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The Subunit of the Saccharomyces cerevisiae Oligosaccharyltransferase Complex Is Essential for Vegetative Growth of Yeast and Is Homologous to Mammalian Ribophorin I

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Abstract. Oligosaccharyltransferase mediates the transfer of a preassembled high mannose oligosaccharide from a lipid-linked oligosaccharide donor to consensus glycosylation acceptor sites in newly synthesized proteins in the lumen of the rough endoplasmic reticulum. The Saccharomyces cerevisiae oligosaccharyltransferase is an oligomeric complex composed of six nonidentical subunits (α–γ), two of which are glycoproteins (α and β). The β and δ subunits of the oligosaccharyltransferase are encoded by the WBPI and SWPI genes. Here we describe the functional characterization of the OST1 gene that encodes the α subunit of the oligosaccharyltransferase. Protein sequence analysis revealed a significant sequence identity between the Saccharomyces cerevisiae Ostl protein and ribophorin I, a previously identified subunit of the mammalian oligosaccharyltransferase. A disruption of the OST1 locus was not tolerated in haploid yeast showing that expression of the Ost1 protein is essential for vegetative growth of yeast. An analysis of a series of conditional ostl mutants demonstrated that defects in the Ost1 protein cause pleiotropic underglycosylation of soluble and membrane-bound glycoproteins at both the permissive and restrictive growth temperatures. Microsomal membranes isolated from ostl mutant yeast show marked reductions in the in vitro transfer of high mannose oligosaccharide from exogenous lipid-linked oligosaccharide to a glycosylation site acceptor tripeptide. Microsomal membranes isolated from the ostl mutants contained elevated amounts of the Kar2 stress-response protein.

Asparagine-linked glycosylation of proteins is a ubiquitous protein modification reaction in eukaryotic organisms that occurs in the lumen of the rough endoplasmic reticulum (Herscovics and Orlean, 1993; Kornfeld and Kornfeld, 1985). Addition of asparagine-linked carbohydrates to many glycoproteins is an obligatory event for folding and assembly of newly synthesized polypeptides (Helenius, 1994). The presence of oligosaccharides is often required for the efficient transport of individual glycoproteins through the secretory pathway (Guan et al., 1985; Riederer and Hinnen, 1991; Winther et al., 1991). Glycan groups contribute to the overall dynamic stability of proteins, in some cases rendering them more resistant to proteolysis in vivo (Barriocanal et al., 1986). Diverse biological roles for asparagine-linked oligosaccharides have been identified including serving as receptors for extracellular ligands, acting as protein-targeting signals, and modulating protein interaction and function (for review see Varki, 1993).

The lumenal enzyme oligosaccharyltransferase catalyzes the transfer of a preassembled high mannose oligosaccharide (Glc3Man9GlcNAc2) from a lipid-linked oligosaccharide donor onto asparagine acceptor sites within the consensus sequon Asn-X-Ser/Thr, where X can be any amino acid except proline (Gavel and Von Heijne, 1990). Despite the evolutionary distance between mammals and yeast, the exact conservation of both the donor and acceptor substrates suggests that the oligosaccharyltransferase in these two diverse organisms should be structurally related. Mammalian and avian oligosaccharyltransferases have been purified as protein complexes consisting of ribophorin I (M, = 66,000), ribophorin II (M, = 63,000), and OST48 (M, = 48,000) (Kelleher et al., 1992; Kumar et al., 1994). Ribophorins I and II are well characterized integral membrane glycoproteins of the rough endoplasmic reticulum (Kreibich et al., 1978; Marcantonio et al., 1982). Consistent with an active site located within the lumen of the endoplasmic reticulum, protein sequence analysis and protease accessibility studies indicate that the ribophorins and OST48 are type I integral
membrane proteins with large amino-terminal luminal domains and shorter carboxy-terminal cytoplasmic domains (Crimaudo et al., 1987; Harnik-Ort et al., 1987; Marcantonio et al., 1982; Silberstein et al., 1992). Protein immunoblot experiments have shown that ribophorin I and II are expressed in all mammalian tissues tested (Marcantonio et al., 1982), and immuno reactive proteins of similar size were detected in avian and amphibian organisms but not in yeast (Crimaudo et al., 1987).

The homology between the mammalian and yeast oligosaccharyltransferase first became evident when canine OST48 protein was found to be 25% identical in sequence to Wbplp, a 45-kD integral membrane protein of Saccharomyces cerevisiae (Silberstein et al., 1992; te Heesen et al., 1991). Wbplp is essential for vegetative growth of yeast and is localized to the yeast endoplasmic reticulum (te Heesen et al., 1991). Phenotypic analysis of a yeast strain bearing a mutant allele of the WBPI gene has shown that the Wbplp is required for asparaginyl-linked glycosylation of proteins in vivo and for oligosaccharride transfer to acceptor peptides in vitro (te Heesen et al., 1992). A second gene (SWPII) encoding a 30-kD polypeptide was identified as an allele-specific high-copy suppressor of the wbpl-2 mutant (te Heesen et al., 1993). Gene product depletion experiments indicate that Swplp is also required for oligosaccharyltransferase activity in yeast (te Heesen et al., 1993). Simultaneous overexpression of both Wbplp and Swplp does not increase the oligosaccharyltransferase activity of yeast microsomes (te Heesen et al., 1993), suggesting that additional subunits remain to be characterized. Indeed, purification of yeast oligosaccharyltransferase as a complex of six subunits (α-γ) was recently reported (Kelleher and Gilmore, 1994). The β and δ subunits of the yeast oligosaccharyltransferase were shown to correspond to Wbplp and Swplp, respectively. Surprisingly, a protein sequence comparison revealed that the 30-kD Swpl protein is related to the carboxy-terminal half of mammalian ribophorin II (Kelleher and Gilmore, 1994).

The finding that two of the yeast oligosaccharyltransferase subunits, Wbplp and Swplp, are homologous to the OST48 and ribophorin II subunits of the mammalian oligosaccharyltransferase, respectively, suggested that the yeast homologue of ribophorin I remained to be identified. Here, we report the isolation and characterization of the yeast OSTI gene that encodes the α subunit of the yeast oligosaccharyltransferase complex. The OstI protein is homologous to mammalian ribophorin I and is shown to be an essential subunit of the yeast oligosaccharyltransferase.

Materials and Methods

Protein Purification and Peptide Sequencing

Yeast oligosaccharyltransferase (50-100 pmol), purified as described previously (Kelleher and Gilmore, 1994), was resolved into subunits by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane or onto a nitrocellulose sheet and stained with Ponceau S. The amino terminal sequence of excised bands corresponding to the 62- and 64-kD forms of the α subunit was determined by the Worcester Foundation for Experimental Biology Protein Chemistry facility. In situ proteolysis of the 64-kD glycoform, peptide purification by narrow-bore reverse phase HPLC, and the sequencing of two peptides were performed by the Harvard University Microchemistry Facility using previously described procedures (Aebi et al., 1987).

Isolation and Sequencing of an OSTI Genomic Clone

PCR (Saiki et al., 1988) was used to amplify DNA encoding a portion of a 30-residue internal tryptic peptide (SFYNTVGIPYEPEHVMSEQEQLL WETNRLPL) derived from the α subunit of the yeast oligosaccharyltransferase. Two degenerate oligonucleotide primers (5GTIGGNATHCCTIACCC and 5GCTCTAGAKRRTTGGTGYTCACC) were synthesized based on the underlined amino acid sequence. The eight nucleotides in bold-faced type in the antisense primer correspond to an XbaI site added as a linker sequence. PCR was performed in a 25-μl reaction volume with 125 pmol of each oligonucleotide primer, 0.4 U Taq DNA polymerase (Perkin Elmer Cetus Corp., Norwalk, CT) and 150 ng of S. cerevisiae genomic DNA. Yeast genomic DNA was to be used as a PCR template and for Southern analysis was isolated as described previously (Hoffman and Winston, 1987). To amplify the 77-bp DNA fragment, 25 cycles of denaturation (94°C, 1 min), annealing (40°C, 1 min), and extension (72°C, 1.5 min), were carried out in an automatic heating/cooling cycle (Programmable Thermal Controller, MJ Research). The PCR product was recovered from an 8% polyacrylamide gel and cloned using TA Cloning System (Invitrogen, San Diego, CA) for DNA sequencing and to prepare hybridization probes.

Approximately 20,000 colonies bearing recombinant plasmids from a S. cerevisiae genomic library in YEp31 were screened by in situ colony hybridization (Sambrook et al., 1989) with a random hexamer 32P-labeled hybridization probe prepared from the PCR product. Filters were hybridized overnight with the probe in 35% formamide, 5× SSC, 5× Denhardt’s solution, 100 μg/ml of denatured salmon sperm DNA, 0.1% SDS at 42°C, washed in 2× SSC, 0.1% SDS at 55°C, and exposed for 4 h at −80°C (Sambrook et al., 1989).

EcoRI-SphI and SphI-XbaI fragments from a hybridization-positive clone designated as pOSTI-1 were subcloned into pUC19 and MI3mp8. DNA sequencing of both strands was by the dideoxy chain termination method (Sanger et al., 1977). DNA sequence analysis and protein sequence comparisons were done with the MacVector (IBI) and DNA Star Acid Align (AANW) software programs.

Genomic Disruption of OSTI

The location of restriction sites and PCR primers used for construction of the plasmid pRS305R-L to disrupt the chromosomal OSTI locus using the transformation procedure (Sikorski and Hieter, 1985) are shown in Table I. A 547-bp Mscl-HindIII fragment from pOSTM was subcloned into Smal-HindIII digested pRS305 (Sikorski and Hieter, 1989), to generate pRS305R. A 320-bp HindIII-DraI fragment derived from pOSTH1 was generated by standard PCR methods using two primers (5’CGCAAGCTT-TAGCTCGGAACAAGACGCAAAC and 5’CTGACATTCCAACGTGC). The sense primer contained a HindIII site and was used to generate a HindIII site. The 508-bp PCR product was digested with HindIII and DraI to obtain a 320-bp fragment which was ligated into plasmid pRS305 that had been digested with XhoI, blunt-ended by filling in, and then digested with HindIII. The resulting construct (pRS305R-L) was linearized at the unique HindIII site that joins the OSTI derived sequences and used to disrupt the genomic OSTI locus in two diploid yeast strains (PRY'238; A 547-bp Mscl-HindIII fragment from pOSTM was subcloned into pUC19 and MI3mp8, M11; trp1-Δ101/ +, his3-A200/ +, +his3-Δ19 and YPH274; M11α, ura3-52/ura3-52, lys2-801/ +, ade2-101/ +, +his3-Δ19 and YPH274; M11α, ura3-52/ura3-52, lys2-801/ +, ade2-101/ +, +his3-Δ19). PRY238 was obtained from P. Robbins (Massachusetts Institute of Technology, Cambridge, MA) (Orlean et al., 1988). YPH274 (Sikorski and Hieter, 1989) was obtained from the Amer. Type Culture Collection (Rockville, MD). Standard laboratory media were used for yeast growth and sporulation (Sherman et al., 1986). Yeast transformations were performed by a modification (Kuo and Campbell, 1983) of the LiOAc transformation procedure (Ito et al., 1983). Staphylococcus aureus cells containing a transforming DNA fragment were used to transform yeast. Transformants were selected on YPD plates supplemented with 0.05 M LiOAc, 1 M glucose, and 100 μg/ml of 5-fluoroorotic acid (5-FOA). Transformants were sporulated, asci were dissected and analyzed for spore viability, and colony formation, and growth on synthetic complete media lacking leucine.
Isolation of Temperature-Sensitive \textit{S. cerevisiae} ostl Mutants

A 2.5-kb EcoRI-XbaI fragment from \textit{pOSTI} was subcloned into the yeast centromeric plasmids \textit{pRS316} and \textit{pRS317} (Sikorski and Boeke, 1991; Sikorski and Hieter, 1989), that were also digested with EcoRI and XbaI, to generate the plasmids designated \textit{pRS315-OST1} and \textit{pRS317-OST1}. The diploid RGY101 was transformed with \textit{pRS316-OST1}, and uracil prototrophs were selected, sporulated, and tetrads were dissected. Viable colonies were screened for growth on selective media and tested for mating type to obtain a haploid segregant with the genotype $\textit{MATa}$, \textit{ura3-52}, \textit{leu2-3,112}, \textit{lys2-801}, \textit{Δostl}:\textit{LEU2} \textit{(pRS316-OST1)} which we designate as RGY116. RGY116 was transformed with \textit{pRS317-OST1}, lysine prototrophs were selected, and subsequently cured of the \textit{pRS316-OST1} plasmid by growth on synthetic complete media plates containing 5-fluoro-orotic acid (Boeke et al., 1987) to generate the recipient strain RGY117 \textit{(MATa}, \textit{ura3-52}, \textit{leu2-3,112}, \textit{lys2-801}, \textit{Δostl}:\textit{LEU2} \textit{(pRS317-OST1)}) for a plasmid-shuffle mutagenesis procedure (Sikorski and Boeke, 1991).

The plasmid \textit{pRS316-OST1} was used as a template for PCR performed under conditions that favor misincorporation of deoxyribonucleotides by Taq polymerase (Leung et al., 1989). Oligonucleotide primers complementary to plasmid sequences flanking the gene were designed to generate mutagenized PCR products consisting of the 2.5-kb EcoRI-XbaI segment containing the \textit{OST1} gene flanked by 165 bp of 5' and 256 bp of 3' vector derived sequence. To enhance deoxyribonucleotide misincorporation, the concentration of one dNTP was reduced fivefold with respect to the other three dNTPs in each of four separate PCRs, in the presence of 1 mM Mg++ and 0.5 mM Mn++. Each 100 µl PCR contained 250 pmol of each primer, 100 ng of plasmid DNA and 1 U of Taq DNA polymerase. After 30 cycles of DNA amplification, the reactions were pooled and the 2,891-bp product was gel purified. The PCR product was reamplified under standard conditions (1.5 mM MgCl2, 0 mM MnCl2, equimolar dNTPs at 200 µM) until µg quantities of DNA were obtained. The resulting DNA fragments were cotransformed with EcoRI-XbaI digested \textit{pRS316} into the \textit{S. cerevisiae} strain RGY117 using a 10:1 ratio of PCR product to gapped plasmid. Transformants that repaired the gapped plasmid by homologous recombination (Ma et al., 1987) were selected at 25°C as \textit{Leu}'' \textit{ura}'' prototrophs. Transformants that could lose the plasmid \textit{pRS317-OST1} bearing the wild-type gene were selected by replica plating onto synthetic minimal media containing DL-α-aminoadipate (Sikorski and Boeke, 1991) while simultaneously selecting for temperature sensitivity by incubation of replica plates at 25°C and 37°C. From ~500 transformants, twelve \textit{Leu}'' \textit{ura}'' \textit{lys}'' colonies that could grow at 25°C but not at 37°C were isolated. Of these twelve colonies, eight were temperature-sensitive for growth when replica plated onto YPD plates. Plasmid DNA was prepared from the temperature-sensitive strains (Hoffman and Winston, 1987) and used to transform \textit{E. coli} for amplification. The resulting plasmids were used to retransform the strain RGY117 and the plasmid-shuffle procedure was repeated to confirm that the temperature-sensitive phenotype was plasmid linked.

Radiolabeling and Immunoprecipitation of Glycoproteins

Before radiolabeling, yeasts were grown for 20 h at 25°C in synthetic minimal media supplemented with the appropriate amino acids until mid-log phase (0.8-1.6 OD at 600 nm). Cultures shifted to 37°C were preincubated for the indicated time before labeling. Cells were collected by centrifugation and resuspended at 5 $\mu$g/ml in minimal medium prewarmed at the corresponding temperature. Cells were labeled for the indicated times with 50 µCi of [35S]methionine (New England Nuclear, Boston, MA) per \textit{A}600 units of cells. Labeling was terminated by the addition of Na2SO4 to 10 mM. To deplete lipid-linked oligosaccharides, tunicamycin was added at a concentration of 10 µg/ml, 15 min before radiolabeling as indicated. Rapid lysis of cells with glass beads and immunoprecipitation of radiolabeled proteins with antibodies to carboxypeptidase Y (CPY) and dipeptidyl aminopeptidase B (DPAP B) were performed as described previously (Rotblatt and Schekman, 1989). Immunoprecipitated proteins were incubated for 20 min at 65°C in SDS-sample buffer and resolved on 8% SDS-polyacrylamide gels.

Membrane Isolation and Oligosaccharyltransferase Activity

Microsomal membranes were isolated from wild-type and \textit{ost1} mutant yeast grown to mid log phase at 25°C in YPD medium using a scaled-down version of the procedure described previously (Kelleher and Gilmore, 1994). After centrifugation for 5 min at 2,000 g in a SS-34 rotor (Sorvall Instruments, Wilmington, DE) to remove broken cells and debris, the supernatant was collected, and cell membranes were pelleted by centrifugation at 120,000 g for 60 min in a Type 50 rotor (Beckman Instrs., Fullerton, CA). The membrane pellets were resuspended in 20 mM Tris-Cl pH 7.4, 1 mM DTT, 10% glycerol and protease inhibitor cocktail (Kelleher and Gilmore, 1994) and centrifuged for 60 min at 120,000 g. Microsomal membranes obtained after the second centrifugation were resuspended in the same buffer and stored at ~80°C. Oligosaccharyltransferase activity in digitonin extracts was assayed as described previously, using an iodinated tripeptide acceptor (Nα-Ac-Asn-[35S]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 Protein Assay (BioRad Labs., Hercules, CA).

Preparation of Antibodies to Ostlp

The expression plasmid pGEXT-Ost1 was constructed by cloning the 1,020-bp DraI-MscI fragment from \textit{pRS316-OST1} into the SmaI site of pGEX-2T (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). \textit{E. coli} TG-1 cells transformed with this plasmid express amino acid residues 93 to 432 as a fusion protein with glutathione-S-transferase (GST). A culture of \textit{E. coli} (100 ml with an \textit{A}600 of 0.7-0.8) was induced to express the GST-Ostlp fusion during a 2-h incubation with 0.5 mM IPTG at 37°C. Inclusion bodies containing the fusion protein were isolated from the cells essentially as described (Sambrook et al., 1989). The washed inclusion bodies containing the fusion protein were solubilized in the presence of urea (SchloB et al., 1988) and purified using glutathione Sepharose 4B beads (Pharmacia LKB Biotechnology) as described (Smith and Johnson, 1988). The purified GST-Ost1 fusion protein was used to immunize rabbits at East Acres Biologicals.

Approximately 3.7 mg of the GST-Ost1 fusion protein was solubilized with 250 µl of 50 mM TEA, pH 7.5 containing 1% SDS. The sample was adjusted to 2.5 µl with 50 mM TEA, pH 7.5, before coupling to Affi-Gel-15 (BioRad Labs.) after the manufacturer's instructions to prepare an affinity column for immunoselection of antibodies to Ostlp. Ostlp specific antibodies were eluted from the Affi-Gel-15 affinity matrix using 100 µM triethylamine (pH 11.5) according to Harlow and Lane (1988).

Protein Immunoblots and Endoglycosidase H Digests

Proteins resolved by polyacrylamide gel electrophoresis in SDS were transferred to PVDF membranes. The membrane blots were probed with antisera that recognize Ostlp, Wbp1p, or Kar2p. Peroxidase-labeled second antibodies were visualized using enhanced chemiluminescence (ECL Western blotting detection kit, Amersham Corp., Arlington Heights, IL). Endoglycosidase H was purchased from New England Biolabs (Beverly, MA); digestions were performed following the manufacturer's recommendations.

Results

Isolation and Sequencing of the OST1 Gene

The α subunit of the yeast oligosaccharyltransferase is resolved into two major forms (62 and 64 kD) and one minor form (60 kD) by SDS gel electrophoresis (Kelleher and Gilmore, 1994). Amino terminal sequencing of the 62- and 64-kD polypeptides yielded a common sequence consistent with previous evidence indicating that these polypeptides are alternative glycoforms of a single protein (Kelleher and Gilmore, 1994). Two tryptic peptides derived from the 64-kD form were also sequenced. Coincidently, the sequence of one tryptic peptide (AQYPEPATWENVDYKR) was identical to the mature amino terminal sequence. Two degenerate oligonucleotide primers were synthesized based on the sequence of a 30-residue tryptic peptide and were used to amplify a yeast genomic DNA template using PCR. DNA sequencing of a PCR product of the predicted size (77 bp)
Figure 1. Restriction endonuclease map, DNA sequence, and gene disruption of the OST1 locus. (A) The OST1 gene is located on the right arm of chromosome X adjacent to PRE3 (gb X78991) and CEN10. The OST1 locus was disrupted by replacement of the Dral–MscI DNA fragment with the yeast integrating plasmid pRS305 bearing the LEU2 gene. The locations of two PCR primers used to amplify a portion of the OST1 locus for construction of the gene disruption plasmid are designated by the arrows flanking the Drai site. Restriction sites used for constructions and mapping of the gene disruption are shown. (B) The nucleotide sequence of an EcoRI–XbaI genomic DNA segment containing the OST1 gene is shown together with the predicted amino acid sequence of Ostlp. Nucleotide residues are numbered on the right starting in the EcoRI site; amino acid residues are numbered on the left. The termination codon is indicated by an asterisk. Two tryptic peptides that were sequenced are underlined. The signal peptidase cleavage site is indicated by an arrow. The dashed line beneath residues 450–467 designates a predicted membrane-spanning segment detected by hydrophathy analysis (Kyte and Doolittle, 1982). Four consensus sites for N-linked glycosylation are enclosed in boxes. These sequence data are available from the EMBL/GenBank/DDBJ under accession number Z6719.
confirmed the isolation of an authentic amplification product of the \textit{OSTI} gene.

The \textit{OSTI} gene was isolated from a yeast genomic library in the YEpl3 vector by colony hybridization using the radiolabeled PCR product as a probe. Sequence analysis of a 2.5-kb EcoRI-XbaI restriction fragment from a hybridization-positive plasmid revealed an open reading frame encoding a protein of 476 amino acids (Ostlp) as well as 5' and 3' flanking sequences (Fig. 1B). The predicted protein sequence contains perfect matches for both tryptic peptides derived from the \alpha subunit of the yeast oligosaccharyltransferase. Hydropathy analysis revealed the presence of two hydrophobic protein segments near the extreme amino and carboxyl termini of Ostlp (Kye and Doolittle, 1982). The amino-terminal hydrophobic segment resembles a cleavable signal sequence for initiating translocation across the endoplasmic reticulum (von Heijne, 1986). The predicted signal peptidase processing site (von Heijne, 1986) is located between residues Ala 22 and Ala 23 consistent with the amino terminal sequence data described above. The second hydrophobic segment located between residues 450 and 467 is predicted to function as a membrane-spanning segment. The arrangement of these two hydrophobic segments suggest that Ostlp is a type I integral membrane protein with the majority of the polypeptide located within the lumen of the endoplasmic reticulum, and with residues 468 to 476 located in the cytoplasm. Four consensus sites for asparagine-linked glycosylation are present in the mature sequence of Ostlp, consistent with endoglycosidase H digestion data showing that the two major glycoforms of Ostlp contain four and three N-linked oligosaccharides, respectively (Kelleher and Gilmore, 1994). Since asparaginyl-linked glycosylation only occurs on lumenally disposed consensus sequons, the presence of four such sites in the deduced protein sequence supports the predicted topology for Ostlp. The calculated molecular weight of 51,448 for mature Ostlp is in reasonable agreement with the \textit{M}. of 54 kD observed for the endoglycosidase H-digested \alpha subunit of the oligosaccharyltransferase on SDS-polyacrylamide gels (Kelleher and Gilmore, 1994).

A search of DNA sequence databases using the BLASTN DNA sequence comparison algorithm (Altschul et al., 1990) revealed that the first 635 nucleotides of the \textit{OSTI} sequence shown here are 96% identical to the 640 nucleotides from the 5' flanking sequence of the yeast \textit{PRE3} gene (Enenkel et al., 1994). Although several discrepancies between the sequence reported here and the 5' flanking sequence of the \textit{PRE3} gene may represent strain differences, others appear to be errors in the latter sequence. The restriction endonuclease map and the chromosomal location of the \textit{OSTI} gene is shown in Fig. 1A. DNA sequence alignment maps the \textit{PRE3} gene 370 bp downstream from \textit{CEN10} (Enenkel et al., 1994). Hence, the ATG codon of the \textit{OSTI} gene is located on the right arm of chromosome X, \sim1.5 kb downstream from the centromere.

\textit{Ostlp Is Homologous to the Mammalian Ribophorin I}

A search of protein sequence databases using the BLASTP protein sequence comparison algorithm (Altschul et al., 1990) disclosed a homology between Ostlp and ribophorin I (Fig. 2). The sequence of ribophorin I has been deduced from the rat and human cDNA clones (Crimaudo et al., 1987; Harnik-Ort et al., 1987). Because the two mammalian ribophorin I sequences are 97% identical, only the human sequence is shown here. Ribophorin I is an integral membrane glycoprotein of the rough endoplasmic reticulum (Kreibich et al., 1978; Marcantonio et al., 1982) that was recently identified as a subunit of the mammalian (Kelleher et al., 1992) and avian (Kumar et al., 1994) oligosaccharyltransferase. Protein sequence analysis, protease accessibility studies, and endoglycosidase H digestions have revealed that ribophorin I is a type I integral membrane glycoprotein with a large amino-terminal luminal domain, a single membrane-spanning segment, and a 150-residue carboxy-terminal cytoplasmic domain (Crimaudo et al., 1987; Harnik-Ort et al., 1987; Marcantonio et al., 1982). The sequence similarity between Ostlp and human ribophorin I extends throughout the luminal domains of both proteins (Fig. 2). Within the overlapping region of 474 amino acids, the overall sequence identity between the two proteins is 28%, whereas sequence similarity was estimated to be 58%. The consensus sites for asparagine-linked glycosylation are not conserved. The most striking difference between human ribophorin I and Ostlp is the abbreviated cytoplasmic domain in the yeast protein relative to mammalian ribophorin I. Recently, a relationship between ribophorin I and the 60/62-kD subunit of the oligosaccharyltransferase was proposed (Knauer and Lehle, 1994) based upon a six-amino acid alignment, after introduction of one gap, between human ribophorin I and the amino terminal 12 residues of the yeast 60/62-kD subunit.

\textit{OSTI Is Essential for Vegetative Growth of Yeast}

To determine whether \textit{OSTI} is required for cell viability, the \textit{OSTI} locus in the diploid yeast strain PRY238 was disrupted using a \gamma transformation procedure (Sikorski and Hieter, 1989). A 1,020-bp segment between the DraI and MscI restriction sites in the \textit{OSTI} gene was replaced with the yeast integrating plasmid pRS305 bearing the \textit{LEU2} gene (Fig. 1A), thereby removing codons 93-432 from the \textit{OSTI} gene. Leucine prototrophs were selected and correct integration of pRS305 into the \textit{OSTI} locus was confirmed by Southern blots using a combination of restriction sites in pRS305 and the DNA sequences flanking the \textit{OSTI} gene (data not shown). Diploid strains heterozygous for the \textit{OSTI} gene disruption (e.g., RGY101) were sporulated and the tetrads dissected. For each tetrad dissected (Table I), a maximum of two viable colonies were produced, both of which were leucine auxotrophs. Identical results were obtained when spores were allowed to germinate at 25°C. Spores bearing the \textit{OSTI} gene disruption germinated and formed microcolonies of 4-8 cells (data not shown), indicating that the Ostl protein is essential for the vegetative growth of yeast. Similar results were obtained when the \textit{OSTI} gene was disrupted in the diploid strain YPH274 (data not shown). The lethal phenotype of an \textit{OSTI} disruption could be rescued by transformation of RGY101 with a centromeric plasmid bearing an intact copy of the \textit{OSTI} gene (Table I). Viable Leu+, Ura+ colonies were obtained upon sporulation and dissection of tetrads from RGY116.

\textit{Isolation of Temperature-Sensitive ostl Mutants}

Conditional \textit{ostl} mutants were generated to determine whether the essential in vivo function of the Ostl protein was
directly related to asparagine-linked glycosylation of proteins. A haploid yeast strain bearing a chromosomal disruption of the OST gene complemented by a plasmid borne copy of the wild-type OST gene served as a recipient for a plasmid shuffle procedure (Sikorski and Boeke, 1991) wherein the plasmid bearing the wild-type OST gene was replaced by a plasmid bearing a mutagenized OST gene. The OST gene was mutagenized by PCR amplification under conditions which enhance misincorporation of deoxyribonucleotides (Leung et al., 1989). Eight independent colonies were isolated that could grow at 25°C, but not at 37°C, and four of these were selected for further analysis. At the non-permissive temperature, the four ostl mutants arrested growth after 1-4 cell divisions (Fig. 3), unlike the strain bearing the wild-type control plasmid (RGY116). In liquid media at 25°C, doubling times similar to RGY116 were observed for ostl-1 and ostl-2, whereas mutants ostl-3 and ostl-4 showed reduced growth rates at the permissive temperature (see legend to Fig. 3). Additionally, the ostl-3 and ostl-4 mutants form visible aggregates when grown in liquid culture at 25°C. Phase contrast microscopy indicates that cultures of ostl-4 contain large clusters of 10-30 cells. Golgi defects that interfere with elongation of N-linked oligosaccharides (i.e., the mnn mutants) also cause this clumping phenotype, apparently due to defective separation of daughter and mother cells (Ballou et al., 1980).

### Table I. Tetrad Analysis of OST1 Gene Disruption

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype and/or plasmid</th>
<th>Tetrads analyzed</th>
<th>Viable colonies per tetrad</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRY238</td>
<td>OST1::OST1</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>RGY101</td>
<td>OST1::ost1::LEU2</td>
<td>24</td>
<td>2†</td>
</tr>
<tr>
<td>RGY103</td>
<td>OST1::ost1::LEU2 [pRS316]</td>
<td>4</td>
<td>2†</td>
</tr>
<tr>
<td>RGY116</td>
<td>OST1::ost1::LEU2 [pPRS316-OST1]</td>
<td>25</td>
<td>3-4†</td>
</tr>
</tbody>
</table>

* Diploid strains were sporulated, tetrads dissected on YPD plates, and incubated for 4 days at 30°C. Colonies obtained were replica plated on selective medium to determine nutritional markers. PRY238 was the recipient for the OST1 disruption to produce RGY101. The diploid RGY101 was transformed with the yeast centromeric vector pRS316 (URAS3, CEN6/ARS4) that contained or lacked the OST1 gene to obtain RGY103 and RGY116.

ostl Mutants Are Defective in Asparagine-linked Glycosylation In Vivo

In vivo synthesis of two yeast vacuolar glycoproteins, CPY and DPAP B, was examined in the four temperature-sensitive strains to determine whether the ostl mutants exhibit defects in asparagine-linked glycosylation. The soluble vacuolar protease CPY is synthesized as a proenzyme that acquires
Figure 3. Growth of the ostl mutants at the restrictive temperature. Wild-type (RGY116) and ostl mutant strains were grown in YPD at 25°C until early logarithmic phase. At time 0, cultures were shifted to 37°C. As needed, cultures were diluted into fresh 37°C media to maintain an 

\[ \text{A}_{600} \] of less than 0.8. The normalized increase in culture density (\( \text{A}_{600} \)) is shown for wild-type ( ), ostl-1 ( ), ostl-2 ( ), ostl-3 ( ), ostl-4 ( ) yeast strains. Doubling times in YPD at 25°C were as follows: wild-type (3.3 h), ostl-1 (3.3 h), ostl-2 (3.6 h), ostl-3 (4.6 h), and ostl-4 (7.1 h).

Figure 4. The ostl mutants are defective in core oligosaccharide transfer to CPY and DPAP B in vivo. Wild-type (RGY116) and ostl mutant cells grown in minimal media were maintained at 25°C or shifted to 37°C for 2 h before labeling with [35S]methionine. As indicated, wild-type cells were incubated for 15 min with tunicamycin before labeling. (A) CPY immunoprecipitates from glass-bead extracts of cells labeled for 1 h with [35S]methionine were resolved by PAGE in SDS. Fully glycosylated vacuolar CPY and unglycosylated vacuolar CPY (ug CPY) are designated by arrows. Underglycosylated variants of CPY lacking between 1 and 4 asparagine-linked oligosaccharides are indicated by labeled arrows on the right side of the panel. (B) DPAP B immunoprecipitates from glass-bead extracts of cells labeled for 10 min with [35S]methionine were resolved by PAGE in SDS. The migration position of the fully glycosylated and unglycosylated (ug DPAP B) forms of DPAP B are indicated by arrows. Underglycosylated forms of DPAP B show intermediate migration rates. Incomplete depletion of lipid-linked oligosaccharides by tunicamycin treatment at 25°C is responsible for the residual glycosylated DPAP B observed in the second lane.

four core oligosaccharides in the endoplasmic reticulum (Stevens et al., 1982). The 67-kD pl form of proCPY is transported to the Golgi complex, where the core oligosaccharides are elongated by the addition of mannose residues to yield the 69-kD p2 form of proCPY. Upon arrival at the vacuole, the propeptide sequence is proteolytically removed to generate the mature 61-kD form of CPY. CPY is an ideal model glycoprotein for this analysis because intracellular transport of CPY is not completely inhibited when assembly of the lipid-linked oligosaccharide donor is blocked by tunicamycin treatment, indicating that defects in glycosylation should not prevent identification of CPY biosynthetic intermediates (Stevens et al., 1982; Winther et al., 1991).

CPY was immunoprecipitated from wild-type and ostl mutant yeast after radiolabeling for 1 h at 25°C or 37°C. As expected, the mature 61-kD vacuolar CPY was the predominant form produced by wild-type yeast at both temperatures (Fig. 4 A). A 51-kD polypeptide, that corresponds to unglycosylated vacuolar CPY, was detected when wild-type cells were treated with tunicamycin. The 59-kD polypeptide designated by the vertical arrow in the lanes derived from tunicamycin-treated yeast is an ER-arrested form of proCPY (Stevens et al., 1982). Transport of unglycosylated proCPY to the vacuole was reduced at 37°C, as observed by previous investigators (Winther et al., 1991). Multiple radiolabeled polypeptides were synthesized by each of the ostl mutants.
at both the permissive and restrictive temperatures (Fig. 4A). Based upon the number of evenly spaced bands that migrate between CPY from untreated and tunicamycin-treated wild-type yeast, we can conclude that the CPY variants labeled in the ostl mutant yeast contain between 0 and 4 N-linked oligosaccharides. Endoglycosidase H digestion of CPY immunoprecipitates from wild-type and ostl mutants yielded a major 51-kD product in each case, confirming that the primary difference between the CPY variants was the number of N-linked oligosaccharides (data not shown). Although low amounts of the p1 and p2 forms of proCPY were observed in immunoprecipitates from the ostl-4 mutant after labeling at 25°C (Fig. 4A), endoglycosidase H digestions showed that ostl-4 cells do not accumulate unglycosylated 59 kD proCPY. The severity of the in vivo glycosylation defect at 25°C correlated with the growth defect displayed by the ostl mutants at 25°C; the mutants designated ostl-3 and ostl-4 showed the most dramatic pattern of underglycosylation of CPY. Likewise, the severity of the in vivo glycosylation defect at both temperatures correlated with the severity of the growth defect after cultures were shifted to 37°C.

In vivo glycosylation of the vacuolar membrane protein DPAP B by the wild-type and ostl mutant cells was also evaluated (Fig. 4B). The antisera used for immunoprecipitation of DPAP B was raised against recombinant DPAP B expressed in E. coli, hence, the antibody recognizes protein rather than carbohydrate epitopes. In a previous study using this antisera, DPAP B was shown to be a type II integral membrane glycoprotein with eight consensus sites for N-linked glycosylation, six to seven of which, on average, are glycosylated in vivo (Roberts et al., 1989). The core glycosylated 110-113 kD ER form of DPAP B is converted to a mature 120-kD glycoprotein by further carbohydrate addition in the Golgi complex (Roberts et al., 1989). Unlike CPY, DPAP B does not undergo a proteolytic maturation reaction upon transport to the vacuole. The fully glycosylated DPAP B synthesized by wild-type cells during a 10-min labeling period at 25°C or 37°C (Fig. 4B) corresponds to the Golgi form of DPAP B based upon pulse-chase studies reported by Roberts et al. (1989). A more rapidly migrating unglycosylated form of DPAP B (ug DPAP B) was detected in wild-type cells labeled in the presence of tunicamycin. DPAP B species that displayed intermediate mobilities, corresponding to different glycoforms of the protein, were synthesized by the ostl mutants at both the permissive and restrictive temperatures. As observed for CPY, the ostl mutants with the most severe growth defects displayed more extensive underglycosylation of DPAP B. Although the individual glycoforms of DPAP B were not well resolved, the protein synthesized by the ostl-4 mutant at 25°C appears to contain, at the most, two N-linked oligosaccharides. Thus, underglycosylation of DPAP B appears to be more severe than underglycosylation of CPY at the permissive temperature. Surprisingly, DPAP B synthesized by the ostl mutants had, on average, more oligosaccharides at the restrictive temperature than at the permissive temperature.

The preceding results show that all four ostl mutants are defective in asparagine-linked glycosylation at both the restrictive and permissive temperatures. Yet, while relatively modest defects in glycosylation are lethal when cultures are shifted to 37°C (e.g., ostl-1 and ostl-2), more severe glycosylation defects (e.g., ostl-3 and ostl-4) are tolerated at 25°C.
cells contained ~70% of the activity observed for extracts from wild-type cells, whereas only 25% of the wild-type activity was detected in extracts from ostl-4 cells. In vivo use of the four glycosylation sequons in CPY at 25°C was quantified by scanning the autoradiogram shown in Fig. 4 A, and these data are expressed as the CPY glycosylation index (Fig. 6, striped bars). For each of the four mutants, the reduction in oligosaccharyltransferase activity correlated reasonably well with the CPY glycosylation index.

Expression of Ostlp, Wbplp, and Kar2p in the ostl Mutant Yeast

The membrane preparations that were assayed for oligosaccharyltransferase activity (Fig. 6) were subjected to protein immunoblot analysis using affinity-purified antibodies to Ostlp to determine whether the expression of Ostlp was altered in the ostl mutants (Fig. 7 A). Polyclonal rabbit antibodies against Ostlp using a fusion protein consisting of codons 93-432 from Ostlp fused to glutathione-S-transferase. The mobility of Ostlp on SDS-polyacrylamide gels was altered in several of the ostl mutants as shown most readily by a comparison of the endoglycosidase H-digested samples (Fig. 7 A). Deglycosylated Ostlp from wild-type cells has a MW of 54 kD, while Ostlp from the mutants had apparent MW of 55 kD (ostl-1), 54 kD (ostl-2), 52 kD (ostl-3), and 53 kD (ostl-4). The alterations in the gel mobility of the mutant proteins are probably not caused by carboxy terminal extensions or truncations. Point mutations that introduce or eliminate charged amino acid residues can influence gel mobility by altering the quantity of protein-bound SDS. However, we have not sequenced the mutant alleles of ostl, so the origin of the altered gel mobility of Ostlp remains undefined. Ostlp from wild-type yeast migrates as a closely spaced glycoform doublet (Fig. 7 A). As shown previously by analysis of endoglycosidase H digestion intermediates, the 64-kD and 62-kD glycoforms of Ostlp contain four and three N-linked oligosaccharides (Kelleher and Gilmore, 1994). In contrast, a single predominant glycoform of Ostlp is present in membranes from the ostl-1, ostl-2, and ostl-3 mutants. Examination of darker exposures of the protein immunoblot revealed the presence of four additional, more rapidly migrating forms of Ostlp in membranes isolated from ostl-2 and ostl-3 cells indicating that the predominant glycoform of Ostlp in these membranes contains four asparagine-linked oligosaccharides. Hence, Ostlp is hyperglycosylated in several ostl mutants relative to the wild-type control yeast. Additional experiments will be required to determine whether Ostlp synthesized by ostl-1 cells contains three or four N-linked oligosaccharides. In contrast, membranes from ostl-4 cells contain underglycosylated forms of Ostlp that contain between 1 and 4 N-linked oligosaccharides. In contrast, membranes from ostl-4 cells contain underglycosylated forms of Ostlp that contain between 1 and 4 N-linked oligosaccharides. In contrast, membranes from ostl-4 cells contain underglycosylated forms of Ostlp that contain between 1 and 4 N-linked oligosaccharides.
Figure 8. Induction of Kar2p expression in ostl mutants at the permissive temperature. Membranes isolated from wild-type or ostl mutant yeast were resolved on a polyacrylamide gel in SDS, transferred to a PVDF membrane, and probed with a rabbit polyclonal antibody to the Kar2 protein. Immunoreactive proteins were detected as described in the Materials and Methods. Each gel lane contained 12.5 μg of membrane protein.

Discussion

Here, we have described the molecular cloning of OST1, the S. cerevisiae gene that encodes the 62/64-kD α subunit of the yeast oligosaccharyltransferase. The OST1 gene was found to encode a 476-residue protein that shares significant sequence identity with ribophorin I, the 66-kD subunit of the mammalian oligosaccharyltransferase. Previous sequence comparisons had revealed that the Wbplp and the Swplp of S. cerevisiae are homologous to the OST48 and ribophorin II subunits of the canine oligosaccharyltransferase, respectively (Kelleher and Gilmore, 1994; Silberstein et al., 1992). Of the three yeast subunits sequenced to date, the Ostl protein has the highest degree of sequence identity to the corresponding mammalian subunit. Although structural similarities between the heterotrimeric canine oligosaccharyltransferase and the hexameric yeast oligosaccharyltransferase have been revealed by sequence comparisons, several noteworthy differences are also evident. Of the three homologue pairs so far identified, only the Wbp1p-OST48 pair shows conservation of both sequence and protein size. Strikingly, ribophorin I contains a 150-residue cytoplasmic domain instead of the abbreviated cytoplasmic domain predicted for Ostl. Although proteolytic digestion of the cytoplasmic domain of canine ribophorin I does not alter in vitro oligosaccharyltransferase activity (Kelleher and Gilmore, unpublished observations), a truncated form of ribophorin I that lacks the cytoplasmic domain was observed to be rapidly degraded in vivo (Tsao et al., 1992). Thus, this portion of ribophorin I may serve a function in vertebrate organisms that is dispensable in yeast, or is instead provided by one of the remaining uncharacterized subunits of the yeast oligosaccharyltransferase (γ, ε, or θ).

As anticipated based upon its postulated function as a subunit of the oligosaccharyltransferase, expression of Ostl was found to be essential for vegetative growth of yeast. The two genes (WBP1 and SWP1) that encode the previously characterized subunits of the oligosaccharyltransferase are also essential for viability (te Heesen et al., 1992, 1993). Likewise, tunicamycin inhibition of lipid-linked oligosaccharide assembly is lethal in yeast (Barnes et al., 1984), as are mutations in the ALGI and ALG2 genes that catalyze early steps in assembly of the lipid-linked oligosaccharide (Huffaker and Robbins, 1982; Jackson et al., 1993).

Yeast bearing mutations in the OST1 gene were unable to grow at elevated temperatures and were defective in glycosylation of both soluble and membrane glycoproteins. Underglycosylation of newly synthesized proteins by the ostl mutants can be directly attributed to a defect in the oligosaccharyltransferase based upon the observed reduction in the glycosylation of an acceptor tripeptide by detergent extracts prepared from the ostl cells. These experiments provide in vivo confirmation for the biochemical identification of Ostl as a subunit of the oligosaccharyltransferase (Kelleher and Gilmore, 1994). By extension, this study provides further support for the proposed role of ribophorin I as a subunit of the mammalian oligosaccharyltransferase (Kelleher et al., 1992) based upon the clearly defined homology between ribophorin I and Ostl.

Notably, all four ostl mutants that were examined displayed defects in glycosylation at both the permissive and restrictive temperatures. Similar results were reported previously for the conditional wbpl mutants (te Heesen et al., 1992). Different explanations for the more pronounced growth defect observed at the elevated temperature should be considered. In the case of the ostl-2 mutant, the glycosylation defect became considerably more severe at later time points after a shift to the restrictive temperature. This gradual decline in oligosaccharyltransferase function at the restrictive temperature would not be consistent with a tem-

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perature-sensitive lesion in enzyme activity, but could be explained by reduced stability of newly synthesized Ostl at the elevated temperature. A second explanation for the more pronounced growth defect at the restrictive temperature is provided by considering the role of N-linked oligosaccharides in protein folding. Inhibition of asparagine-linked glycosylation interferes with folding and intracellular transport of many, but not all, glycoproteins (Helenius, 1994). Malfolded proteins that accumulate upon tunicamycin treatment often contain aberrant disulfides, can become stably associated with the luminal ER chaperone BiP (Kar2p), and are eventually degraded in the endoplasmic reticulum (Helenius, 1994). Protein folding is temperature dependent, hence, underglycosylation of proteins leads to more pronounced folding defects at higher temperatures. For example, in tunicamycin-treated cells the unglycosylated G protein of vesicular stomatitis virus accumulates in a Triton X-100 insoluble aggregate at 37°C, but not at 30°C (Gibson et al., 1979). Extended incubation of ostl cells at the restrictive temperature should result in the accumulation of multiple defective proteins, at least some of which will be required for cell viability.

Expression of the Kar2 protein was induced in the ostl mutants at the permissive temperature, consistent with the synthesis of folding-impaired proteins by the ostl mutants. Previous studies have shown that Kar2 mRNA expression is induced by tunicamycin or 2-deoxy-glucose treatment of wild-type yeast (Rose et al., 1989), or by shifting sec53 mutant strains of yeast to the restrictive temperature, thereby causing a block in assembly of the lipid-linked oligosaccharide (Rose et al., 1989). Consistent with this observation, kar2 wbpl double mutants show markedly reduced growth rates at the permissive temperature due to a synthetic interaction between these two gene products (te Heesen and Aebi, 1994).

Each of three glycoproteins examined (CPY, DPAP B and Wbplp) was underglycosylated by the ostl mutants. Glycosylation of the two integral membrane proteins appeared to be more severely reduced than glycosylation of CPY by mutations in Ostl at 25°C. Enhanced glycosylation of DPAP B at the restrictive temperature by the ostl mutants may be indicative of prolonged exposure of unfolded forms of DPAP B to the oligosaccharyltransferase. Hence, we speculate that folding of DPAP B is temperature-sensitive, and may be contingent upon acquisition of a minimum number of N-linked oligosaccharides at the restrictive temperature.

The protein immunoblot experiments suggest that the glycosylation defect in the two most severely impaired ostl mutants arise by diverse mechanisms. The reduced membrane content of Ostlp in membranes prepared from ostl-3 cells correlated with the reduction in the in vitro oligosaccharyltransferase activity. The protein immunoblot experiments also showed hyperglycosylation of the α subunit (Ostlp) relative to that observed in wild-type yeast in two of four mutants analyzed (ostl-2 and ostl-3). Malformed proteins can be modified at glycosylation sequences that are not used, or are under-used in the wild-type protein resulting in hyperglycosylation of a folding-impaired protein (Bulleid et al., 1992; McGinnies and Morrison, 1994). We postulate that the basis for the reduced oligosaccharyltransferase activity in ostl-3 is a defect in the assembly of the mutant protein into the oligosaccharyltransferase. Interestingly, the membrane content of the Wbplp subunit of the oligosaccharyltransferase was only slightly reduced in membranes prepared from ostl-3 cells. Thus, the endoplasmic reticulum of ostl-3 cells may contain both fully assembled active oligosaccharyltransferase complexes and inactive complexes that lack the α subunit. In contrast, the Ostl protein synthesized by the ostl-4 mutant is underglycosylated, yet apparently stable. The latter observation would be expected if oligomeric assembly of the α subunit in the ostl-4 cell results in a stable, yet catalytically impaired oligosaccharyltransferase.

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