, F), which migrated during SDS-PAGE electrophoresis at about half the molecular weight of the full-length protein (Fig. 4A). To check whether this shorter product could also be used for topology mapping, we first determined whether it originates from the N- or C-terminal part of STT3-A. We thus translated a series of C-terminally truncated version (198, 280, 310, 498, 543 residues long) of STT3-A in the absence of microsomes (Fig. 4B). As the fragment was not seen for the three shortest constructs but was present in the longer ones, it clearly represents the N-terminal ~340 residues of STT3-A. Whether it results from proteolysis or premature termination of the full-length protein is unclear, but since it is produced in the absence of microsomes and thus cannot result from an ER-bound proteolytic activity, we favor the latter possibility. The fragment is not glycosylated when STT3-A is translated in the presence of microsomes (Fig. 4A (lanes 2 and 3)), again as expected for an N-terminal fragment, since the first potential glycosylation site in STT3-A is at Asn381.

For the topology study, an acceptor site (Asn-Ser-Thr) for N-linked glycosylation was inserted into predicted loops at residues 2, 56, 74, 78, 185, 340, 385, 441, and 515 (constructs N2, N56, etc.) (Fig. 4A and Table I). Since the mobility shift resulting from the addition of one extra glycan to the full-length protein is somewhat difficult to resolve in the gel, we used both the F-fragment and, for some of the constructs, the...
versions truncated at residue 538 in the luminal C-terminal domain (and thus lacking the three endogenous glycosylation sites in this domain) (Fig. 4C) to assess the glycosylation status of the engineered sites. To verify that the observed mobility shifts in both the full-length protein and the F-fragment are caused by N-linked glycosylation, Endo H digestion was carried out on wild-type STT3-A and construct N56 (Fig. 4D).

The F-fragment was found to be glycosylated in constructs N56, N74, N78, and N340 but not in wild-type STT3-A or in constructs N2, N385, and N515 (Fig. 4A). These results were confirmed using C-terminally truncated constructs for wild type, N340, and N515 (Fig. 4C). Note that the N340 site is rather inefficiently glycosylated in the ~340-residue-long F-fragment (but efficiently glycosylated in the longer F1 product), consistent with earlier observations that glycosylation sites located close to the C terminus of a protein are only inefficiently modified (29). The lack of glycosylation of the F1-fragment of wild-type STT3-A also shows that the endogenous Asn381 site mentioned above is not glycosylated in STT3-A.

The predicted loop around residue 441 was also targeted. This loop is in itself too short to be glycosylated even if located in the ER lumen (28). For this reason it was extended to a total length of 43 residues, with the engineered glycosylation site placed in the middle. No glycosylation was seen in the context of the truncated construct (F1-fragment) (Fig. 4C). Since the engineered glycosylation site is placed well beyond the C-terminal end of the F-fragment, this should not be modified, as is indeed the case (Fig. 4, A and C).

**DISCUSSION**

This study reports a detailed topology mapping of STT3, the catalytic subunit of OT, from two different organisms, yeast and mouse. STT3 is highly conserved throughout its length (see Supplementary Material S1), and the yeast and mouse proteins exhibit striking similarities in hydrophobicity profiles. Thus, it seems reasonable to assume that the membrane topology of the yeast and mouse STT3 homologues will be the same. Despite the sequence similarity, the topology predicted by TMHMM is different for the two proteins (Fig. 1). The topology predicted for the yeast protein has the large C-terminal domain located in the ER lumen, in agreement with previously published experimental data (2). We have used a combination of glycosylation site engineering and reporter fusion techniques both in S. cerevisiae and in a mammalian in vitro transcription/translation system to determine the topology of the two STT3 proteins; the results are summarized in Table I.

Glycosylation sites placed before the first predicted TM of both the yeast and the mouse proteins are not modified, pointing to a cytosolic location of the N terminus. Three engineered glycosylation sites in the first predicted luminal loop (constructs N56, N74, N78) are efficiently modified, and the Suc2/His4C reporter fusions at residues 78, 105, and 113 in the same loop also have a luminal location. The TMHMM predicts the first TM of Stt3p as a possible cleavable signal sequence; however, the results showing that a Suc2 insertion at the N terminus of yeast Stt3p rescued the loss of wild type (Fig. 2A) and that the fusion protein migrated more slowly than the wild type during SDS-PAGE (Fig. 2B) indicate that it is not cleaved. Although TMHMM predicts a TM between residues 79 and 101 of yeast Stt3p and residues 81 and 103 of mouse STT3-A with the following loop in the cytosol, the results for both the invertase insertions after residues 105 and 113 of yeast Stt3p and the engineered glycosylation sites (constructs N74 and N78) of mouse STT3-A indicate that this loop remains in the lumen (a TM in this location would bring the glycosylation site in con-

![Fig. 3. Investigation of yeast Stt3p topology by C-terminal Suc2/His4C fusions. A, the glycosylation status of the Suc2/His4C fusions assayed by Endo H digestion and Western blotting with a hemagglutinin antibody. B, the growth of the Suc2/His4C fusions on -His plates supplemented with histidinol.](https://www.jbc.org/content/20265/1/20265/F1.large.jpg)
struct N78 too close to the membrane for glycosylation to occur (26–28)).

The invertase insertion after residue 139 is not glycosylated, indicating a cytosolic orientation of this loop, but a reporter fusion to the neighboring residue 140 is glycosylated and does not support growth on histidinol, suggesting a luminal orientation. The location of the corresponding loop thus cannot be deduced with certainty. The location of the loop region around residue 385 must likewise be regarded as uncertain, as again the results for the insertion after residue 385 and the reporter fusion at residue 386 do not agree. Nevertheless, since the invertase insertions after residues 113 and 385 do not inactivate the function of Stt3p (Fig. 2A), they more likely represent the correct membrane topology as compared with the truncation data in which a functional assay cannot be carried out. The results for all the remaining engineered glycosylation sites, invertase insertions, and Suc2/His4C reporter fusion in both the yeast and the mouse proteins agree with the TMHMM prediction for yeast Stt3p.

Using all the experimental data (but excluding the results for the truncations at residues 140 and 386 of yeast Stt3p and the N185 construct for mouse STT3-A; see below) as constraints on the TMHMM predictor (30), we obtained the 11 TM model shown in Fig. 5A. It is of interest that this model places all the highly conserved sequence elements in the lumen of the ER, where the enzymatic catalysis occurs.

A recent genetic study by Chavan et al. (31) has reported that the N-terminal domain of Stt3p is important for interactions with the protein kinase C cascade in yeast and that the conserved segment 158–168 (Fig. 5, star) may interact with some cytosolic component in the Pkc1p cascade. In the model shown in Fig. 5A, this segment is located in the ER lumen. If, however, a cytosolic location of residues 158–168 is included as an additional constraint, TMHMM predicts the topology shown in Fig. 5B, with TM3 shifting from residues 140 to 157 to residues 169 to 186. In an attempt to differentiate between these two models, we engineered a glycosylation acceptor site into positions 185–187 (construct N185). As seen in Fig. 4A, the F-

FIG. 4. Investigation of mouse STT3-A topology by in vitro translation. The position of the Asn residue in the engineered NST glycosylation acceptor sites is indicated above each lane. S indicates the insertion of a 10-residue-long spacer sequence at residue 441. A, in vitro translation in the absence (−) or presence (+) of rough microsomes (RM) of full-length protein containing both the three endogenous acceptor sites at Asn537, Asn544, and Asn548 and an engineered site in different position (constructs N2, N56, N74, N78, N185, N340, N515, and N441). WT, wild type; G, glycosylated product; UG, unglycosylated product; FL, full-length product, F, N-terminal fragment corresponding approximately to residues 1–340. B, in vitro translation in the absence of microsomes of N-terminal fragments with lengths ranging from 189 residues to the full-length protein (705 residues). C, in vitro translation in the absence (−) or presence (+) of rough microsomes of constructs WT, N340, N441, and N515 truncated at amino acid residue 538. FL, product corresponding to residues 1–538. D, in vitro translation in the absence (−) or presence (+) of rough microsomes of constructs WT and N56 followed by Endo H digestion.
The ER membrane. Most of the highly conserved segments on the luminal side of the N terminus in cytosol and the C terminus in lumen, and membrane topology of STT3 from yeast and mouse. It has 11 TMs, among which residues 158–168 are cytosolic. We thus favor the model presented in Fig. 5A to TM3 to be glycosylated (26–28), whereas if the model in Fig. 5B is correct, construct N185 should be glycosylated. This is the expected result for the model in Fig. 5B in which N185 is too close to TM3 to be glycosylated (26–28), whereas if the model in Fig. 5A is correct, construct N185 should be glycosylated. On balance, we thus favor the model presented in Fig. 5B.

In summary, we have derived a common model for the membrane topology of STT3 from yeast and mouse. It has 11 TMs, the N terminus in cytosol and the C terminus in lumen, and most of the highly conserved segments on the luminal side of the ER membrane.

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