Oligosaccharyltransferase Subunits Bind Polypeptide Substrate to Locally Enhance N-glycosylation*

M. Fairuz B. Jamaluddin‡, Ulla-Maja Bailey‡, and Benjamin L. Schulz‡§

Oligosaccharyltransferase is a multiprotein complex that catalyzes asparagine-linked glycosylation of diverse proteins. Using yeast genetics and glycoproteomics, we found that transient interactions between nascent polypeptide and Ost3p/Ost6p, homologous subunits of oligosaccharyltransferase, were able to modulate glycosylation efficiency in a site-specific manner \textit{in vivo}. These interactions were driven by hydrophobic and electrostatic complementarity between amino acids in the peptide-binding groove of Ost3p/Ost6p and the sequestered stretch of substrate polypeptide. Based on this dependence, we used \textit{in vivo} scanning mutagenesis and \textit{in vitro} biochemistry to map the precise interactions that affect site-specific glycosylation efficiency. We conclude that transient binding of substrate polypeptide by Ost3p/Ost6p increases glycosylation efficiency at asparagine residues proximal and C-terminal to sequestered sequences. We detail a novel mode of interaction between translocating nascent polypeptide and oligosaccharyltransferase in which binding to Ost3p/Ost6p segregates a short flexible loop of substrate polypeptide to the oligosaccharyltransferase active site for efficient modification. 

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Asparagine (N)-linked glycosylation is an essential post-translational modification of secretory and membrane proteins in eukaryota and also occurs in archaea and some bacteria (1). Oligosaccharyltransferase (OTase)\(^{1}\) is an integral membrane protein that catalyzes N-glycosylation of nascent polypeptides in the lumen of the endoplasmic reticulum (ER) (2). Eukaryotic OTase is physically associated with the translocon (3) and transfers oligosaccharide from a dolichyl-phosphate carrier onto asparagine side-chains of substrate polypeptides (2). The efficiency of glycosylation of asparagine residues is dramatically increased if they are present in glycosylation “sequons” (N-x-T/S; x ≠ P), the peptide recognition motifs of the catalytic site of OTase (4). After the transfer of glycan to protein, the presence of N-glycosylation assists efficient glycoprotein folding in the ER intrinsically and by locally recruiting the disulfide isomerase EErp57 through the lectins calnexin and calreticulin (5). Correctly folded glycoproteins are free to traffic through the Golgi, where further modification and extension of glycan structures can occur (6). The precise glycan structures present on mature glycoproteins are often vital for their biological functions, including those involved in immune response, embryonic development, and cancer (6–8).

OTase in Baker’s yeast, \textit{Saccharomyces cerevisiae}, is a multiprotein complex consisting of eight subunits (2). The catalytic site is located in Stt3p, and other protein subunits whose functions have been investigated are required for the integrity of the complex and for regulation of substrate specificity. It has been proposed that the requirement of Ost1p (human ribophorin I) for efficient glycosylation of a subset of integral membrane proteins (9) is due to direct physical association (10) to retain potential substrates in close proximity to Stt3p (11). The details and mechanisms of this association are not known. Ost3p and Ost6p are homologous proteins, and the incorporation of either into OTase defines two isoforms of the enzyme with distinct protein substrate specificities (Fig. 1) (12–14). Efficient glycosylation of some asparagine residues requires Ost3p-OTase, whereas others require Ost6p-OTase (15). A model of Ost3p/Ost6p function in N-glycosylation site selection has been proposed (16) in which the ER-lumenal peptide-binding grooves transiently tether nascent polypeptide non-covalently or through mixed disulfides, inhibiting local protein folding and increasing the efficiency of glycosylation of nearby asparagine residues. Aspects of this model, including mixed-disulfide formation (17) and non-covalent peptide binding (18), have been tested \textit{in vitro}. Although it has been established that Ost3p-OTase and Ost6p-OTase have different polypeptide substrate preferences \textit{in vivo} (12–15, 19), the physiological relevance and details of any interactions between Ost3p/Ost6p and substrate polypeptide are unclear.

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\footnotesize{\textsuperscript{1}}The abbreviations used are: OTase, oligosaccharyltransferase; ER, endoplasmic reticulum; GlcNAc, N-acetylglucosamine; ESI, electrospray ionization; ANOVA, analysis of variance.

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A Wild type  
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\begin{array}{c}
\text{ER lumen} \\
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\(\text{Ost6p} \)

B \(\Delta ost3/\Delta ost6\) pOST3  
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\[\text{Fig. 1. Overview of experimental manipulation of yeast OTase isoforms.}\]

Here, we used in vivo yeast genetics, glycoproteomics, and in vitro biochemistry to identify the precise sites of interaction between Ost3p/Ost6p and substrate polypeptides that affect the efficiency of N-glycosylation at diverse asparagines. Based on these data, we present a model in which transient binding of nascent polypeptide by Ost3p/Ost6p isolates newly translocating polypeptide from pre-translocated polypeptide, resulting in the formation of a short flexible loop of glycosylation-competent polypeptide substrate in close proximity to the active site of OTase for efficient modification.

**EXPERIMENTAL PROCEDURES**

**Cloning and Mutagenesis**—DNA encoding OST3 or OST6 including 1 kb up- and downstream were amplified using PCR and cloned into the YEp352 vector using BamHI and Sall restriction enzymes, yielding pOST3 and pOST6. DNA encoding GAS1 including 1 kb up- and downstream was amplified via PCR and cloned into the pRS415 vector using BamHI and SalI restriction enzymes, yielding pGAS1.

**Peptide Affinity Chromatography**—MBP-Ost6L, MBP-Gas1, and MBP-Gas1R90Q were expressed in *Escherichia coli* carrying plasmids pMAL-OST6L, pMAL-GAS1, and pMAL-GAS1R90Q, respectively, and purified via amylose-agarose affinity chromatography as described (18). MBP-Gas1 and MBP-Gas1R90Q were eluted in column buffer (20 mM Tris HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA) with 1% SDS, and MBP-Ost6L was retained on the amylose-agarose resin. MBP-Gas1 and MBP-Gas1R90Q were reduced, alkylated, precipitated, and digested with AspN as described (21). Residual AspN was inactivated by the addition of EDTA to 1 mM. Peptide affinity chromatography was performed as described (18). Briefly, peptides from equal amounts of MBP-Gas1 and MBP-Gas1R90Q in column buffer were combined and applied to amylose-agarose beads containing bound MBP-Ost6L, with the Ost6p CxxC motif either oxidized or reduced by prior incubation with DTT. Peptides were washed from the affinity matrix by means of sequential gravity flow washing with column buffer, and wash fractions were collected. Peptides in the load and wash fractions were then detected via LC-ESI-MS/MS.

**Mass Spectrometry and Data Analysis**—Peptides were analyzed via LC-ESI-MS/MS using a Prominence nanoLC system (Shimadzu, Kyoto, Japan) and TripleToF 5600 mass spectrometer with a Nanospray III interface (AB SCIEX, Washington, D.C.) as described (21, 22). Glycosylation occupancy was measured at previously identified glycosylation sites (15, 21) using unmodified and GlcNAc-modified versions of the same peptide. This identification used ProteinPilot (AB SCIEX) searching the LudwigNR database with standard settings: sample type, identification; cysteine alkylation, acrylamide; instrument, TripleToF 5600; species, *S. cerevisiae* with common contami-
nants; I.D. focus, biological modifications; enzyme, trypsin or AspN; and search effort, thorough I.D. False discovery rate analysis using ProteinPilot was performed on all searches, and peptides identified with greater than 99% confidence and a local false discovery rate of less than 1% were included for further analysis. The glycosylation occupancy at a given sequon was defined by the abundance as measured by the peak intensity of the GlcNAc-modified peptide as a fraction of the sum of the GlcNAc-modified and unmodified versions of the same peptide (15). For in vitro peptide affinity chromatography analysis, the relative enrichment of each peptide in each wash fraction was determined as described (18) based on the ratio of the abundance of each peptide. Interactions with the peptide-binding groove of Ost6p were determined for each peptide based on the log of the relative abundance of each peptide in each wash fraction between the oxidized and reduced versions of Ost6p. Experiments were performed in biological triplicate.

RESULTS AND DISCUSSION

Ost3p and Ost6p Peptide Binding Controls Glycosylation—In vitro, Ost3p and Ost6p can transiently bind selected peptides with complementary characteristics to their peptide-binding grooves (16). The Ost6p groove is hydrophobic and lined by basic residues (K96 and K99), and it binds peptides enriched in hydrophobic and acidic residues. Point mutations removing these basic residues in Ost6p (Ost6K96Q,K99Q) abolish in vitro peptide binding, whereas the addition of basic residues in place of the natively neutral residues at the equivalent positions in the Ost3p groove (Ost3Q103K,Q106K) allows binding of a hydrophobic and acidic peptide that also binds to wild-type Ost6p (18). This demonstrates that mutations to the groove of Ost3p and Ost6p affect peptide binding in vitro. To investigate the physiological relevance of these in vitro interactions to in vivo site-specific N-glycosylation efficiency, we constructed yeast strains expressing only a single isoform of OTase, wild-type Ost3p-OTase, Ost6p-OTase, or variants of each with point mutations at their peptide-binding grooves (Ost3Q103K,Q106K or Ost6K96Q,K99Q). We then used mass spectrometry to measure the site-specific glycosylation occupancy of cell wall glycoproteins from these cells. As this method analyzes mature glycoproteins in the yeast cell wall that have successfully folded and trafficked from the ER, it likely underestimates the effect of deleterious mutations to OTase (15, 16). The analysis used endoglycosidase H to release N-glycans, leaving a single GlcNAc “tag” on previously glycosylated asparagines. LC-ESI-MS/MS analysis could then detect GlcNAc-modified (glycosylated) and unmodified (non-glycosylated) forms of the same peptide to measure site-specific glycosylation occupancy at many different asparagines. For example, this approach detected both GlcNAc-modified and unmodified (non-glycosylated) forms of peptide V91-K105 from Gas1p, which contains a potential glycosylation site at N95 (Figs. 2A and 2B).

We first compared yeast with only Ost3p-OTase or with only Ost6p-OTase. Consistent with previous reports (12, 14, 15), this analysis confirmed that certain asparagines required Ost3p-OTase for efficient glycosylation equivalent to that of Ost6p-OTase (12, 14, 15). LC-ESI-MS/MS analysis showed that GlcNAc-N, a single GlcNAc "tag" on previously glycosylated asparagines, was released from Gas1p, consistent with previous reports (12, 14, 15). LC-ESI-MS/MS analysis could then detect GlcNAc-modified (glycosylated) and unmodified (non-glycosylated) forms of the same peptide to measure site-specific glycosylation occupancy at many different asparagines. For example, this approach detected both GlcNAc-modified and unmodified (non-glycosylated) forms of peptide V91-K105 from Gas1p, which contains a potential glycosylation site at N95 (Figs. 2A and 2B).

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OTase Subunits Generate Substrate Competence

A relative occupancy at different glycosylation sites (e.g. N79 in Sag1p) in Δost3/Δost6 cells with pOST3 relative to pOST6 (pOST3:pOST6), pOST6K96Q,K99Q relative to pOST6 (pOST6Q:pOST6), and pOST3Q101K,Q103K relative to pOST3 (pPOST3K:pPOST3). The ratio is mapped from yellow (increase) to blue (decrease) or black (no change). *p < 0.05 ANOVA. Occupancy of (A) N57 in Gas1p, (C) N95 in Gas1p, (D) N253 in Gas1p, and (E) N328 in Ecm33p in various yeast strains. Values are mean. Error bars are S.E. *p < 0.05 ANOVA. F, cartoon surface representation of proposed binding of hydrophobic and neutral (black) but not hydrophobic and basic (blue) stretches of nascent polypeptide by the basic peptide binding groove of Ost6p (PDB I.D. 3G7Y (16, 18)).

wild-type cells, whereas others required Ost6p-OTase. For example, glycosylation occupancy at N95 in Gas1p was significantly lower in yeast with only Ost6p-OTase than in yeast with only Ost3p-OTase, indicating a requirement for Ost6p-OTase for efficient glycosylation at this site (Figs. 2C and 2E). Equivalent analyses were performed in biological triplicate for the other robustly detected glycosylation sites in cell wall glycoproteins (Fig. 3A, column “pOST3:pPOST6”; supplemental Table S1). This showed that efficient glycosylation at N253 in Gas1p required Ost6p-OTase, as has been previously reported (15). In contrast, Ost3p-OTase was required for efficient glycosylation at N79 in Sag1p; N40, N57, and N95 in Gas1p; N177 in Crh1p; N304 and N328 in Ecm33p; N491 in Pb2p; and N60 in Gas5p. Although qualitatively consistent, our analysis here showed a stronger requirement for Ost3p-OTase than previous reports (15). Indeed, all asparagines that were glycosylated in wild-type cells required the presence of either Ost3p or Ost6p for efficient glycosylation (supplemental Table S1), highlighting the importance of Ost3p/Ost6p-mediated glycosylation. Further, Gas1p contained three sites (N40, N57, and N95) that required Ost3p and one site (N253) that required Ost6p. As all of these sites are efficiently glycosylated in wild-type yeast (with both Ost3p and Ost6p), this result implies that both Ost3p-OTase and Ost6p-OTase isoforms are located concurrently at the same translocon. This is consistent with previous genetic and biochemical studies (13, 15, 16), although split-ubiquitin studies have proposed that Ost3p and Ost6p interact differentially with the Sec61 and Shs1 translocons (23).

We next tested the effect of mutations in the peptide-binding grooves of Ost3p and Ost6p on in vivo site-specific glycosylation. For this, we compared site-specific glycosylation occupancy in yeast with only Ost3Q101K,K103K-OTase to that in yeast with only Ost3p-OTase, and we compared that in yeast with only Ost6K96Q,K99Q-OTase to that in yeast with only Ost6p-OTase. These point mutations did affect in vivo glycosylation efficiency at selected sites. For example, glycosylation occupancy at N95 in Gas1p was higher in yeast with only Ost6K96Q,K99Q-OTase than in yeast with only Ost3p-OTase (Figs. 2C and 2D), whereas occupancy at this site was lower in yeast with only Ost3Q103KQ106K-OTase than in yeast with only Ost6p-OTase (Figs. 2C and 2F). We performed equivalent analyses in biological triplicate for the other robustly detected glycosylation sites in cell wall glycoproteins (Fig. 3A, columns pOST6Q:pOST6 and pOST3K:pOST3; supplemental Table S1). N57 and N95 in Gas1p and N328 in Ecm33p required Ost3p for efficient glycosylation and were underglycosylated with Ost6p (Figs. 3A–3C and 3E; supplemental Table S1). However, this requirement for Ost3p was dependent on a neutral Ost3p/Ost6p peptide binding groove, as occupancy at Gas1p N57 and N95 was increased in the Ost6K96Q,K99Q variant and occupancy at all three sites was decreased in the Ost3Q101K,Q103K variant relative to wild-type proteins (Figs. 3A–3C and 3E; supplemental Table S1). In contrast, N253 in Gas1p required Ost6p for efficient glycosylation, and occupancy at this site was increased in the Ost3Q103K,Q106K variant (Figs. 3A and 3D, supplemental Table S1). These effects on site-specific glycosylation occupancy were consistent with growth of the corresponding yeast strains. Whereas the growth defect of Δost3/Δost6 yeast was fully complemented by Ost3p and partially complemented by Ost6p, expression of the Ost3Q101K,Q103K variant reduced growth relative to Ost3p (supplemental Fig. S1), consistent with inefficient glycosylation at several asparagines in this strain (Fig. 3). In summary, glycosylation by Ost6p-OTase at sites requiring Ost3p-OTase could be increased with point mutation to make Ost6p more like Ost3p, and vice versa. These results showed that point mutations that affect in vitro peptide binding by Ost3p and Ost6p also affected in vivo site-specific glycosylation occupancy, consistent with nascent polypeptide interacting directly with the grooves of Ost3p/Ost6p.
Mapping Sites of Interaction between Substrate and Ost6p—Efficient glycosylation at N57 and N95 of Gas1p and N328 of Ecm33p required the absence of basic lysine residues in the peptide-binding groove of Ost3p/Ost6p (Figs. 3A–3C, supplemental Table S1). This suggested that specific basic amino acid residues in the Gas1p and Ecm33p nascent polypeptides caused electrostatic repulsion with Ost6p or Ost3Q101K,Q103K, which had basic peptide-binding grooves (Fig. 3F). We used this effect to identify the precise sites of interaction between the Gas1p substrate nascent polypeptide and the Ost6p peptide-binding groove in vivo.

We performed scanning mutagenesis of basic to neutral residues through the Gas1p sequence surrounding N57 and N95 in Gas1p variants expressed in Δost3Δost6 pOST6 cells. Color as in Fig. 3A. *p < 0.05 ANOVA. Occupancy of (B) N57 in Gas1p and (C) N95 in Gas1p in various yeast strains. Values are mean. Error bars are S.E. *p < 0.05 ANOVA. D, relative occupancy at N57 and N95 in Gas1p variants expressed in Δost3Δost6 pOST6*K96Q,K99Q compared with Δost3Δost6 pOST6. Color as in Fig. 3A. Occupancy of (E) N57 in Gas1p and (F) N95 in Gas1p in various Gas1p variants expressed in Δost3Δost6 pOST6 (black) or Δost3Δost6 pOST6*K96Q,K99Q (white). Values are mean. Error bars are S.E. *p < 0.05 ANOVA. G, position of basic-neutral point mutations (blue) in Gas1p that increased occupancy at specific glycosylation sites (green).
Fig. 5. Non-covalent peptide binding by Ost6p in vitro. A, aligned AspN peptides from MBP-Gas1 and MBP-Gas1R90Q detected via LC-ESIQ-MS/MS. Extracted ion chromatograms of peptide from MBP-Gas1 (solid) and MBP-Gas1R90Q (dashed) in (B) load, and second wash fraction with (C) reduced MBP-Ost6 and (D) oxidized MBP-Ost6. E, relative enrichment of the Gas177–108 peptide by columns with attached oxidized MBP-Ost6. Data are average of triplicates. Error bars indicate range.

occupancy increased with Ost6pK96Q,K99Q relative to Ost6p for Gas1R76Q and Gas1R90Q but not for Gas1R47Q (Figs. 4D and 4E; supplemental Table S3). This indicated that binding near R47 of Gas1p to Ost6p was required for efficient glycosylation of N57. Consistent with point mutations at R76 and R90 increasing glycosylation of N57 by destabilizing Gas1p, N57 is in a surface exposed loop of Gas1p, in contrast to N95, which is relatively inaccessible (24) (supplemental Fig. S3). Together, the data showed that transient binding to Ost3p/Ost6p of stretches of Gas1p nascent polypeptide proximal and N-terminal to N57 and N95 allowed efficient glycosylation at these sites (Fig. 4G).

In Vitro Validation of in Vivo Peptide Binding by Ost6p—To confirm that the increased glycosylation we observed in the Gas1p variants was due to increased physical association of stretches of Gas1p with Ost6p, we performed in vitro peptide binding experiments. We expressed and purified Gas1p and Gas1R90Q as MBP-fusion proteins in E. coli and digested the proteins with AspN to obtain unglycosylated peptides similar to nascent polypeptide entering the ER lumen, which is the physiological substrate of OTase (Fig. 5A). Peptides were applied to resin containing bound MBP-Ost6L, with the active-site CxxC motif either oxidized or reduced through preincubation with DTT. The peptide-binding groove is only present when the CxxC motif is oxidized, and peptides do not bind when this motif is reduced (16, 18). We measured the MBP-Gas1 peptides present in each wash fraction via LC-ESIQ-MS/MS and observed increased retention of the robustly detected Gas1p77–108 peptide from the Gas1R90Q variant relative to wild-type Gas1p with oxidized (Fig. 5D) but not reduced (Fig. 5C) Ost6p, relative to the load (Fig. 5B). This indicated that Gas1R90Q mutation increased in vitro interaction with the Ost6p peptide-binding groove (Fig. 5E), consistent with our in vivo results (Figs. 3 and 4).

To gauge the generality of our results, we inspected the sequence N-terminal and proximal to glycosylation sites dependent on the presence of basic residues lining the peptide binding groove of either Ost3p or Ost6p (Fig. 3A) and compared the characteristics of these sequences (Fig. 6). Although Ost3p and Ost6p have different charge preferences, both require the presence of short stretches of hydrophobic amino acids in peptides for efficient binding (18, 25). The closest hydrophobic sequence stretches N-terminal to N57 and N95 in Gas1p and N328 in Ecm33p were flanked by basic amino acids, consistent with efficient glycosylation of these sites requiring neutral peptide-binding grooves in Ost3p or Ost6p. In contrast, N253 in Gas1p had a hydrophobic stretch with acidic amino acids nearby, consistent with its preference for basic Ost3p or Ost6p.

Model of Ost3p/Ost6p Function in Vivo—Together, our data showed that Ost3p/Ost6p transiently bound hydrophobic stretches of nascent polypeptide substrate proximal and N-terminal to specific asparagines to increase their glycosylation efficiency. Based on these findings, we propose a mechanistic model of Ost3p/Ost6p function in N-glycosylation (Fig. 7). In this model, short hydrophobic stretches of nascent polypeptide exiting the translocon into the ER lumen (Fig. 7A) bind to the peptide-binding groove of Ost3p/Ost6p independent of the location of nearby glycosylation sites (Fig. 7B). This binding is non-covalent (18) or through a mixed disulfide (17) and depends on complementarity between the stretch of nascent polypeptide and the peptide-binding groove of Ost3p/Ost6p (Figs. 4–6). Mutations at the thioredoxin-like CxxC active site motif of Ost3p/Ost6p in vivo in yeast affect glycosylation at only ~10% of sites (16), suggesting that the non-covalent mode of binding is dominant, and mixed disulfides form only when cysteines are appropriately positioned in stretches of bound nascent polypeptide. In either mode, this sequestration physically separates polypeptide that has already translocated into the ER lumen from newly translocating sequence. Continued translocation therefore produces a loop of nascent polypeptide with its ends constrained by the translocon exit and the Ost3p/Ost6p groove (Fig. 7C). As OTase is physically associated with the translocon (3) and Ost3p/Ost6p is adjac-
OTase Subunits Generate Substrate Competence

![Diagram](image)

Fig. 7. Mechanistic model of Ost3p/Ost6p function in N-glycosylation. A, partially translocated and folded nascent polypeptide enters the ER lumen from the translocon. B, a stretch of nascent polypeptide (red) with affinity for Ost3p/Ost6p is sequestered at the peptide-binding groove. C, continued translocation produces a flexible loop of nascent polypeptide kept physically separate from previously translocated polypeptide. D, glycosylation sequons in the loop are presented to the acceptor-binding site of Stt3p in a glycosylation-competent form and (E) efficiently glycosylated. F, non-covalent or mixed-disulfide binding of nascent polypeptide to Ost3p/Ost6p is transient, and (G) translocation and folding continues. Ost3p/Ost6p and Stt3p are physically adjacent in OTase (2) but are shown separately for clarity. See text for details.

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