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Cotranslational and posttranslocational N-glycosylation of proteins in the endoplasmic reticulum

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Abstract

Asparagine linked glycosylation of proteins is an essential protein modification reaction in most eukaryotic organisms. N-linked oligosaccharides are important for protein folding and stability, biosynthetic quality control, intracellular traffic and the physiological function of many N-glycosylated proteins. In metazoan organisms, the oligosaccharyltransferase is composed of a catalytic subunit (STT3A or STT3B) and a set of accessory subunits. Duplication of the catalytic subunit gene allowed cells to evolve OST complexes that act sequentially to maximize the glycosylation efficiency of the large number of proteins that are glycosylated in metazoan organisms. We will summarize recent progress in understanding the mechanism of (a) cotranslational glycosylation by the translocation channel associated STT3A complex, (b) the role of the STT3B complex in mediating cotranslational or posttranslocational glycosylation of acceptor sites that have been skipped by the STT3A complex, and (c) the role of the oxidoreductase MagT1 in STT3B-dependent glycosylation of cysteine-proximal acceptor sites.

Keywords

asparagine linked glycosylation; endoplasmic reticulum; oligosaccharyltransferase; congenital disorders of glycosylation

1. Introduction

Asparagine-linked glycosylation of proteins in the rough endoplasmic reticulum (RER) is one of the most common protein modification reactions in eukaryotic cells. Most proteins that enter the secretory pathway are N-glycosylated including secretory proteins, the majority of cellular integral membrane proteins and the luminal resident proteins of the endoplasmic reticulum (ER), Golgi, nuclear envelope and lysosome. Asparagine linked oligosaccharides perform important roles in protein folding, quality control, and sorting

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events in the secretory pathway [1]. In the RER, N-linked oligosaccharides are essential for the calnexin-calreticulin chaperone cycle that promotes protein folding and entry into ER-to-Golgi transport vesicles (as reviewed by [2]). After trimming by mannosidases in the ER, high mannose oligosaccharides direct misfolded proteins into the ER associated degradation (ERAD) pathway [3]. Thus, the biosynthetic pathway of many cellular proteins is dependent upon the accurate and efficient addition of N-linked oligosaccharides.

The oligosaccharyltransferase (OST) catalyzes the transfer of a preassembled oligosaccharide from a lipid linked oligosaccharide (LLO) donor onto the asparagine residue of glycosylation acceptor sites (typically N-X-T/S, where X = P) or sequons in newly synthesized proteins. N-linked glycosylation is an ancient and conserved pathway that is present in most archaeobacteria, a subset of eubacteria, and almost all eukaryotes. Unlike mammalian organisms which have several thousand N-glycosylated proteins [4], N-glycosylation in bacteria is limited to a small number of cell surface proteins including the S-layer glycoprotein, archaeellins and pilin [5]. Archaeobacterial and eubacterial OSTs are single subunit enzymes designated as AglB and PglB respectively. Although certain eubacterial OSTs recognize an extended acceptor site (D/E-Z-N-X-S/T, where X, Z = P [6]) the conservation of the hydroxyamino acid residue (T/S) at the +2 position relative to asparagine is indicative of conservation of the acceptor peptide-binding site.

The catalytic subunit (STT3) of the eukaryotic OST complex is homologous to AglB and PglB [7, 8]. Eukaryotic OST complexes range in size between the single subunit OSTs of trypanosomes and the heterooctameric OSTs that are present in higher eukaryotes [9]. The multi-subunit OSTs of fungi and metazoan organisms allow more precise selection of the fully assembled oligosaccharide donor [10-12]. With the exception of many protist organisms, most eukaryotes synthesize the dolichol pyrophosphate (Dol-PP) linked oligosaccharide Dol-PPGlcNAc₂Man₉Glc₃ as the oligosaccharide donor. Metazoan organisms express two STT3 proteins (STT3A and STT3B) that are incorporated into distinct OST complexes that have partially overlapping roles in N-linked glycosylation [11, 13, 14].

The physiological significance of N-linked glycosylation is highlighted by the family of human diseases referred to as congenital disorders of glycosylation (CDG, as reviewed by [15, 16]). Mutations that cause defects in the assembly of the LLO donor, or reduce the transfer of the oligosaccharide onto proteins in the endoplasmic reticulum are grouped as CDG-1. Mutations that impact the processing of protein bound oligosaccharides in the Golgi constitute CDG-2. The CDG-1 family of diseases is characterized by hypoglycosylation of cellular glycoproteins resulting in multi-system pathological abnormalities.

2. Mammalian oligosaccharyltransferases

The two mammalian OST complexes, which we will refer to as the STT3A complex and the STT3B complex are composed of one copy of a catalytic subunit (STT3A or STT3B), a shared set of five non-catalytic subunits (ribophorin I, ribophorin II, OST48, DAD1 and OST4) as well as isoform specific subunits (MagT1, TUSC3, KCP2 and DC2).

In recent years, insight into the in vivo roles of the mammalian OST complexes has been obtained by siRNA-mediated depletion of OST subunits in cultured cells, and by the analysis of fibroblasts from human patients with congenital disorders of glycosylation (CDG-1). First, we will briefly summarize results concerning OST subunits that are shared by the both the STT3A and STT3B complexes before embarking on a more detailed discussion of the in vivo roles of the STT3A and STT3B complexes.

One copy of each of the shared subunits is present in both the STT3A and STT3B complexes. Yeast homologues of ribophorin I (Ost1p), ribophorin II (Swp1p), OST48 (Wbp1p), DAD1 (Ost2p) are encoded by essential yeast genes and are necessary for the stability and function of the OST complex [17-19]. Yeast *ost4* mutants are temperature sensitive for growth and have a defect in N-glycosylation linked to a reduced association between the oxidoreductase subunits (Ost3p or Ost6p) and STT3 [20-22].

A mutation in the human DDOST gene, encoding OST48, causes a form of CDG-1 [23]. The DDOST mutation reduces the expression of OST48 and caused a general deficiency in N-linked glycosylation. At the restrictive temperature for tsBN7 cells, a point mutation in DAD1 destabilizes the OST complex, causing a glycosylation defect that results in apoptosis [24]. siRNA mediated depletion of OST48 or DAD1 destabilizes the STT3A and STT3B complexes and causes hypoglycosylation [25]. Depletion of OST4 by siRNA treatment causes a reduction in the steady state levels of the STT3A complex and a defect in glycosylation of prosaposin [26]. A homozygous point mutation in the ribophorin II gene has been identified in a CDG-1 patient, suggesting that a ribophorin II mutation causes a general glycosylation defect [27].

Conflicting results have been reported concerning ribophorin I. Depletion of ribophorin I by siRNA causes a reduction in both STT3A and STT3B, and a general defect in protein glycosylation that mimics simultaneous depletion of STT3A and STT3B [13]. Other investigators have concluded that ribophorin I depletion causes selective hypoglycosylation of specific glycoproteins [28, 29] and have proposed that ribophorin I delivers substrates to the active site of the OST complex. Current cryoelectron microscopy derived structures of the OST lack sufficient resolution to indicate how ribophorin I might impact substrate delivery [30, 31].

Malectin, an ER-localized lectin that binds the terminal Glc α -1,3 Glc disaccharide on GlcNAc₂Man₉Glc₂ [32, 33], is thought to interact with the OST complex via ribophorin I [34]. Malectin coprecipitates with both the STT3A and STT3B complexes [35], but may not be present in equal stoichiometry to the core subunits of the STT3A and STT3B complexes. The association of malectin with the OST is an enigma given that malectin is proposed to be involved in a quality control pathway for malformed glycoproteins [36, 37].

MagT1 and TUSC3 are orthologs of the Ost3p and Ost6p subunits of the yeast OST [9]. Human STT3B complexes contain either MagT1 or TUSC3 [35], just as the yeast OST complex contains either OST3 or OST6 [21, 22, 38]. The structures of the luminal domains of Ost6p and TUSC3 have been solved revealing a thioredoxin fold with an active site CXXC motif that is required for function [39, 40]. Phylogenetic analysis of eukaryotic STT3

proteins revealed that fungal Stt3 proteins fall within the STT3B clade consistent with the presence of an oxidoreductase subunit in the yeast OST and mammalian STT3B complexes [14, 35].

KCP2 and DC2 were first identified as subunits of the STT3A complex when ribosome associated membrane proteins (RAMPs) were resolved by two dimensional blue-native gel electrophoresis [41]. Subsequent studies using cultured cells demonstrated that KCP2 is needed for full activity of the STT3A complex [25, 42]. Although DC2 reportedly assembles into STT3A and STT3B complexes [42], we favor the view that DC2 is an STT3A specific subunit based on the absence of readily identifiable DC2 and KCP2 orthologs in fungal organisms.

3. Cotranslational glycosylation by the STT3A complex

Although N-glycosylation is frequently referred to as a posttranslational modification, it has long been known that vacant acceptor sites in fully-folded proteins cannot be modified by the OST. Hence, glycosylation of proteins is spatially restricted to the lumen of the RER and temporally restricted by the formation of protein secondary and tertiary structures that prevent access of acceptor sequons to the enzyme active site. The earliest evidence that most glycoproteins are modified by a cotranslational process was obtained in radiolabeling experiments of intact cells, where it was observed that newly synthesized proteins were fully glycosylated after a relatively brief pulse. Using short pulses (90 sec) and two-dimensional gel electrophoretic analysis of nascent polypeptides, the Helenius lab showed that glycans are added in a highly synchronized process to the elongating influenza hemagglutinin protein before disulfide bond formation [43]. Acceptor sites in a nascent polypeptide first have access to the OST active site when the asparagine residue in a sequon is 65-75 residues from the peptidyltransferase site on the translating ribosome [44, 45]. The 65-75 residues of polypeptide are in an extended conformation, first passing through the ribosome exit tunnel in the large ribosomal subunit (~35 amino acid residues), then traversing the membrane through the central pore in the protein translocation channel (Sec61 complex, ~25 amino acid residues) before having access to the OST active site. The OST active site, which is located approximately 30Å from the luminal surface of the membrane [46, 47], is quite near (~20-30Å) the luminal face of the protein translocation channel. Photocrosslinking experiments employing nascent polypeptides with a photoreactive amino acid at the X-position of a cryptic glycosylation site (QXT instead of NXT) exclusively yielded crosslinks to STT3A when the QXT site was located 70-90 residues from the peptidyltransferase site on the ribosome [48].

Biochemical evidence that the oligosaccharyltransferase is associated with membrane bound ribosomes and the protein translocation channel was deduced from cell fractionation experiments showing a 1:1 correlation between the content of membrane bound ribosomes and ribophorin I [49]. Antibodies that recognize the cytoplasmic C-terminus of ribophorin I block protein translocation by sterically interfering with ribosome binding to the protein translocation channel [50]. Detergent treatment of microsomal membranes yields a ribosome-associated membrane protein (RAMP) fraction that includes the protein translocation channel as well as ribophorin I, ribophorin II and OST48 [41, 51-53]. Mass

spectrometric analysis revealed that the RAMP fraction contains the STT3A complex [41]. Differential treatment of microsomes with digitonin at increasing salt concentrations preferentially solubilizes the STT3B complex while leaving the STT3A complex in a RAMP fraction together with the protein translocation channel [13]. Taken together these results indicate that the STT3A complex is associated with the protein translocation channel, while the STT3B complex localizes to the RER but is not directly associated with the Sec61 complex (Fig. 1A)

Blue native gel electrophoresis of microsomal membrane proteins can resolve the OST into 470 and 500 kD STT3A complexes and a 550 kD STT3B complex [25]. Although the 470 kD STT3A complex lacks KCP2 [25], it is not clear whether KCP2 dissociates from the 500 kD complex during RAMP isolation and electrophoresis, or instead indicates that cells assemble an alternative version of the STT3A complex. We favor the former explanation as STT3A complexes isolated by a conventional protein purification procedure lacked DC2 and KCP2 [11] indicating that these small subunits can dissociate from the STT3A complex in detergent solution.

Localization of the STT3A complex adjacent to the translocation channel allows the OST to scan the growing nascent polypeptide for the presence of acceptor sites and transfer oligosaccharides before protein folding occurs (Fig. 1A). An important question is whether cotranslational glycosylation by the STT3A complex is important for high occupancy of acceptor sites in mammalian glycoproteins. Specific depletion of the STT3A complex in HeLa cells by siRNA treatment reduces glycosylation of specific proteins [13, 25]. Human glycoprotein substrates that are particularly sensitive to STT3A depletion include prosaposin, progranulin and transferrin [13, 14, 54]. Acceptor sites that are strongly dependent upon the STT3A complex must correspond to suboptimal substrates for the STT3B complex. Prosaposin and progranulin are polypeptides consisting of small cysteine-rich autonomous folding domains separated by spacer segments [55, 56]. Alkylation with PEG-maleimide indicates that the 32 cysteine residues in prosaposin are oxidized within a few minutes after synthesis in HeLa cells [13], consistent with rapid folding of the four 82 residue saposin domains. Transferrin is a cysteine-rich protein and appears to fold quickly based upon relatively rapid acquisition of complex oligosaccharides in the Golgi [57]. Cotranslational glycosylation of nascent proteins by the STT3A complex provides a mechanism to glycosylate acceptor sites that are near cysteine residues (Fig. 1B) before disulfide bond formation stabilizes secondary or tertiary protein structures that would prevent access to the OST active site [35]. Hypoglycosylation of transferrin, as detected by isoelectric focusing (IEF), is the standard diagnostic marker for CDG-1 [58]. The obligate cotranslational glycosylation of transferrin by the STT3A complex causes transferrin to be prone to hypoglycosylation when the assembly pathway for the lipid-linked oligosaccharide is perturbed.

Crystal structures of monomeric bacterial OSTs have been solved in the presence (*C. lari* PgIB, [47]) and absence (*A. fulgidus* AgIB, [59]) of acceptor peptides. The requirement for a hydroxyamino acid at the +2 position relative to asparagine and the preference for NXT sequons relative to NXS sequons is explained by the structure of the peptide-binding pocket [47]. Interestingly, the asparagine residue in the N-X-T/S acceptor site projects through a

narrow porthole in the active site that is closed by a flexible loop linking two of the PglB transmembrane spans. Thus, binding of an acceptor site to the OST active site and glycopeptide product release requires conformation changes in the OST [47].

The location of the STT3A complex adjacent to the protein translocation channel raises the possibility that acceptor sites in a nascent glycoprotein are recognized by an N-terminal to C-terminal scanning mechanism instead of a stochastic binding mechanism. Insight into this question has been provided by bioinformatic and biochemical analysis of closely spaced acceptor sites in glycoproteins [60]. STT3B independent glycosylation of adjacent sites (N-X-T/S-N-XT/S) or gap-1 sites (N-X-T/S-Z-N-X-T/S) is efficient when both sites have threonine as the +2 residue. Sequon skipping by STT3A, and incomplete modification by STT3B occurs when closely spaced sites contain serine as the +2 residue [60]. The analysis of reporter proteins that had tandem arrays of gap1 sites (e.g. (N-X-T-A)_N) provided evidence for an N-terminal to C-terminal scanning mechanism by STT3A. The third sequon in arrays containing either 4 or 5 sequons was uniformly skipped, presumably due to steric constraints. Uniform skipping of the third sequon can only be explained by an N-terminal to C-terminal mechanism of acceptor site modification [60].

HeLa cells that have been treated with siRNAs specific for STT3A, but not STT3B, express higher amounts of the luminal ER chaperone BiP [13] consistent with induction of the unfolded protein response (UPR) pathway in response to hypoglycosylation of proteins. This observation suggests that the STT3A complex is responsible for glycosylating most acceptor sites as the nascent polypeptide enters the ER lumen. The STT3A complex displays a higher stringency than the STT3B complex in selection of the fully assembled oligosaccharide donor [11] thereby assuring that most newly synthesized glycoproteins will carry the correct glycans for entry into the glycoprotein quality control pathways that are directed by ER-localized glycosidases and lectin chaperones.

An important role for the STT3A complex in human health was provided by the analysis of fibroblasts from a STT3A-CDG patient [54]. The V626A mutation in STT3A reduces stable expression of STT3A. The patient has an abnormal IEF pattern for transferrin, and has severe CDG symptoms [54]. Pulse labeling of the patients fibroblasts revealed defects in N-linked glycosylation that were very similar to results obtained in STT3A depleted HeLa cells.

4. Posttranslocational glycosylation by the STT3B complex

Glycosylation sites that are poorly modified in STT3B depleted cells relative to untreated cells must correspond to acceptor sites that are skipped at a high frequency by the STT3A complex (Fig. 1C) necessitating glycosylation by STT3B. Acceptor sites that have been observed to be skipped by the STT3A complex include the following: (a) acceptor sites that are within five residues of the signal sequence cleavage site, (b) acceptor sites located in the C-terminal 50 residues of proteins, (c) closely spaced NXS acceptor sites, (d) NXS sites in very small type I membrane proteins, (e) internal acceptor sites with non-optimal sequons including a subset of acceptor sites that are close to cysteine residues.

The analysis of glycosylation in STT3B depleted cells indicates that the major cellular role for the STT3B complex is to maximize sequon occupancy in glycoproteins by modification of sites that are skipped by the STT3A complex. STT3B can modify skipped sites by cotranslational (Fig. 1A) or posttranslational pathways (Fig. 2). We will discuss the basis for site skipping by STT3A in more detail and summarize current knowledge concerning the STT3B dependent pathway for modification of skipped sequons.

Bioinformatics analysis indicated that the N-terminal and C-terminal regions of glycoproteins contain fewer N-linked glycans than internal regions [61, 62]. The N-terminal region of glycoproteins have a lower glycan density in part due to the absence of acceptor sites in N-terminal signal sequences and the membrane spanning segments of type 2 ($N_{\text{cyt}}-C_{\text{lum}}$) membrane proteins. Glycosylation acceptor sites that are within 5 residues of the signal sequence are not accessible to the OST active site prior to signal sequence cleavage [13, 63, 64] hence these sites are frequently skipped by the STT3A complex that is scanning the growing chain (Fig. 1A).

Analysis of large glycopeptide databases [4, 65] revealed that the C-terminal 65-75 residues of metazoan glycoproteins contain a lower glycan density than internal segments. Moreover, glycopeptides from the C-terminal region of proteins have a higher NXT:NXS ratio than internal segments of proteins [14]. The C-terminal reduction in glycan density is caused by a lower density of acceptor sequons and a reduced efficiency of acceptor site modification [14]. The C-terminal region of low glycan density correlates nicely with the experimentally determined distance between the OST active site and the peptidyltransferase site on the ribosome [14, 44, 45].

Following chain termination, the rate at which an acceptor site will move past the STT3A complex will no longer be limited by the protein synthesis rate, which in mammalian cells is approximately six residues per second [66]. Glycosylation of extreme C-terminal sites is strongly dependent upon STT3B (Fig. 2A), indicating that rapid movement of acceptor sites past STT3A promotes acceptor site skipping, particularly for sites located in the C-terminal 50 residues of proteins [14]. The rates and efficiency of posttranslational glycosylation are higher for NXT sequons than for NXS sequons consistent with the higher affinity of the OST active site for NXT sequons [67] and the elevated NXT:NXS ratio among extreme C-terminal glycopeptides. Posttranslational glycosylation of an extreme C-terminal site in a typical 50 kD soluble protein is slow ($t_{1/2}$ of 3-10 min) relative to the time required for synthesis (~ 70 sec) but more rapid than the $t_{1/2}$ for ER to Golgi transport.

Posttranslational glycosylation is likely limited by the rates of competing reactions including protein folding and diffusion of the glycoprotein away from the luminal surface of the RER membrane. Posttranslational glycosylation by the STT3B complex does not involve a polypeptide scanning mechanism (Fig. 2A) as extreme C-terminal sites in a single protein are modified at different rates [14, 60].

Extreme C-terminal sites that are glycosylated by STT3B have been identified in type II membrane proteins [14] and multi-spanning membrane proteins [68]. Glycosylation of extreme C-terminal sites in type II membrane proteins was less sensitive to STT3B depletion suggesting that protein retention in the vicinity of the protein translocation channel reduces

acceptor site skipping by STT3A [14]. Glycosylation sites that are inserted into C-terminal epitope tags of membrane and soluble proteins are modified with high efficiency [69] by the STT3B complex [14].

Certain glycosylation sites in low molecular weight type I (N_{lum} - C_{cyt}) membrane proteins are posttranslocationally glycosylated. This class of posttranslocational glycosylation site was first identified in KCNE1 [70], a potassium channel β -subunit with two glycosylated asparagine residues (N5 and N26) located in the 43 residue luminal domain. Cotranslational glycosylation of the N5 site promotes posttranslocational glycosylation of the N26 site. Replacing the $N_{26}MS$ site with a $N_{26}MT$ site allowed cotranslational glycosylation of both sites [70]. Further analysis of four other KCNE family members showed that cotranslational glycosylation of type I membrane proteins is favored by the presence of longer cytoplasmic domains (>100 residues) and NXT acceptor sites [71]. Thus, posttranslocational glycosylation of small type I membrane proteins by the STT3B complex is dependent upon sequon skipping by STT3A, which is enhanced by suboptimal acceptor sites and a brief residence time within the protein translocation channel [71, 72].

A fourth class of STT3B dependent sequon is exemplified by the posttranslocational glycosylation sites in blood coagulation factor VII [13, 73] and hemopexin [60]. These acceptor sites are skipped at a high frequency (>50%) by the STT3A complex despite being located more than 75 residues from the C-terminus of a protein (Fig. 1C). Protein sequence factors that promote skipping of internal acceptor sites by STT3A complex include the following: (a) close spacing between NXS acceptor sites [60], (b) NXS sites that have bulky hydrophobic or acidic X residues [72, 74, 75], (c) a subset of NCT/S sites, (d) and NXT/S sites that are closely bracketed by a disulfide in the folded protein (Fig. 1C). Additional local sequence context factors promote acceptor sequon skipping by the oligosaccharyltransferase, as determined by bioinformatic analysis of modified and unmodified acceptor sites [61, 62]. Although the clearest example is the strong bias against proline residues at the +3 position relative to asparagine in glycosylated acceptor sites [61, 62], statistical analysis of glycopeptide databases suggests that additional nearby residues influence glycosylation efficiency [62].

Due to a higher affinity for the OST active site [76], NXT sites are more efficiently glycosylated than NXS sites which in turn are much more efficiently glycosylated than NXC sites [77]. Experimentally verified NXC sites that are glycosylated are relatively uncommon [4, 78] and are prone to low occupancy by glycans [79]. Skipping of internal glycosylation sites by the STT3A complex can be reduced or eliminated by acceptor site optimization. Replacing the STT3B dependent NCS sequon in hemopexin with a NCT site eliminated sequon skipping by STT3A [60]. Elimination of the cysteine residues that bracket the $N_{360}IT$ site in factor VII reduces, but does not eliminate, sequon skipping by STT3B [35] indicating that multiple factors promote skipping of internal sites.

The physiological importance of posttranslocational glycosylation role was revealed by the discovery that reduced expression of STT3B can cause a newly discovered form of CDG-1 [54]. An important open question is whether recognition of acceptor sites by the STT3B complex occurs simply by diffusion, or whether unfolded proteins are delivered to the

STT3B complex by luminal chaperones or ER lectins. Glycosylation of the STT3B dependent sequons in factor VII, KNCE1 and β -glucuronidase decreases when the cotranslational glycosylation sites in the same proteins are eliminated [13, 14, 70] indicating that the glycosylation status of the substrate impacts delivery to the STT3B complex.

A role for the STT3B complex in the ER associated degradation (ERAD) pathway was recently described [80]. A folding defective form of transthyretin undergoes slow posttranslocational glycosylation by STT3B at a normally unmodified C-terminal glycosylation site prior to entry into the glycan dependent branch of the ERAD pathway. It will be interesting to see if transthyretin glycosylation by STT3B is a unique example or a more general mechanism for targeting misfolded proteins into the ERAD pathway.

5. Posttranslocational glycosylation of cysteine proximal acceptor sites

The initially described roles for MagT1 and TUSC3 in protein glycosylation [11], became controversial when several groups reported that these proteins were cell-surface proteins needed for magnesium transport activity [81, 82]. However, cell-surface biotinylation and immunofluorescence microscopy experiments established that MagT1 is an RER protein [35].

The observation that TUSC3 expression is more limited than MagT1 expression [82, 83] allowed the analysis of MagT1 function in HeLa cells, which do not express TUSC3. Glycosylation of all tested STT3B-dependent acceptor sites was reduced in MagT1-depleted HeLa cells [35]. The MagT1 depletion induced hypoglycosylation can be suppressed by expression of TUSC3. Thus, as expected based on the high sequence identity, TUSC3 and MagT1 have overlapping functions.

Why does the oligosaccharyltransferase have an accessory subunit with oxidoreductase activity? Results obtained in the yeast experimental system indicated that deletion of the MagT1 orthologs (Ost3p or Ost6p) selectively reduces glycosylation of a subset of acceptor sites that on average are closer to a cysteine residue in the protein sequence [39, 84]. Ost3p and Ost6p are proposed to form mixed disulfides with free thiols in glycoprotein substrates, thereby delaying disulfide bond formation until nearby acceptor sites have been glycosylated [39]. In vivo, the majority of MagT1 is in the oxidized state [35], consistent with formation of mixed disulfides with protein thiols. Indeed, glycosylation of the STT3B dependent site in factor VII is dependent upon both active site cysteine residues in MagT1. Reaction of MagT1 or TUSC3 with free thiols in nascent glycoproteins should be the predominant pathway by which MagT1 facilitates N-glycosylation by the STT3B complex (Fig. 2B).

Analysis of the N₂₉CT glycosylation site in cathepsin C was particularly informative. This acceptor site, which is four residues from the signal sequence ceases to be STT3B dependent under the following conditions: (a) cells are pulse labeled in the presence of sufficient dithiothreitol to reduce the RER lumen, (b) the cysteine residue in the sequon is replaced by serine, or (c) the distance between the signal sequence and the acceptor sequence is increased by 25 residues [35]. Thus, skipping of the N₂₉CT site by STT3A is dependent upon the location next to the signal sequence, while MagT1 is required because C30 can form a disulfide that interferes with glycosylation of N29. Glycosylation of the N₂₉CT site

in cathepsin C is partially rescued by a MagT1 mutant containing a single active cysteine residue, indicating that a transiently reduced form of MagT1 can react with a non-native disulfide in cathepsin C to allow access of STT3B to the glycosylation site [35].

Both the yeast (Ost3p/Ost6p) and mammalian (MagT1/TUSC3) proteins have oxidoreductase dependent and independent functions. As shown first in the yeast system [39], mutation of the active site disulfide (CXXC motif) in Ost3p or Ost6p causes a less severe defect in glycosylation than the corresponding deletion mutant. Moreover, siRNA mediated depletion of MagT1 interferes with STT3B-dependent glycosylation of an SHBG derivative that lacks cysteine residues near the extreme C-terminal glycosylation sites [35]. Conceivably, the peptide bonding pocket of the oxidoreductase may help direct acceptor sites to the OST active site [40] regardless of whether cysteine chemistry is involved.

In mammalian glycoproteins, the presence of cysteine as the X residue in a sequon or the presence of a bracketing disulfide cannot be used to predict whether a sequon is MagT1 and STT3B dependent. Instead, sequon skipping by the STT3A complex is a prerequisite for MagT1 or TUSC3 dependent glycosylation sites.

Mutations in the human MagT1 gene cause an X-linked immunodeficiency disease (XMEN) characterized by chronic Epstein-Barr virus infections [85]. An earlier report that a point mutation (V311G) in MagT1 can cause X-linked mental retardation (XLMR, [83]) has been challenged by more recent evidence that the V311G variant of MagT1 is present in unaffected individuals [86]. Mutations in TUSC3 cause non-syndromic autosomal recessive mental retardation (ARMR) a disease characterized by intellectual impairment [83, 87, 88]. Consistent with transferrin being a STT3A dependent substrate, TUSC3-ARMR patients do not hypoglycosylate transferrin [83]. TUSC3-ARMR and MagT1-XMEN patients have milder symptoms than a STT3B-CDG patient [54], presumably because MagT1 and TUSC3 are semi-redundant in tissues where both mRNAs are expressed.

6. Conclusions and perspectives

Duplication of the STT3 gene gave rise to a second oligosaccharyltransferase complex in metazoan cells that allowed a division of labor between the translocation channel associated STT3A complex that scans the growing polypeptide chain for acceptor sites, and the STT3B complex that modifies acceptor sites that have been skipped by STT3A. As such, the two complexes cooperate to maximize glycosylation site occupancy in newly synthesized glycoproteins.

The concept of site skipping by the STT3A complex sheds light on the longstanding enigma that certain acceptor sites in glycoproteins show partial glycan occupancy. Postranslocational glycosylation of skipped sites by the STT3B complex is limited by protein folding rates and movement of the acceptor site away from the luminal surface of the RER. We suspect that sequon skipping by the STT3A complex is more prevalent than suggested by current experiments that rely on siRNA-mediated depletion of the STT3B complex.

Incomplete glycosylation site occupancy can also be caused when the pool of Dol-PPGlcNAc₂Man₉Glc₃ is reduced as observed in ALG7-CDG fibroblasts [89], or when LLO assembly is blocked at a late stage leading to the accumulation of truncated oligosaccharide donors (e.g., ALG6-CDG fibroblasts [90, 91]). The stringent selection of the LLO donor by the STT3A complex is predicted to enhance site skipping in cells with LLO assembly defects. The reduced stringency of LLO selection by the STT3B complex may partially compensate for sequon skipping in proteins that are good STT3B substrates. As the STT3B:STT3A ratio may vary between human tissues, the severity of protein hypoglycosylation that is caused by defects in oligosaccharide donor assembly may show tissue specific differences.

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Abbreviations

ARMR	autosomal recessive mental retardation
CDG	congenital disorders of glycosylation
ERAD	ER-associated degradation
IEF	isoelectric focusing
LLO	lipid linked oligosaccharide
OST	oligosaccharyltransferase
RAMP	ribosome associated membrane proteins
RER	rough endoplasmic reticulum
SHBG	sex hormone binding globulin
XLMR	X-linked mental retardation

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Fig. 1.

Cotranslational glycosylation of proteins by the STT3A complex. (A) The STT3A complex is associated with the protein translocation channel and scans the growing polypeptide for glycosylation acceptor sites. The STT3B complex occupies a more distal position, but is able to modify skipped glycosylation sites by a cotranslational or posttranslocational mechanism. (B) Cotranslational scanning of the nascent polypeptide allows efficient glycosylation of acceptor sites in cysteine-rich proteins before disulfide bond formation stabilizes protein tertiary structure. (C) Examples of N-terminal, internal and extreme C-terminal acceptor sites (red asterisks) that have been skipped by the STT3A complex.

Fig. 2.

Posttranslocational glycosylation of skipped acceptor sites by the STT3B complex. (A) The STT3B complex mediates posttranslocational glycosylation of acceptor sites that are skipped by the STT3A complex. The MagT1 subunit of the STT3B complex is necessary for glycosylation of all tested STT3B dependent sites including those adjacent to cysteine residues. (B) The oxidized form of MagT1 is proposed to form a mixed disulfide with an acceptor site proximal free cysteine residue thereby delaying disulfide bond formation and promoting acceptor site recognition by the STT3B active site. Disulfide chemistry releases the nascent glycoprotein to regenerate the oxidized form of MagT1.