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ISOFORMS OF OST SUBUNIT STT3

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ABSTRACT

Oligosaccharyl Transferase (OST) N-glycosylates nascent polypeptides as they are being translocated into the endoplasmic reticulum (ER). Mammalian OST contains eight subunits, of which STT3 is believed to be the catalytic one. There are two isoforms of STT3, STT3-A and STT3-B, which have different kinetic and substrate recognition properties. Interestingly, it was found that both isoforms are present in cells from a variety of tissues. However, it is unclear if both are needed for proper functioning of OST. RNAi will be used to knockdown expression of one of the isoforms in HEK293 cells to measure differing effects in N-glycosylation of proteins.

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BACKGROUND

Endoplasmic Reticulum

The endoplasmic reticulum, abbreviated ER, is a vital component in eukaryotic cells. It has several primary functions, including calcium ion storage, membrane integration of proteins, synthesis of steroids and phospholipids, translocation of proteins, and folding and modification of proteins. The ER extends through the cell, from the nuclear envelope to the cell membrane, as one continuous membrane system. The ER is divided into smooth and rough domains, in which different processes occur. These different regions are created by localization of certain proteins in specific areas of the ER. While there are many proteins that are shared between the smooth ER (SER) and the rough ER (RER), those that are involved in translocation and protein processing are concentrated in the RER (Voeltz *et al.*, 2002).

The SER has a different function depending upon cell type, with only one type being present in all cell types. A region called the transitional ER is a form of SER that is found in all cells and is involved in packaging proteins for export to the Golgi. Cell specific types of SER are involved in calcium ion storage and secretion in muscle, steroid and phospholipids production, or detoxification of hydrophobic substances (Voeltz *et al.*, 2002).

The RER is so called because ribosomes localize there as part of the protein translocation pathway, causing this region to appear granular. Once translocated, proteins are modified, most often by glycosylation. This modification is used to aid in protein folding and targeting for secretion or retention (Voeltz *et al.*, 2002).

Glycosylation

There are two classes of glycosylation, O-glycosylation and N-glycosylation. Glycosylation has previously been well characterized as a signal for membrane or extracellular proteins. However, O-glycosylation occurs on proteins that remain in the cytoplasm or the nucleus. Proteins that are O-glycosylated usually have a single N-acetylglucosamine (GlcNAc) covalently attached to hydroxyl groups on serine or threonine residues. These proteins also tend to be phosphorylated in addition to being O-glycosylated. This post-translational modification seems to serve multiple purposes, depending on the protein. It has been implicated in protecting a protein from degradation by the proteasome, being recognized in the major histocompatibility complex class I antigen presentation pathway, and lymphocyte activation, among others. It is believed to generally serve a regulatory function in cells, similar to that of phosphorylation (Comer *et al.*, 2000).

The enzyme that catalyzes the O-glycosylation is a uridine diphospho-N-acetylglucosamine:polypeptide β -N-acetylglucosaminyltransferase or O-GlcNAc transferase (OGT). Studies have indicated that OGT is localized in the cytosol and the nucleus. OGT is essential for viability of embryonic stem cells, signifying that O-glycosylation is necessary for eukaryotic cellular physiology (Comer *et al.*, 2000).

O-glycosylation has an important role in nuclear trafficking, transcription, and translation. OGT O-glycosylates the exposed surfaces of nucleoporins, which control active transport of molecules into and out of the nucleus. Monoclonal antibodies or lectins that bind to O-GlcNAc have been shown to block nuclear transport. Studies have been done to determine exact role O-glycosylation plays in nuclear transport. While it is

still unclear how O-glycosylation affects nuclear trafficking, there are two major theories to explain it. The first hypothesis is that O-GlcNAc is an alternant nuclear transport signal. Second, O-glycosylation could have a direct role in regulation of the translocation pathway (Comer *et al.*, 2000).

In addition to nuclear trafficking, O-glycosylation is also important in regulation of transcription. RNA polymerase II, along with transcription factors, are O-glycosylated. The C-terminal domain (CTD) of RNA polymerase II is modified, inducing a conformational change, which affects interactions with other transcription elements. O-glycosylation may also contribute to controlling turnover of transcription factors or aid in the assembly of transcription complexes. However, its precise role is still unclear (Comer *et al.*, 2000).

O-glycosylation also affects the regulation of protein translation. Eukaryotic initiation factor 2 (eIF-2) is a vital component of the translation complex that is inactivated by phosphorylation. However, p67 is a protein that, when O-glycosylated, associates with eIF-2 and blocks phosphorylation. When the cell is placed under stress, a deglycosylase is activated which removes the O-GlcNAc from p67, allowing inhibition of eIF-2 (Comer *et al.*, 2000).

The final main function of O-glycosylation is to modify cytoskeletal proteins. Proteins that bridge the cytoskeleton to cell membranes are O-glycosylated. O-GlcNAc modification has been implicated in intermediate filament fibrillogenesis (Comer *et al.*, 2000).

While O-glycosylation has a variety of functions related to intracellular regulation, the primary roles of N-glycosylation are stabilizing proteins against

denaturation or proteolysis, increasing solubility, aiding in immune responses, orienting proteins in relation to a membrane, granting structural rigidity, regulating turnover, adjusting the isoelectric point of proteins, and mediating interactions with pathogens. N-glycosylation is a far more common post-translational modification than O-glycosylation, as evidenced by the wide variety of functions it serves (Helenius *et al.*, 2004).

N-glycosylation occurs in the ER, but glycosylated proteins are further modified in the Golgi. Polypeptides and the oligosaccharide that is transferred to the protein are synthesized at the ER. It is also the location where glycans are covalently linked to polypeptides and where initial modification of proteins occurs. After the ER quality control mechanisms ensure that the glycoprotein is folded correctly, it is transported to the Golgi, where the oligosaccharide is further modified and trimmed. In many cases, the initial oligosaccharide added is partially disassembled and new saccharides are added (Helenius *et al.*, 2004).

Initially, the purpose of assembling one oligosaccharide just to disassemble it and place different saccharides on the polypeptide was unclear. However, it appears that each configuration of oligosaccharides on the protein serves a separate function, instead of just being an intermediate in the final processing. The initial oligosaccharide added is important for protein folding and quality control. The partially trimmed oligosaccharide is involved in intracellular transport and targeting. After extensive modification in the Golgi, the glycan on the protein serves a role in its function (Helenius *et al.*, 2004).

N-linked glycans are attached to the nitrogen on side chains of asparagine residues and are exposed on the surface of proteins. Only the first two GlcNAcs form interactions with the surface of the protein; the other saccharides are free. This has

several important consequences. First, N-linked glycans can be modified without severe effects on the protein. Second, it allows cells to imprint N-glycans on cell surface proteins with markers for use in cell recognition without altering protein function. N-linked glycans are regularly used in signaling pathways because they are exposed on the surface of proteins, polar, and can be present in multiple copies per protein. Also, the addition or loss of a single saccharide or an alteration of a single bond can dramatically affect binding to lectins, causing it to function differently as a signal (Helenius *et al.*, 2004).

In the endoplasmic reticulum, the same presynthesized initial oligosaccharide is added to proteins. This core oligosaccharide is highly conserved in eukaryotes and is composed of two N-acetylglucosamines, nine mannoses, and three glucoses ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) (Figure 1). This core oligosaccharide is then modified in the Golgi to the mature form. The ER has to synthesize this macromolecule before transferring it to protein. Each individual saccharide is added to the lipid carrier dolichol-pyrophosphate by monosaccharyl-transferases in the ER membrane and synthesis occurs on both sides of the membrane (Figure 2). The two GlcNAcs and the first five mannoses are added to the dolichol-pyrophosphate before it is flipped into the luminal side of the ER for further assembly. The translocation across the membrane is catalyzed by an ATP-independent bidirectional flippase. Each enzyme has strong preference for a single substrate, which yields a step-wise assembly of the oligosaccharide. The final glucose residue that is placed in the oligosaccharide is needed for efficient recognition by oligosaccharyl transferase (OST), which is the enzyme that transfers the dolichol-oligosaccharide to the protein (Helenius *et al.*, 2004).

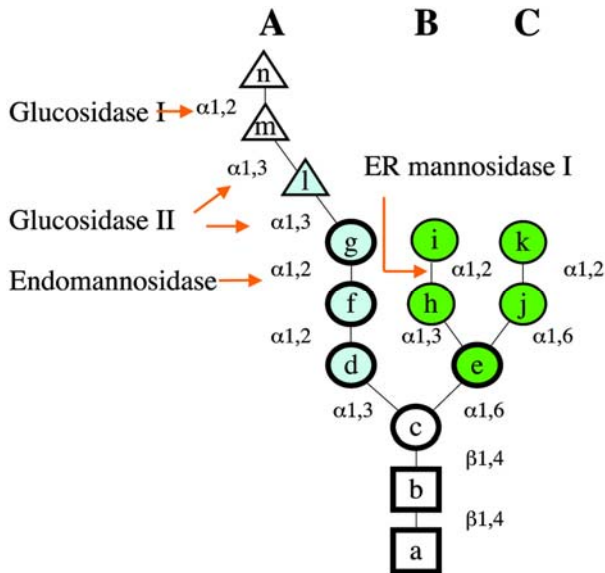


Figure 1 The N-linked core oligosaccharide. The core glycan has 14 saccharides: 3 glucoses (triangles), 9 mannoses (circles), and 2 N-acetylglucosamines. Each saccharide has a letter assigned to it, and these are used in the text to identify the saccharide (3). There are three branches named A, B, and C. The cleavage sites of some glycosidases involved in trimming are indicated. Residues a-g (shown by bold symbols) are added to the glycan on the cytosolic surface of the ER-membrane during biosynthesis; the rest are added lumenally. The blue residues (d, f, g, and l) are involved in the interaction of monoglucosylated glycans with calnexin and calreticulin. The green residues, with the exception of i, are likely to interact with EDEM. (Helenius *et al.*, 2004)

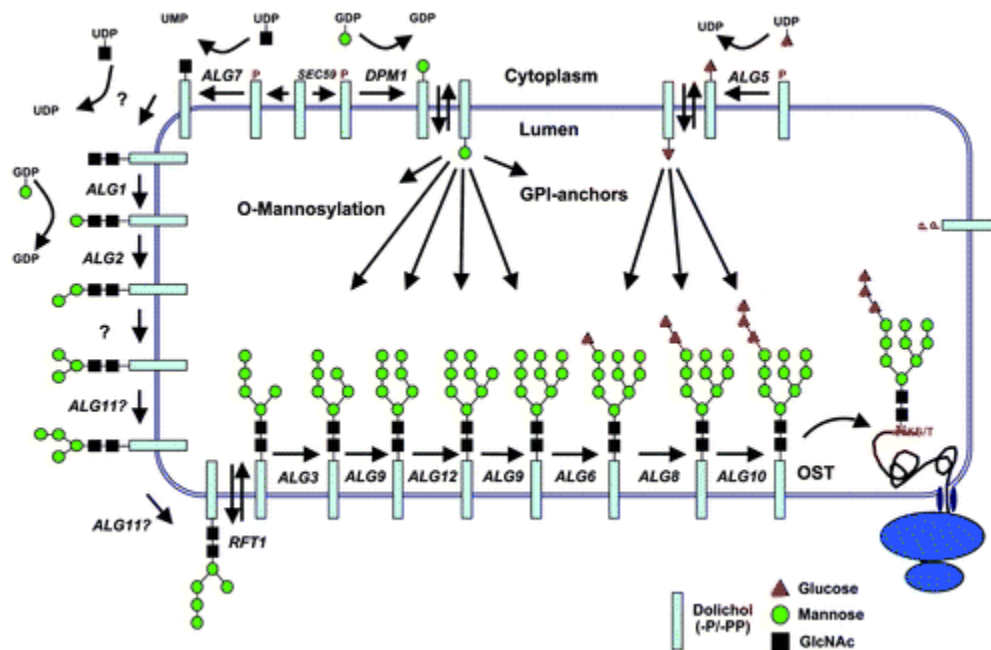


Figure 2 Synthesis of the N-linked core oligosaccharide and its transfer to a polypeptide chain. Biosynthesis occurs on both sides of the ER membrane. The yeast loci required for the individual biosynthetic steps are indicated. Synthesis starts on the cytoplasmic side where GlcNAc-1-phosphate is transferred from UDP-GlcNAc to dolicholpyrophosphate, followed by an additional GlcNAc and five mannose residues. The Man₅ GlcNAc₂ oligosaccharide, thus generated, is translocated (flipped) into the lumen of the ER (13). On the lumenal side, the lipid-linked Man₅ GlcNAc₂ is extended by the addition of four mannose and three glucose residues. The enzymes involved differ from most other glycosyltransferases in spanning the membrane several times and in being quite hydrophobic (16, 17, 34179, 180, 181). Unlike the cytosol-oriented glycosyltransferases that initiate core oligosaccharide assembly and Golgi-localized glycosyltransferases, these use lipids (dolichol-P-Man and dolichol-P-Glc) as saccharide donors. Little is known about the catalytic mechanism of these interesting enzymes. However, based on sequence similarity, a common origin has been suggested (182). (Helenius *et al.*, 2004)

Oligosaccharyl Transferase

OST is an octamer that is a component of the translocon complex. All nascent polypeptides pass through OST, among other factors, upon exiting the translocon and entering the ER. OST recognizes the glycosylation sequence, Asn-X-Ser/Thr, on nascent polypeptides and adds the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharide to the nitrogen on the side chain of Asn. X can not be proline. The polypeptide remains unfolded as it passes through OST, at a rate of five residues per second. The N-glycosylation reaction requires a loop to be formed in the polypeptide so that the hydroxyl groups of the Ser/Thr residues can interact with the amide on the Asn sidechain to make it more nucleophilic. Proline prevents formation of this loop, which is why X can not be proline (Helenius *et al.*, 2004).

OST is composed of eight subunits in yeast, Ost1p, Ost2p, Wbp1, Swp1, Stt3p, Ost3p/Ost6p, Ost4p, and Ost5p. Ost1p, Ost2p, Wbp1, Swp1, and Stt3p are essential for OST function. Ost3p and Ost6p are homologues and only one is present in any given OST molecule. There are mammalian homologues for all of the yeast subunits except Ost4p and Ost5p. Ribophorin I, ribophorin II, Ost48, Dad1, STT3-A/STT3-B, and N33/IAP are mammalian homologues for OST subunits (Kelleher *et al.*, 2003).

It is difficult to determine the exact function of each of the subunits because OST is so complex. However, it is believed that STT3, ribophorin I, and OST48 interact directly with the substrate. STT3 is believed to be the catalytic subunit because different isoforms affect kinetics and that a bacterial homologue, PglB, is sufficient for OST activity (Helenius *et al.*, 2004).

STT3

STT3-A and STT3-B are isoforms that confer different kinetic properties to the OST complexes that they are a component of. STT3-A and STT3-B have 59% sequence homology, with STT3-B having extensions at the N-terminal and C-terminal ends (Figure 3). Both isoforms of STT3 are themselves glycosylated (Figure 4). OST containing

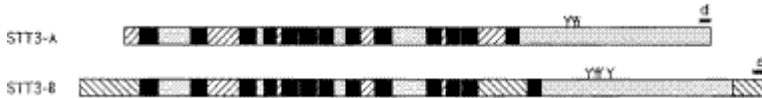


Figure 3 – STT3 isoform homology

Sequence identity between signal sequences (horizontal hatch), lumenal segments (gray), TM spans (black), and cytosolic loops (diagonal hatch) of STT3-A and STT3-B. Nonhomologous extensions and an insertion in the STT3-B sequence are indicated by alternate hatching relative to STT3-A. The letters d and e designate the region on the polypeptide that antibodies were raised against (Kelleher *et al.*, 2003).

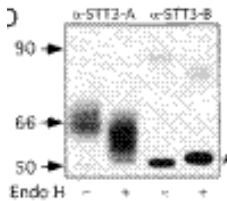


Figure 4 – Endo H digestion of STT3 isoforms

Immunoblot of STT3-A and STT3-B in the absence (-) or presence (+) of Endo H. The asterisk indicates a polypeptide recognized by the secondary antibody (Kelleher *et al.*, 2003).

STT3-A as a subunit has a higher affinity for fully assembled $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ substrate, but a lower V_m than OST containing STT3-B (Figure 5). In Figure 5, OST I contains STT3-B, OST II contains a mix of both STT3-A and STT3-B, and OST III contains STT3-A. OST I and OST II have a five to six fold higher turnover number than OST III when $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ - PP- Dol is used as a substrate. They have a seventeen to twenty fold higher turnover number when $\text{Man}_9\text{GlcNAc}_2$ - PP-Dol is used as a substrate. It is believed that binding of an oligosaccharide donor to an activator or regulatory site is necessary for binding of donor and acceptor substrates to the catalytic site. The K_m for the tripeptide acceptor (K_p) in OST I is unaffected by the structure of the

donor substrate. However, OST III has a higher affinity for Glc₃Man₉GlcNAc₂- PP- Dol than Man₉GlcNAc₂- PP-Dol. Figures 5C and 5D show OST activity assays. When Glc₃Man₉GlcNAc₂- PP- Dol is used as a substrate, both OST I and OST III have similar

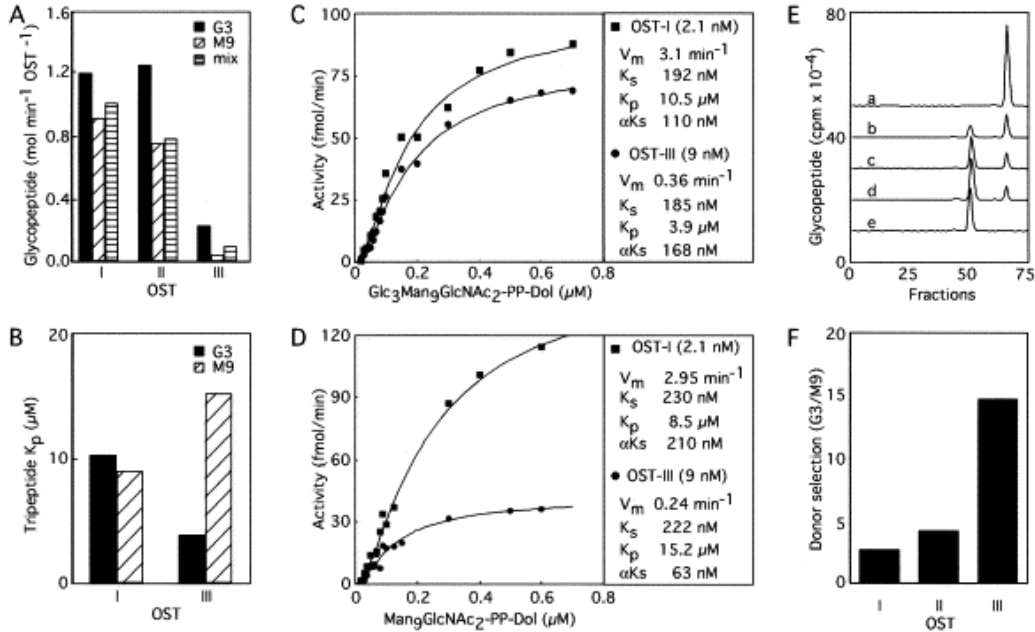


Figure 5. Kinetic Properties of OST Isoforms

(A) The activity of OST-I, OST-II, and OST-III was determined in assays that contained 5 μ M *N* α -Ac-Asn-[¹²⁵I]Tyr-Thr-NH₂ as the tripeptide acceptor and either Glc₃Man₉GlcNAc₂-PP-Dol (G3; 700 nM or 1 μ M), Man₉GlcNAc₂-PP-Dol (M9; 700 nM or 1 μ M), or a donor substrate mixture (mix; 100 nM Glc₃Man₉GlcNAc₂-PP-Dol plus 1 μ M Man₉GlcNAc₂-PP-Dol). The rate of glycopeptide formation for the 700 nM and 1 μ M assays were averaged to obtain apparent V_{max} values, which are expressed as the turnover number. (B) The apparent K_m for the tripeptide acceptor (K_p) was determined in assays that contained 5–90 μ M tripeptide acceptor and either 600 nM Glc₃Man₉GlcNAc₂-PP-Dol or 600 nM Man₉GlcNAc₂-PP-Dol. (C and D) Donor substrate saturation curves for assays that contained 2.1 nM OST-I or 9 nM OST-III were obtained for Glc₃Man₉GlcNAc₂-PP-Dol (C) or Man₉GlcNAc₂-PP-Dol (D) in assays that contained 5 μ M (C) or 10 μ M (D) acceptor tripeptide. The K_p values from (B) were used for curve fitting. (E) Glycopeptides from selected assays in (A) were resolved by HPLC to separate Glc₃Man₉GlcNAc₂-NYT from Man₉GlcNAc₂-NYT. The HPLC profiles (offset for clarity) are from the following assays: (a) OST-II, 1 μ M Glc₃Man₉GlcNAc₂-PP-Dol; (b) OST-III, donor mix; (c) OST-II, donor mix; (d) OST-I, donor mix; (e) OST-II, 1 μ M Man₉GlcNAc₂-PP-Dol. (F) The HPLC-resolved glycopeptides (E) were quantified to calculate the donor preference ratio (DP) using the following equation: $DP = (M_9-PP-Dol/G_3-PP-Dol) \times (G_3-P/M_9-P)$. $M_9-PP-Dol$ and $G_3-PP-Dol$ are the concentrations of the donor substrates, and G_3-P and M_9-P are the quantities, in cpm, of the glycopeptide products (Kelleher *et al.*, 2003)

activities. However, when Man₉GlcNAc₂- PP- Dol is used, the activity of OST III significantly decreased, while the activity of OST I increased slightly. Figure 5F shows donor preference ratios for OST I, OST II, and OST III. OST I prefers the

Glc₃Man₉GlcNAc₂- PP- Dol only slightly over that of Man₉GlcNAc₂- PP- Dol.

However, OST III has a much higher preference for Glc₃Man₉GlcNAc₂- PP- Dol than for Man₉GlcNAc₂- PP- Dol (Kelleher *et al.*, 2003).

While there are kinetic differences between OST enzymes containing STT3-A or STT3-B, both types are found in a variety of tissues (Figure 6). Ribophorin I is found in

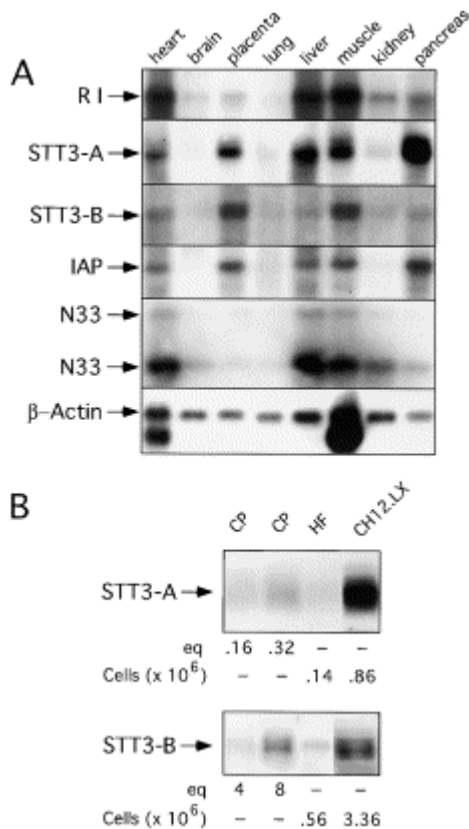


Figure 6. Differential Expression of OST Subunits

(A) Multiple tissue Northern blots were hybridized with probes specific for ribophorin I, STT3-A, STT3-B, IAP, N33, and β -actin. The hybridization positive bands are consistent with the expected mRNA sizes for ribophorin I (2.5 kb), STT3-A (2.7 kb), STT3-B (4.4 kb), IAP (2.2 kb), N33 (1.8 and 2.1 kb), and β -actin (2.0 kb). The β -actin mRNA probe served as a gel loading control. (B) Microsomes isolated from canine pancreas (CP), human fibroblasts (HF), and CH12.LX cells (CH12.LX) were resolved by PAGE in SDS and probed with antibodies to STT3-A or STT3-B. The amount of CP microsomes loaded per gel lane is expressed in eq (400 fmol OST/eq of CP microsomes). The quantity of HF and CH12.LX microsomes loaded per gel lane is derived from the indicated number of cells. Densitometry was used to estimate the amount of STT3-A and STT3-B in the HF and CH12.LX microsomes relative to the CP microsome standard. (Kelleher *et al.*, 2003)

all OST complexes and, therefore, is an indicator of OST expression. STT3-A is expressed highly in placenta, liver, skeletal muscle, and pancreas. It is found in lower levels in brain, lung, and kidney. STT3-B is expressed in more even levels in the different tissues. Both isoforms are expressed in human fibroblasts and CH12.LX cells as shown in Figure 6B. Experiments have shown that both STT3-A and STT3-B containing OST complexes are present within a single cell type (Kelleher *et al.*, 2003).

PROJECT PURPOSE

The STT3 subunit of oligosaccharyl transferase has been previously characterized to have two isoforms, STT3-A and STT3-B. These different OST isoforms have different kinetic and substrate recognition properties. However, different cell types contain both the STT3-A and STT3-B form of OST. It is unclear why a single cell type would contain both isoforms. The goal of this project was to knockdown expression of one isoform of STT3, using RNAi, in order to study the effect that would have on glycosylation. However, due to time constraints, the knockdown was not able to be achieved.

METHODS

Cell Culture

HEK293 cells were grown in DMEM (containing 4.5 g/L glucose and 110 mg/L sodium pyruvate and 2 mM L-glutamine), supplemented with 10% (v/v) heat-inactivated fetal bovine serum and incubated at 37° C with 5% CO₂.

Preparation of Cell Lysates

Cells were lysed using three different techniques. In the sonication method, cells were suspended in sonication buffer composed of:

50 mM TEA pH 7.5
0.1 M KOAc
0.1% Nikkol
1 mM DTT

Then, the cells were frozen at -80° C for a minimum of 1 hour, thawed, and sonicated.

The lysate was then centrifuged and the pellet discarded.

In the lysis buffer method, cells were suspended in lysis buffer composed of:

50 mM Tris-HCl pH 7.5
5 mM EDTA
1% SDS

Then, cells were centrifuged and the pellet discarded. In Dan's method, cells were

suspended in Dan's buffer composed of:

20 mM Tris-HCl pH 7.5
0.25 M Sucrose
50 mM KOAc
6 mM Mg(OAc)₂
1 mM EDTA
1 mM DTT

Then, the cells were frozen at -80°C for a minimum of 1 hour, thawed, and homogenized. The lysate was then centrifuged and the pellet discarded.

Western Blots

Proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. Primary antibodies for STT3-A and STT3-B were polyclonal and had been previously made in the lab from a rabbit using the C-terminal domain of each isoform as their epitopes. The antibody for LAMP-2 was a monoclonal mouse antibody and was purchased from Santa Cruz Biotechnology. The secondary antibodies used were goat anti-rabbit or goat anti-mouse with horseradish peroxidase (HRP) covalently attached. Immunoblots were visualized using ECL.

Protein Quantification

Protein samples run on SDS-PAGE gels were quantified using two methods. In order to measure equivalents, samples were incubated at room temperature for twenty minutes in 1% SDS. Then, the absorbance of each sample was measured at OD280. One equivalent is 50 OD280 units/mL.

Proteins were also quantified using the BioRad Protein Quantification Assay.

PNGase F Digestion

Samples were incubated with 1 μL of PNGase F at 37°C for 1 hour.

RESULTS

In order to eventually perform the STT3 isoform knockdown experiments, preliminary technique development had to be performed first. An antibody against STT3-B had previously been created in the lab, but it had never been tested. When an immunoblot was performed using this polyclonal rabbit antibody, many nonspecific bands appeared on the blot. In order to confirm that the band believed to be STT3-B was in fact STT3-B, an immunoblot was run, probing the left half with the STT3-B antibody and the right half with an antibody for ribophorin I (RI) (Figure 7). Liver rough microsomes (LRM638) extracted from dog liver were used as a partially purified ER membrane sample. The RI antibody was characterized previously and, while unspecific bands are present, it is known where it migrates on an SDS-PAGE gel. STT3-B was projected to migrate just above RI, at ~85 kDa, so its position is confirmed on the immunoblot.

After confirming that the band recognized by the STT3-B antibody was indeed STT3-B, it was necessary to develop a method of cell lysis for both isoforms for further study. Three methods were tested, the first being sonication of the cells. The cells were also placed in Lysis buffer containing a strong detergent or mechanically homogenized (see Methods). HEK293 cells were subjected to each of the three methods of lysis and an immunoblot was performed, probing for either STT3 isoform (Figure 8). The STT3-A signal was the strongest in the pellets and the lysis buffer supernatants (Figure 8A). The pellets contained nuclear components, including DNA, so the supernatant samples were more desirable to use in further experiments. The STT3-B signal was more uniformly distributed between the different methods in either the supernatant or the pellet. Since it

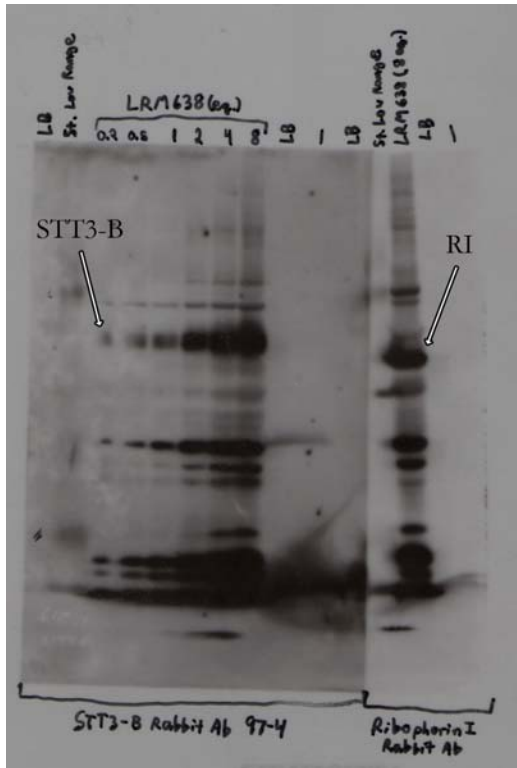


Figure 7 – Confirmation of STT3-B Detection
 Increasing amounts of equivalents of Liver Rough Microsomes (LRM638) from dog liver were run on an 8% SDS-PAGE gel. The blot was probed for STT3-B on the left and RI on the right. The relative position of the STT3-B band to the RI band confirms that that band is STT3-B.

did not make a significant difference which method was used to lyse cells to detect STT3-B, but STT3-A signal was much higher in lysis buffer supernatant, it was decided to use the lysis buffer method in further experiments.

Once STT3 detection was perfected, a control protein had to be chosen as a marker for N-glycosylation when knockdown experiments were performed. Lysosome associated membrane protein 2 (LAMP-2) was chosen because it contains sixteen N-glycans, making it highly glycosylated (Mane *et al.*, 1989). Detection of LAMP-2 in relation to STT3-A was performed to confirm that migration position of LAMP-2 on a gel and to ensure that LAMP-2 is expressed in HEK293 cells normally (Figure 9). Fully glycosylated LAMP-2 is ~100 kDa, so it appears much higher on the gel than STT3-A, which is ~60 kDa. The LRM lanes are negative controls on the LAMP-2 side of the gel.

LAMP-2 is lost if the purification process of obtaining the LRM samples, so it should not be present on an immunoblot.

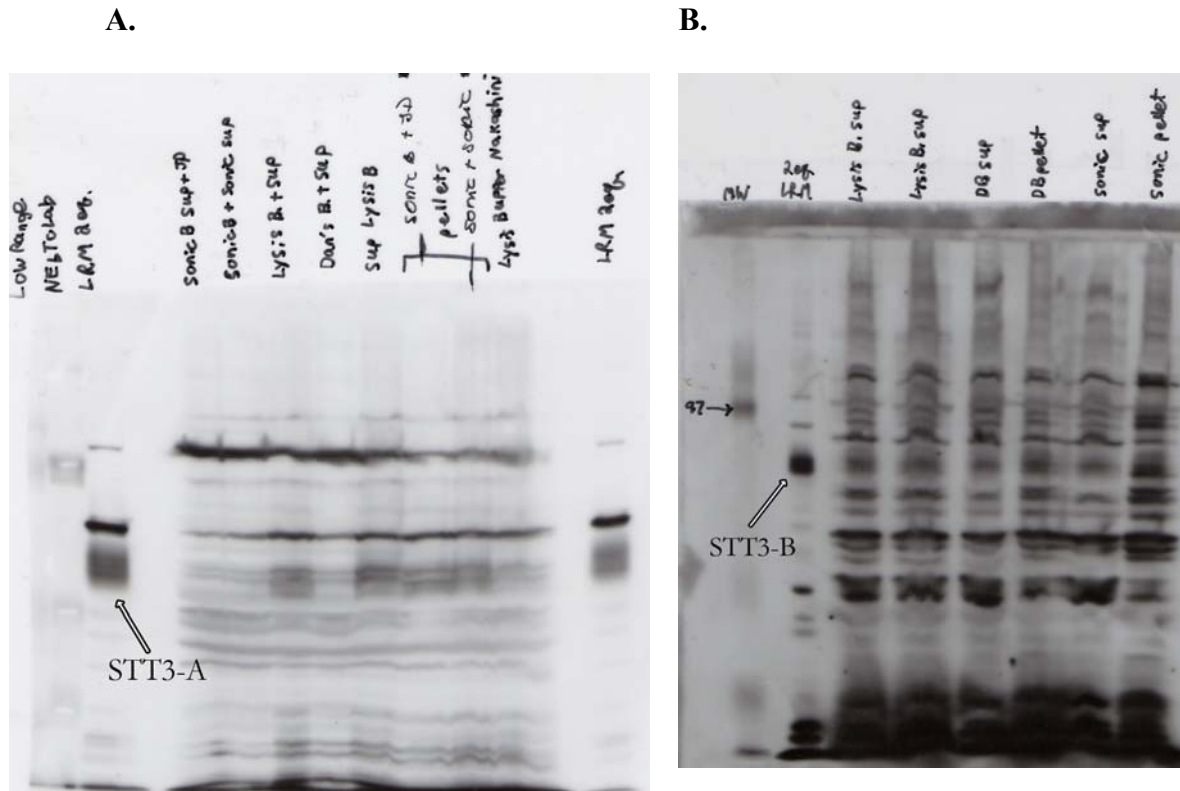


Figure 8 – HEK293 Cell Lysis Methods

Three different methods of cell lysis, sonication, lysis buffer, and Dan's method, were used to determine which yielded the highest amount of (A) STT3-A or (B) STT3-B. Samples were resolved by SDS-PAGE on an 8% gel. The Lysis B. + sup and sup Lysis B. lanes are duplicates.

In order to determine the size of unglycosylated LAMP-2, HEK cell samples were digested with PNGase F to remove all N-linked glycans from the protein (Figure 10). PNGase F cleaves the bond between the nitrogen atom on the asparagine side chain and the first GlcNAc of the oligosaccharide. Based on the sequence of LAMP-2, the unglycosylated form should be ~45 kDa. However, no signal is detected in the lanes digested with PNGase F. In order to ensure that the PNGase F digestion was working properly, half of the immunoblot was probed for STT3-A, which is also glycosylated. A

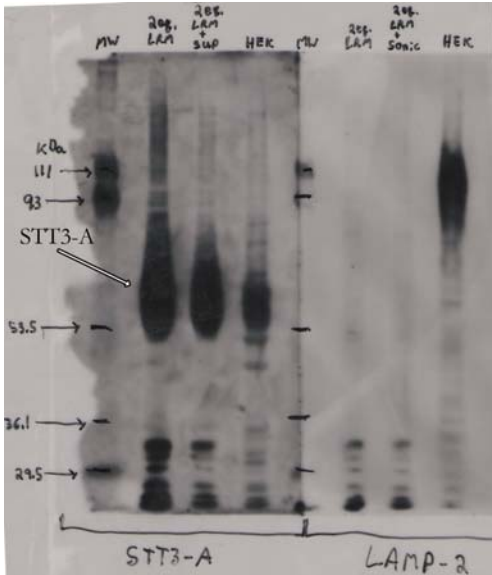


Figure 9 – Detection of LAMP-2 by Immunoblot
An 8% SDS-PAGE gel was run and probed for STT3-A (left) and LAMP-2 (right).

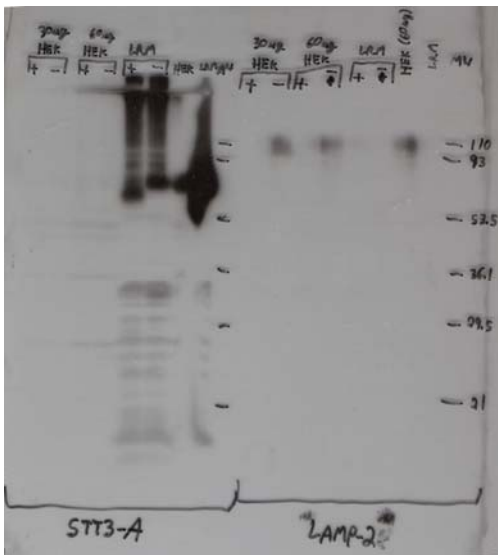


Figure 10 – PNGase F Digestion of HEK Cell Extracts
Cell extracts were incubated in the presence (+) or absence (-) of PNGase F and probed with an antibody for STT3-A (left) or LAMP-2 (right).

shift in the band for STT3-A was observed when it was digested with PNGase F, confirming that the PNGase F digestion worked.

Since no unglycosylated LAMP-2 signal was detected in the previous experiment, it was repeated with addition of extra protease inhibitors (PI) during the PNGase F digestion (Figure 11). It was thought that unglycosylated LAMP-2 may be highly unstable and rapidly degraded and that that was the cause of the lack of signal. Adding

DISCUSSION

In order to reach the ultimate goal of knockdown of STT3 isoforms using RNAi, detection methods of this OST subunit had to be perfected. It was found that placing the cells in lysis buffer with strong detergent to lyse them was the best method of detecting both STT3 isoforms in HEK293 cells.

LAMP-2 is a protein found in HEK293 cells that is highly glycosylated, with 16 N-glycans on its surface. It is desirable to use as a marker for glycosylation in the cell because of its larger number of glycosylated sites. However, detection of the unglycosylated form of LAMP-2 has been nonexistent to date. Since it has been shown that the PNGase F was deglycosylating properly, two major theories remain that can explain the lack of signal.

The first hypothesis is that LAMP-2 is unstable and rapidly degraded when it is unglycosylated. The lack of signal on the immunoblots may be due to this degradation of the protein, so none is there to be detected. If this is the case, then adding protease inhibitors during the digestion could help stabilize LAMP-2 so that it may be detected. An experiment was conducted where extra protease inhibitors were added in the PNGase F digestion, but the results were inconclusive. This experiment would have to be repeated in order to conclude if LAMP-2 is unstable when it is deglycosylated.

The second major theory is that the antibody used against LAMP-2 contains part of an oligosaccharide in its epitope. In this case, the unglycosylated form of the protein is present, but not detectable. The monoclonal LAMP-2 antibody used was purchased from Santa Cruz Biotechnology and was raised against adherent spleen cells of human origin. If experiments show that addition of protease inhibitors does not result in detection of

unglycosylated LAMP-2, then a new antibody would have to be obtained to detect it. Otherwise, a new marker of glycosylation could be chosen.

Once a marker protein is able to be properly detected, RNAi experiments will be conducted to knockdown one isoform of STT3 and measure its effect on the cell. In order to accomplish this, a plasmid containing DNA that would produce dsRNA would be transfected into HEK293 cells. This dsRNA would have one strand complementary to the mRNA of the STT3 isoform that will be knocked down and would activate the post transcriptional silencing regulation pathway.

After knocking down one isoform of STT3, there are two hypotheses as to what the results would be. First, alternative glycosylation of the marker protein could be observed, which would indicate that both isoforms are necessary for normal cell function. Further studies could then be conducted to determine the interaction of each isoform in the N-glycosylation mechanism of the cell.

Alternatively, no defect in glycosylation could be detected, which would indicate that only one isoform is necessary for proper N-glycosylation in the cell. Further studies must be conducted in order to determine the role of each isoform in N-glycosylation of proteins in the ER.

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