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Functional characterization of Ost3p. Loss of the 34-kD subunit of the Saccharomyces cerevisiae oligosaccharyltransferase results in biased underglycosylation of acceptor substrates

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**Abstract.** Within the lumen of the rough endoplasmic reticulum, oligosaccharyltransferase catalyzes the en bloc transfer of a high mannose oligosaccharide moiety from the lipid-linked oligosaccharide donor to asparagine acceptor sites in nascent polypeptides. The *Saccharomyces cerevisiae* oligosaccharyltransferase was purified as a heterologeric complex consisting of six subunits (α-ζ) having apparent molecular masses of 64 kD (Ostlp), 45 kD (Wbplp), 34 kD, 30 kD (Swplp), 16 kD, and 9 kD. Here we report a structural and functional characterization of Ost3p which corresponds to the 34-kD γ-subunit of the oligosaccharyltransferase. Unlike Ostlp, Wbplp, and Swplp, expression of Ost3p is not essential for viability of yeast. Instead, *ost3* null mutant yeast grow at wild-type rates on solid or liquid media irrespective of culture temperature. Nonetheless, detergent extracts prepared from *ost3* null mutant membranes are twofold less active than extracts prepared from wild-type membranes in an in vitro oligosaccharyltransferase assay. Furthermore, loss of Ost3p is accompanied by significant underglycosylation of soluble and membrane-bound glycoproteins in vivo. Compared to the previously characterized *ostl-1* mutant in the oligosaccharyltransferase, and the *alg5* mutant in the oligosaccharide assembly pathway, *ost3* null mutant yeast appear to be selectively impaired in the glycosylation of several membrane glycoproteins. The latter observation suggests that Ost3p may enhance oligosaccharide transfer in vivo to a subset of acceptor substrates.

**Asparagine-linked glycosylation of proteins within the lumen of the rough endoplasmic reticulum (RER)** occurs via a highly conserved pathway in all eukaryotic organisms (Herscovics and Orlean, 1993; Kornfeld and Kornfeld, 1985). An increasing body of evidence articulates the crucial role of N-linked oligosaccharides in the physicochemical properties and biological function of many secreted and integral membrane glycoproteins (Varki, 1993). The oligosaccharyltransferase (OST) catalyzes the en bloc transfer of a core unit of 14 saccharides (Glc3Man9GlcNAc2) from a dolichol pyrophosphate donor, onto the nitrogen of an asparagine side chain in a Asn-X-Ser/Thr consensus sequon (Kornfeld and Kornfeld, 1985). Studies in *Saccharomyces cerevisiae* have provided important information concerning the biosynthesis of asparagine-linked oligosaccharides (Herscovics and Orlean, 1993; Kukuruzinska et al., 1987). In particular, the *alg* (asparagine-linked-glycosylation) mutants have been invaluable in defining the assembly pathway for the dolichol-linked oligosaccharide donor and in identifying the individual glycosyltransferases responsible for the sequential transfer of monosaccharides onto dolichol phosphate (Huffaker and Robbins, 1982; Kukuruzinska et al., 1987).

Insight into the membrane organization and subunit composition of the oligosaccharyltransferase was first disclosed upon purification of the canine and avian oligosaccharyltransferase as protein complexes consisting of ribophorin I (66 kD), ribophorin II (63 kD), and OST48 (48 kD) (Kelleher et al., 1992; Kumar et al., 1994). As shown previously for ribophorins I and II (Crimaudo et al., 1987; Harnik-Ort et al., 1987), protein sequence analysis and protease accessibility studies revealed that OST48 is oriented so that the majority of the polypeptide is located within the ER lumen (Silberstein et al., 1992). The protein sequence of OST48 is homologous to Wbplp (Silberstein et al., 1992), a 45-kD yeast ER membrane protein shown to be required for in vivo and in vitro expression of oligosaccharyltransferase activity (Herscovics and Orlean, 1993). Subsequently, the oligosaccharyltransferase of *S. cerevisiae*
Asparagine-linked glycosylation of proteins occurs during or shortly after transport of acceptor sites into the lumen of the endoplasmic reticulum (Rothman and Lodish, 1977). One poorly understood aspect of oligosaccharide transfer is that some N-X-S/T consensus acceptor sites in proteins that enter the ER lumen are not used, or are instead used with low efficiency in vivo, while other sites are quantitatively glycosylated. Glycosylation sites located within 13 amino acid residues of a membrane spanning segment are not modified in vivo, suggesting that the active site of the OST does not have access to such sites for steric reasons (Nilsson and von Heijne, 1993). A statistical analysis of non-utilized glycosylation sequons revealed a significant bias against glycosylation of sequons with the sequence N-X-T/S-P (Gavel and Von Heijne, 1990) in addition to the known lack of glycosylation at N-P-S/T sites. Although the presence of proline at the +1 or +3 position relative to asparagine can explain why many N-X-S/T sites are not utilized, or used with low efficiency (Allen et al., 1994), the latter observation has been resolved by denaturing gel electrophoresis and isolated as described (Silberstein et al., 1995). Tryptic digestion of the 34-kD γ-subunit, purification of tryptic peptides by reverse phase high pressure liquid chromatography, and sequencing of the amino terminus and internal tryptic peptides were performed by the Protein Chemistry Facility of the Worcester Foundation for Experimental Biology.

### Materials and Methods

#### Protein Purification and Peptide Sequencing

The oligosaccharyltransferase was purified from *S. cerevisiae* as described previously (Kelleher and Gilmore, 1994) and the six subunits were resolved by denaturing gel electrophoresis and isolated as described (Silberstein et al., 1995). Tryptic digestion of the 34-kD γ-subunit, purification of tryptic peptides by reverse phase high pressure liquid chromatography, and sequencing of the amino terminus and internal tryptic peptides were performed by the Protein Chemistry Facility of the Worcester Foundation for Experimental Biology.

#### Disruption of the OST3 Gene

The plasmid pRS305ΔOST3 (Fig. 1) was constructed to replace the chromosomal OST3 locus with the LEU2 gene using the y transformation procedure (Sikorski and Hieter, 1989). A 197-bp HindIII-Seq fragment from pOST3-1 was subcloned into pRS305 which had been first digested with XhoI, blunt-ended by filling in, and then digested with HindIII to generate pRS305ΔOST3R. A 210-bp BamHI fragment derived from pOST3-1 was generated by PCR using two primers (5′GGTTATCGGGCAGTTACAGGATGATGGGC-3′) and ligated to pRS305ΔOST3R which had been digested with NotI and blunt-ended prior to digestion with Xbal. The resulting construct, pRS305ΔOST3, was linearized at the unique SmaI site and transformed into two diploid yeast strains (PRY238 and YPH274) using a lithium acetate transformation protocol (Ito et al., 1983). Standard laboratory media were used for yeast growth and sporulation (Sherman, 1991). Leu + transformants were selected, and genomic DNA was isolated (Hoffman and Winston, 1987) to perform Southern analysis to confirm allele replacement. From each genetic background, diploid transformants were sporulated. asc were dissected and analyzed.

#### Isolation and Sequencing of an OST3 Genomic Clone

Two degenerate oligonucleotide primers (5′GGNTYCCARTTTYCARYT and 5′GGNWSRWTMOYTTYTRA) were synthesized by selecting regions with the least degeneracy (underlined) from the sequence of two tryptic peptides (APOFFQOLNVPVR and LLFKPSNSHLDHSSXS) and were used to amplify a yeast genomic DNA template using the PCR (Saiki et al., 1988). The amino acid residues designated X could not be unambiguously assigned from the tryptic peptide sequence data. PCR was performed in a 25 μl reaction volume with 125 pmol of primers, 2.5 U Taq DNA polymerase (Perkin Elmer Cetus Corp., Norwalk, CT) and 100 ng of *S. cerevisiae* genomic DNA. 25 amplification cycles using an automatic heating/cooler cycle (programmable thermal controller; MJ Research, Watertown, MA) were conducted as follows: 1 min at 94°C, 1 min at 40°C, and 1.5 min at 72°C. A 63-bp PCR product was recovered from a 8% polyacrylamide gel and subjected to direct DNA sequencing as described (Kusukawa et al., 1990).

A *S. cerevisiae* genomic DNA library in YEp24 was plated and two sets of nitrocellulose replicas were screened by in situ colony hybridization (Sambrook et al., 1989) using a random hexamer 32p-labeled 63-bp PCR product as a probe (Sambrook et al., 1989). Positive clones were isolated and subjected to restriction analysis. A HindIII–SplI fragment (2.5 kb) from a hybridization positive genomic clone was subcloned into pGEM-4Z (Promega Biotech, Madison, WI) to generate pOST3-1. Bacterial transformation was carried out using Escherichia coli DH5α as described (Sambrook et al., 1989). The DNA sequence of both strands of a 1,544-bp (HindIII–BspHI) fragment containing the coding sequence of the Ost3 protein was determined using the dideoxy chain termination method (Sanger et al., 1977). DNA and protein sequence analysis was done with the MacVector (IBI, New Haven, CT) software program.
Figure 1. Partial restriction endonuclease map, DNA and protein sequences, and gene disruption of the OST3 locus. (A) The OST3 locus was disrupted by replacement of the ScaI-BanI DNA fragment with the yeast integrating plasmid pRS305 carrying the LEU2 gene. The arrows flanking the BanI site designate PCR primers used to amplify a portion of the OST3 locus for construction of the gene disruption plasmid. Restriction sites used for constructions and mapping of the gene disruption are shown. (B) The nucleotide sequence of an HindIII-BspHI genomic DNA fragment containing the OST3 gene is shown together with the predicted amino acid sequence of Ost3p. Nucleotide residues are numbered on the right; amino acid residues are numbered on the left. The termination codon is indicated by an asterisk. Solid lines beneath the protein sequence designate sequences determined by gas phase sequencing of NH2-terminal and internal peptides. The signal peptidase cleavage site is designated by an arrow. The calculated molecular weight of 37,046 for mature Ost3p is in reasonable agreement with the Mr of 34 kD for the 7-subunit of the OST complex estimated by SDS-PAGE (Kelleher and Gilmore, 1994). Dashed underlining of the protein sequence designates four predicted membrane spanning segments (Kyte and Doolittle, 1982). A consensus site for N-linked glycosylation is enclosed in a box. These sequence data are available from the EMBL/GenBank/DDBJ under accession number U25052.
tion of 10 μg/ml 15 min before radiolabeling. Rapid lysis of cells with glass beads and immunoprecipitation of radiolabeled proteins with antibodies to carboxypeptidase Y (CPY), dipeptidyl aminopeptidase B (DFAP B) and invertase were performed as described previously (Rothblatt and Schekman, 1989). Immunoprecipitated proteins were incubated for 30 min at 55°C in SDS-sample buffer, boiled for 3 min, and resolved on 8% SDS–polyacrylamide gels.

Membrane Isolation and Oligosaccharyltransferase Assay

Microsomal membranes were isolated from Δost3, ostl-1, alg5-1 mutants and wild-type yeast grown to mid log phase at 25° or 30°C in YPD medium by a scaled-down version (Silberstein et al., 1995) of a procedure described previously (Kelleher and Gilmore, 1994). Oligosaccharyltransferase activity in digitonin extracts was assayed using an iodinated tripeptide acceptor ([N-acetyl-32]Tyr-Thr-NH2) and bovine lipid-linked oligosaccharide as a donor (Kelleher and Gilmore, 1994; Kelleher et al., 1992). The protein concentration of the microsomal membranes was determined using the Bio-Rad Protein Assay.

Immunological Methods

Equal amounts of microsomal membrane protein were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (TransBlot; Bio-Rad Laboratories, Hercules, CA). Blots were incubated for 1–16 h in TBS buffer (20 mM Tris-C1, pH 7.5,150 mM NaCl) containing either 1% Tween-20 or 2% nonfat dried milk plus 0.1% Tween-20 with antibodies against Ost1p, Wbplp, Swplp, Ost2p, or the Golgi GDPase. The preparation of antibodies against Ost2p, Wbplp, Swplp, Ost3p, or the Golgi GDPase will be described elsewhere (Silberstein, S., P. G. Collins, D. J. Kelleher, and R. Gilmore, manuscript in preparation). Preparation and characterization of the antibody to the GDPase has been described (Bernhince et al., 1994). Antiserum specific for CPY was raised in rabbits using a scaled-down version (Silberstein et al., 1995) of a procedure described (Raymond et al., 1992). The anti-peptide antibodies were used in a Western blot assay.

Quantification of Glycosylation

The average number of oligosaccharides for each of the glycoproteins was determined by radioanalytic scanning of fluorographs using a Molecular Dynamics Phosphorimager, or by scanning ECL images using a densitometer. The ratio between the average number of oligosaccharides on a given glycoprotein in the Δost3 mutant and the average number of oligosaccharides for that glycoprotein in a wild-type strain was expressed as the percent glycosylation relative to the wild-type.

Results

Isolation and Sequence Analysis of the OST3 Gene

The amino acid sequence of the mature NH2-terminus of the 34-kD γ-subunit of the yeast oligosaccharyltransferase, as well as four internal tryptic peptides, was determined by gas phase sequencing. These peptide sequences were not present in the current releases of the protein sequence databases indicating that the 34-kD subunit was a novel protein. Two pairs of degenerate oligonucleotide primers were designed based upon the sequence of two internal tryptic peptides (AFQQFQLNVP and LFIFKPNPSX-ILDHSXXX), where X designates residues that could not be unambiguously assigned. PCR was used to amplify a yeast genomic DNA template. Sequencing of a primer-specific 63-bp amplification product obtained with one primer pair confirmed that we had obtained an authentic amplification product. A genomic clone encoding the 34-kD protein, henceforth, designated Ost3p, was obtained by screening a S. cerevisiae YEp24 library with the radiolabeled PCR product. Southern analysis of a hybridization-positive clone showed that a 2.5-kb HindIII–SpI fragment hybridized with the PCR probe.

Subsequent subcloning and sequencing revealed an open reading frame encoding a protein of 350 amino acids (Fig. 1 B). The predicted protein sequence contains matches for all five peptides derived from the γ-subunit of the yeast oligosaccharyltransferase. Notably, the mature NH2-terminal methionine residue of Ost3p corresponds to residue 23 of the predicted open reading frame. Amino termini of proteins are subject to several cotranslational protein modifications, including signal peptidase, Nα-acetyltransferase and methionine aminopeptidase (Kendall et al., 1990). The specificity of modification of the amino terminus by the latter two enzymes is largely controlled by the identity of the penultimate amino acid in the protein. If Met 23 were the functional initiation codon, then the amino-terminal residue of the mature Ost3 protein would be an acetylated serine rather than a methionine, based upon the experimentally determined specificity of Nα-acetyltransferase and methionine aminopeptidase (Kendall et al., 1990). The amino-terminal sequence data is
The protein sequence was analyzed for the presence of hydrophobic segments using the algorithm of Kyte and Doolittle (1982). Five hydrophobic segments were detected, the first of which resembles a typical amino terminal cleavable signal sequence (von Heijne, 1986). The location of the most probable signal peptidase processing site using the predictive method of von Heijne (1986) is in agreement with the amino terminal sequence of the mature protein. Four additional hydrophobic segments, denoted by dashed underlining in Fig. 1 B, are located towards the carboxy terminus of Ost3p. These four segments are predicted to function as membrane spanning domains such that the majority of the protein is located within the lumen of the endoplasmic reticulum. Although a consenss site for N-linked glycosylation is located at Asn 33, Con A blot, and Endo H digestion data indicate that Ost3p is not a glycoprotein (Kelleher and Gilmore, 1994). The potential glycosylation sequon at Asn 33 is followed by proline residues (NKSP). Statistical analysis of utilized and nonutilized glycosylation sites in proteins that are translocated into the endoplasmic reticulum has disclosed a statistically significant bias against modification of sites followed by proline residues (Gavel and Von Heijne, 1990). Synthetic peptide substrates with proline at this position are not detectably glycosylated in vitro (Bause, 1983).

A search of protein sequence databases using the BLASTP protein sequence comparison algorithm (Altschul et al., 1990) revealed a homology between Ost3p and an open reading frame in chromosome 3 of Caenorhabditis elegans (P34669) that encodes a 37.7-kD protein. The C. elegans sequence has also been recovered as expressed sequence tags (T01933 and M88869). Despite the relatively modest sequence identity (21%) between Ost3p and the 37.7-kD C. elegans protein, the two proteins have an identical arrangement of four predicted membrane spanning segments. To our knowledge, vertebrate homologues of Ost3p have yet to be identified.

Disruption of the OST3 Gene

The OST3 locus was disrupted in two diploid yeast strains as an initial step towards analyzing the function of the Ost3 protein. An 1,158-bp Scal-BanI segment of the OST3 gene was replaced with the yeast integrating plasmid pRS305 bearing the LEU2 marker thereby deleting the complete open reading frame (Fig. 1 A). Leucine prototrophs were selected, and correct integration at the OST3 locus was confirmed by Southern analysis (Fig. 2 A). An integrant from each genetic background was sporulated, asci were dissected, and spores were tested for viability on YPD plates at 30°C (Table II). Four viable spores were obtained from each tetrad, and these gave rise to four colonies of identical size indicating that the OST3 gene is not essential for growth. Replica plating onto selective media established that the nutritional marker used for the disruption (LEU2) segregated 2:2 in all cases. Southern analyses of three tetrads, which was performed using a combination of restriction sites in pRS305 and DNA sequences flanking the OST3 gene, confirmed the replacement of the OST3 gene by pRS305 in those haploids that were leucine pro-

Figure 2. Disruption of the OST3 gene and its effect upon N-linked glycosylation. (A) Southern blot analysis of the OST3 disruption. Genomic DNA was isolated from PRY238, RGY301, and four haploid segregants of a RGY301 tetrad (RGY311-RGY314). HindIII-BspHI digests were resolved by agarose gel electrophoresis, and Southern transfers were probed with a 32P-labeled HindIII-Scal 5’ flanking fragment from the OST3 gene. The labeled arrows designate hybridization positive bands of 1,532 and 1,330 bp that correspond to the intact and pRS305-disrupted alleles of the OST3 gene, respectively. Disruption of the OST3 locus was also confirmed using a 32P-labeled BanII-XbaI PCR product as a 3’ flanking hybridization probe (data not shown). Growth of the six strains on synthetic minimal media lacking leucine (SD-Leu) is indicated below A. Genotypes of RGY311-RGY314 are in Table I. (B) The haploid segregants analyzed in A (RGY311-RGY314) were grown in minimal media at 30°C and were pulse-labeled for 30 min as described in Materials and Methods. As indicated, wild-type cells (RGY314) were incubated for 15 min with tunicamycin (TM) prior to labeling. CPY-specific immunoprecipitates from glass-bead extracts were resolved by PAGE in SDS. Fully glycosylated vacuolar CPY (mCPY) and underglycosylated variants of CPY are indicated by labeled arrows. (C) DPAP B immunoprecipitates from glass-bead extracts of cells labeled for 10 min at 30°C with TRAN35S were resolved by PAGE in SDS. The migration positions of fully glycosylated DPAP B from wild-type cells and unglycosylated DPAP B from tunicamycin-treated cells are designated by arrows.

The four haploid segregants (RGY311-RGY314) de-
Table II. Tetrad Analysis of OST3 Gene Disruption

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Tetrad Analyzed</th>
<th>Viable colonies per Tetrad</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRY238</td>
<td>OST3/OST3</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>RGY301</td>
<td>OST3/∆ost3::LEU2</td>
<td>8</td>
<td>4*</td>
</tr>
<tr>
<td>YPH274</td>
<td>OST3/OST3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>RGY302</td>
<td>OST3/∆ost3::LEU2</td>
<td>7</td>
<td>4*</td>
</tr>
</tbody>
</table>

* Diploid strains were sporulated, tetrads dissected on YPAD plates, and incubated for 2-3 days at 30°C. Colonies obtained were replica plated on selective medium to determine nutritional markers. PRY238 and YPH274 were the recipients for the OST3 disruption to produce RGY301 and RGY302, respectively. Detailed genotypes for each strain are given in Table I.

Table III. Relative Glycosylation of Proteins Expressed in Δost3 Mutants

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Topology</th>
<th>Average number of sites glycosylated by wild-type yeast*</th>
<th>Percentage of wild-type glycosylation by Δost3$^B$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25°C</td>
<td>30°C</td>
</tr>
<tr>
<td>CPY</td>
<td>Soluble</td>
<td>4</td>
<td>91$^a$</td>
</tr>
<tr>
<td>Invertase</td>
<td>Soluble</td>
<td>9.7</td>
<td>66$^a$</td>
</tr>
<tr>
<td>DPAP B</td>
<td>Type II membrane</td>
<td>6.5</td>
<td>ND</td>
</tr>
<tr>
<td>Ostlp</td>
<td>Type I membrane</td>
<td>3.7</td>
<td>ND</td>
</tr>
<tr>
<td>GDPase</td>
<td>Type II membrane</td>
<td>1.5</td>
<td>20$^a$</td>
</tr>
<tr>
<td>Wbp1lp</td>
<td>Type I membrane</td>
<td>2</td>
<td>11$^a$</td>
</tr>
</tbody>
</table>

*See text for an explanation of the average sites glycosylated in vivo by wild-type yeast.

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DPAP B synthesized by the ost3 null mutant strains consisted of a diverse set of glycoforms of intermediate mobility. Despite the incomplete resolution of the intermediate glycoforms of DPAP B, underglycosylation of DPAP B by the ost3 null mutant was clearly more severe than underglycosylation of CPY. Quantification by radioanalytic scanning indicated that DPAP B synthesized by the ost3 null mutants contains, on average, roughly 4 N-linked oligosaccharides rather than 6.5 for the wild-type strain (Table III).

Defects in lipid-linked oligosaccharide assembly also cause reduced in vivo glycosylation of yeast glycoproteins (Huffaker and Robbins, 1983). To determine whether the reduction in glycosylation of CPY and DPAP B could be directly attributed to a defect in the oligosaccharyltransferase, we assayed digitonin-solubilized microsomal membranes for oligosaccharyltransferase activity using dolichol-linked oligosaccharide isolated from bovine pancreas as an exogenous donor and the synthetic tripeptide N\(^\alpha\)-Ac-Asn\(^{1251}\)Tyr-Thr-NH\(_2\) as an exogenous oligosaccharide acceptor (Kelleher and Gilmore, 1994; Kelleher et al., 1992). Because both the lipid-linked oligosaccharide donor and the tripeptide acceptor are supplied in the in vitro assay, any in vivo defect in oligosaccharide donor assembly should not be detected using the in vitro oligosaccharyltransferase assay. Detergent extracts of membranes isolated from cultures of the two ost3 null mutant strains (RGY311 and RGY313) exhibited a 50% reduction in oligosaccharyltransferase activity relative to the two wild-type strains (Fig. 3).

**Expression and Glycosylation of the Oligosaccharyltransferase Subunits in the ost3 Null Mutant**

Protein immunoblots of membranes isolated from wild-type and Δost3 mutant strains were probed with antisera to the Ostlp, Wbplp, Swplp, and Ost2p to determine whether loss of Ost3p results in a reduced membrane content of the other subunits of the yeast oligosaccharyltransferase (Fig. 4). Previous studies have shown that the 64- and 62-kD glycoforms of Ostlp isolated from wild-type cells contain four and three N-linked oligosaccharides respectively (Kelleher and Gilmore, 1994; Silberstein et al., 1995). Underglycosylated forms of Ostlp were readily detected in membranes isolated from ost3 null mutant yeast relative to the wild-type (Fig. 4 A). The extent of underglycosylation of Ost3p was similar to that observed for DPAP B (Fig. 2 C and Table III). The comparable intensity of the Ostlp band after endo H digestion suggests that microsomal membranes isolated from wild-type and ost3 null mutants contain similar concentrations of Ost3p.

The Wbpl protein has two consensus sites for N-linked glycosylation (te Heesen et al., 1991) both of which are used in vivo (Kelleher and Gilmore, 1994). Protein immunoblot analysis of membranes isolated from wild-type and ost3 null mutant yeast showed that Wbplp was severely underglycosylated in the ost3 null mutant yeast (Fig. 4 B and Table III). The predominant form (~60%) of Wbplp detected in membranes from the ost3 null mutant comigrated with Endo H digested Wbplp. A comparison of the intensity of the Endo H digested samples suggests that the membrane content of Wbplp is not reduced in the ost3 null mutant strain. As observed for Ostlp and Wbplp, a reduction in the membrane content of Swplp and Ost2p was not apparent in the Δost3 mutant yeast (Fig. 4, C and D). Together, these results suggest that loss of Ost3p does not lead to a reduced synthesis, membrane incorporation or stability of any of the known subunits of the oligosaccharyltransferase complex, but instead reduces the in vivo and in vitro activity of the oligosaccharyltransferase.

**Figure 4. Glycosylation and expression of four subunits of the yeast oligosaccharyltransferase complex.** Microsomal membrane proteins that were isolated from wild-type (RGY314) and Δost3 mutant (RGY313) cells were resolved by SDS-PAGE and transferred to PVDF membranes. Each gel lane contained the following amounts of membrane protein: Ostlp (40 μg), Wbplp (5 μg), Swplp (60 μg), and Ost2p (60 μg). As indicated, the membrane proteins were treated with Endo H prior to SDS-PAGE analysis. Antibodies specific for Ostlp (A), Wbplp (B), Swplp (C), or Ost2p (D) were used as probes and visualized as described in the Materials and Methods. Fully glycosylated, underglycosylated and deglycosylated forms of Ostlp (A) and Wbplp (B) are indicated by labeled arrows.

*Figure 3. In vitro oligosaccharyltransferase activity of ost3 null mutants. Microsomal membranes were isolated from cultures of two wild-type (RGY312 and RGY314) and two Δost3 mutants (RGY311 and RGY313) after growth in YPD media at 30°C. The oligosaccharyltransferase activity of digitonin extracts was assayed in vitro as described in the Materials and Methods. Specific activity values (pmol mg\(^{-1}\) min\(^{-1}\)) are the average of two determinations.*
Selective Glycosylation Deficiency of the Δost3 Mutant

The different glycoproteins analyzed in the preceding experiments showed considerable variability in the extent of underglycosylation, ranging between 21% of wild-type glycosylation for Wbp1p to 87% of wild-type for CPY (Table III). We next asked whether this variability in the extent of underglycosylation was peculiar to the ost3 mutant, or was instead a general property of yeast with defects in N-linked glycosylation. For the following experiments we compared the ost3 null mutant to two other yeast mutants deficient in asparagine-linked glycosylation; alg5-1 (Huffaker and Robbins, 1983; te Heesen et al., 1994) and ostl-1 (Silberstein et al., 1995). The alg5-1 mutant accumulates the lipid-linked oligosaccharide Man₉GlcNAc₂-P-P-dolichol as the largest oligosaccharide donor, due to a deficiency in UDP-glucose/dolichyl-phosphate glucosyltransferase (Huffaker and Robbins, 1983; te Heesen et al., 1994). Synthesis of underglycosylated proteins by the alg5-1 mutant can be ascribed to the preference of the oligosaccharyltransferase for the fully assembled dolichol-linked oligosaccharide donor (Trimble et al., 1980).

When grown at the permissive temperature (25°C), ostl-1 cells show reduced transfer of core oligosaccharides to soluble and membrane-bound glycoproteins, yet grow at a wild-type rate (Silberstein et al., 1995). The extent of underglycosylation of CPY by the alg5-1 and ostl-1 mutant yeast was quite similar; in both cases the predominant glycoform lacks one N-linked oligosaccharide (Fig. 5 A). Thus, underglycosylation of CPY by the alg5-1 and ostl-1 mutants was more pronounced than that observed for the ost3 null mutant. Invertase was examined as a second soluble glycoprotein (Fig. 5 B). Three major forms of invertase can be identified when yeast cells induced to synthesize invertase are labeled for a brief time period (Esmon et al., 1981). Both the 60-kD cytoplasmic form of invertase and unglycosylated invertase synthesized in the presence of tunicamycin (Fig. 5 B, lane b) are readily resolved from several core-glycosylated ER glycoforms of invertase (Fig. 5 B, lane a). The predominant glycoform of invertase secreted by wild-type cells contains, on average, 9-10 N-linked oligosaccharides (Reddy et al., 1988). Mature invertase migrates as a 100-150-kD smear due to extensive elongation of core oligosaccharide chains in the Golgi apparatus (Esmon et al., 1981). Essentially identical distributions of underglycosylated forms of invertase were observed when two ost3 null mutants (RGY311 and RGY322) were analyzed (Fig. 5 B, lanes c and d). As RGY322 is a haploid derived from a different diploid strain (YPH274), we can conclude that disruption of OST3 in two different genetic backgrounds yields a similar underglycosylation phenotype. The ER form of invertase synthesized by alg5-1 cells contains, on average, 3.7 less N-linked oligosaccharides than invertase synthesized by a wild-type strain (Fig. 5 B). When invertase was immuno-precipitated from Δost3, and ostl-1 mutant cells grown at 25°C, the ER form of invertase respectively lacked, on average, 3.3 and 3.0 oligosaccharides relative to the wild-type strain (Fig. 5 B and Table III). Thus, the extent of underglycosylation by alg5-1, ostl-1, and Δost3 was fairly similar when invertase was used as the test glycoprotein.

Membranes that were isolated from 25°C cultures of Δost3, ostl-1, alg5-1 and two wild-type control strains were subjected to protein immunoblot analysis using antibodies to Wbp1p (Fig. 5 C). As observed previously (Silberstein et al., 1995), glycosylation of Wbp1p is reduced in the ostl-1 mutant, as indicated by the asterisk (*).
mutant relative to a wild-type control strain. The Wbp1p glycoforms detected in the ostl-1 and alg5-1 membranes contain, on average, 1.1 and 0.9 oligosaccharides, respectively. In contrast, the predominant form of Wbp1p synthesized by both Aost3 mutants lacks both oligosaccharides (Table III). Since Wbp1p is a subunit of the oligosaccharyltransferase, we evaluated the glycosylation of an unrelated Golgi membrane protein, the GDPase, which contains three consensus sites for N-linked glycosylation (Abeijon et al., 1993; Berninsone et al., 1995). Two discrete GDPase glycoforms were observed in both wild-type strains (Fig. 5 D, lanes a and k), indicating that the oligosaccharides on the GDPase are not subject to extensive outer chain elongation. Based upon the mobility increase caused by Endo H digestion, we tentatively conclude that one of the three consensus sites in the GDPase is not modified in vivo, while a second site is subject to partial glycosylation. One of the consensus sites in the GDPase is located 17 residues from the signal-anchor sequence in this type II membrane protein (Abeijon et al., 1993). Although the canine OST has been shown to modify consensus sites within 13 residues of a membrane-spanning segment (Nilsson and von Heijne, 1993), comparable experiments have not been performed with yeast membranes. The predominant form (~80%) of the GDPase detected in membranes prepared from both Aost3 null mutant strains was not glycosylated (Fig. 5 D, lanes c and i; Table III). In contrast, the predominant form of the GDPase synthesized by the alg5-1 and ostl-1 mutants contained a single oligosaccharide. Hence, the GDPase, like Wbp1p, is more severely underglycosylated by the Aost3 null mutant than by two other yeast strains that have lesions in the assembly (alg5-1) or transfer (ostl-1) of N-linked oligosaccharides.

Discussion

We have isolated and characterized the S. cerevisiae OST3 gene that encodes a 34-kD protein from yeast oligosaccharyltransferase preparations obtained using three different procedures (Kelleher and Gilmore, 1994; Knauer and Lehle, 1994; Pathak et al., 1995). However, the Ost3 protein may be present in substoichiometric amounts compared to the other OST subunits as judged by a reduced relative staining intensity with silver or Coomassie blue (Kelleher and Gilmore, 1994; Pathak et al., 1995). Additional studies will be required to address subunit stoichiometry in the intact endoplasmic reticulum membrane, and to determine whether the Ost3 protein is an accessory or a regulatory subunit. Expression of the OST3 gene is required for normal glycosylation of each of the six glycoproteins that we have tested. While underglycosylation of proteins in vivo can be caused by several mechanisms, the reduced in vitro oligosaccharyltransferase activity of the ostl-1 mutant is most readily explained by a lesion in the oligosaccharyltransferase. Since none of the other OST subunits was present in reduced amounts in membranes prepared from the ostl-1 mutant, we conclude that Ost3p is not required for synthesis, assembly or stability of the OST complex.

The Ost3 protein sequence does not show homology to the sequence of any of the three subunits of the mammalian oligosaccharyltransferase, nor does it appear to be related to any of the previously characterized subunits of the yeast OST. The existence of a potential homologue in C. elegans raises the possibility that vertebrate organisms might contain a related protein. As noted previously, the purified yeast oligosaccharyltransferase is roughly five-fold more active than the heterotrimeric canine OST complex (Kelleher and Gilmore, 1994). Based upon the in vitro OST assays described here, we would anticipate that dissociation of a putative canine Ost3p homologue during purification would lead to a reduction in OST activity. Alternatively, a putative Ost3 protein in a vertebrate organism might act as a stimulatory factor that is not stably associated with the oligosaccharyltransferase. Studies to differentiate between these alternatives using canine microsomes are in progress.

Ost3 null mutant yeast are able to grow at 25\textdegree, 30\textdegree, or 37\textdegree without showing a detectable reduction in growth rate relative to appropriate control strains. This observation was unexpected, as the genes encoding the three previously characterized subunits of the yeast oligosaccharyltransferase are all essential (Silberstein et al., 1995; te Heesen et al., 1992, 1993). When we evaluated glycosylation of secretory and membrane proteins in vivo, the Ost3 strains exhibited underglycosylation at 25\textdegree (Fig. 5), 30\textdegree (Figs. 2 and 4), and at 37\textdegree (data not shown). Thus, even at elevated temperatures, yeast cells will tolerate considerable underglycosylation of proteins. In this regard, Ost3 mutants are phenotypically similar to a subset of the alg mutants (e.g., alg5, alg6, and alg8) that effect late stages in assembly of the lipid-linked oligosaccharide, underglycosylate proteins, yet are viable at 37\textdegree (Huffaker and Robbins, 1983).

Interestingly, glycosylation of proteins was differentially reduced upon disruption of the OST3 gene. The extent of underglycosylation, relative to wild-type, ranged between 13\% for CPY and 79\% for Wbp1p (Table III). Although fewer glycoproteins were examined in previous characterizations of oligosaccharyltransferase mutants (Silberstein et al., 1995; te Heesen et al., 1992, 1993), a pronounced bias against glycosylation of specific substrates was not reported. As this property was not anticipated, we directly compared the ostl-1 mutant with a second oligosaccharyltransferase mutant (ostl-1) and a yeast strain bearing a defect in lipid-linked oligosaccharide assembly (alg5-1). Previous results indicate that ostl-1 mutants are defective in core oligosaccharide transfer to CPY, DPAP B, and Wbp1p (Silberstein et al., 1995). We confirmed and extended those results using invertase and the Golgi GDPase as additional test substrates. As observed here, yeast that are defective in donor assembly (alg5-1) or oligosaccharide transfer (ostl-1) underglycosylate all glycoproteins tested, without showing a dramatic bias against selected acceptor substrates (e.g., Wbp1p and GDPase). Control experiments indicate that the observed bias in glycosylation of some proteins is not a trivial artifact caused by differences in growth temperature, genetic background or detection method, but is instead an inherent property of all ostl-1 mutants isolated in this study. However, it would be premature to conclude that biased underglycosylation will not be displayed by any other mutant that affects the donor assembly pathway or the oligosaccharyltransferase.

Due to the limited number of glycoproteins analyzed, we can not draw an unequivocal conclusion concerning
what feature or features of the acceptor substrates are responsible for biased underglycosylation. However, we considered several properties of the nascent glycoproteins that might contribute to underglycosylation of a subset of substrates. Analysis of 427 used glycosylation sites has revealed that N-X-S sites are roughly twice as common as N-X-S sites, while these two sequences occur with similar frequency in cytosolic proteins, or non-used consensus sequences within secreted proteins (Gavel and Von Heijne, 1990). In vitro assays using synthetic peptides indicate that N-X-S peptides are more rapidly glycosylated than N-X-S peptides (Bause, 1984). Could biased underglycosylation due to the Ost3 mutants be explained by reduced recognition of one type of acceptor sequence? The observed twofold reduction in the in vitro OST activity is not consistent with a N-X-S consensus site-specific lesion given that the assay substrate is N²-Ac-Asn-[¹⁵¹]Tyr-Thr-NH₂. The two proteins that were most heavily underglycosylated (Wbp1p and GDPase) each contain one N-X-S site, while CPY lacks N-X-S sites. However, the relative absence of interactors underglycosylation of invertase, which contains three N-X-S sites out of thirteen total sites, does not provide strong support for the hypothesis that Ost3 mutants are selectively deficient in glycosylation of N-X-S sites. A thorough analysis of the carbohydrate content of each of the glycosylation sequons in invertase has revealed that seven sites are modified in all invertase molecules synthesized by wild-type yeast, while five additional consensus sites are subject to variable glycosylation ranging between 30 and 80% (Reddy et al., 1988). We have not determined which sites in invertase are subject to underglycosylation by the Ost3 mutant, so it is not clear whether the reduction in glycosylation is a consequence of reduced modification at all normally used sites, or is instead due to selective underglycosylation of a subset of consensus sites.

Perhaps the most obvious structural difference between the marginally and severely underglycosylated proteins was that the latter category consists of membrane proteins while the former category is comprised primarily of soluble proteins. Whether or not this correlation is significant, the fundamental question to be addressed is how or why the Ost3 mutation exhibits biased underglycosylation of certain proteins (i.e., Wbp1p and the GDPase). Although membrane proteins appear to be slightly more prone to underglycosylation when the oligosaccharyltransferase is impaired (Silberstein et al., 1995), the biased underglycosylation of membrane proteins by the Ost3 mutant cannot be readily explained by a reduced catalytic efficiency of the oligosaccharyltransferase. How might the Ost3 protein enhance glycosylation of certain classes of nascent glycoproteins? Perhaps the Ost3 protein interacts with subunits of the yeast translocon to localize the oligosaccharyltransferase in the vicinity of a translocation site. Protein translocation across the yeast endoplasmic reticulum can occur via a cotranslational signal recognition particle (SRP)-dependent pathway, or a posttranslational chaperone dependent pathway (for a review see Sanders and Schekman, 1992). Interestingly, translocation or integration of some proteins is most severely affected by lesions in SRP or SRP receptor (Hann and Walter, 1991; Ogg et al., 1992), while translocation of other proteins is most severely impaired by mutations in the components of the Sec63p complex (Feldheim et al., 1993; Stirling et al., 1992). Notably, translocation of CPY is not affected in a ΔSRP54 yeast strain, in contrast to integration of DPAP B which is impaired (Hann and Walter, 1991; Ogg et al., 1992). Yeast bearing mutations in the Sec63p complex are markedly deficient in translocation of CPY, yet show essentially wild-type integration of DPAP B (Stirling et al., 1992). Taken together, these results suggest that different proteins are translocated through Sec61p translocation channels (Müsch et al., 1992; Sanders et al., 1992) that have different auxiliary components that are specific for the cotranslational or posttranslational targeting pathway (Hann and Walter, 1991). Conceivably, biased underglycosylation in the Ost3 mutant may be due to an impaired interaction between the oligosaccharyltransferase and the auxiliary proteins that comprise the SRP-dependent subset of Sec61p translocation channels. Alternatively, the Ost3 protein may enhance glycosylation of some acceptor substrates by increasing the time window that the nascent polypeptide is in contact with the oligosaccharyltransferase prior to the initiation of folding reactions that will render unused glycosylation sites refractory to modification.

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