The Essential OST2 Gene Encodes the 16-kD Subunit of the Yeast Oligosaccharyltransferase, a Highly Conserved Protein Expressed in Diverse Eukaryotic Organisms

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Abstract. Oligosaccharyltransferase catalyzes the transfer of a preassembled high mannose oligosaccharide from a dolichol-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 non-identical subunits (α-ζ). The α, β, γ, and δ subunits of the oligosaccharyltransferase are encoded by the *OST1*, *WBP1*, *OST3*, and *SWP1* genes, respectively. Here we describe the functional characterization of the *OST2* gene that encodes the ε-subunit of the oligosaccharyltransferase. Genomic disruption of the *OST2* locus was lethal in haploid yeast showing that expression of the Ost2 protein is essential for viability. Overexpression of the Ost2 protein suppresses the temperature-sensitive phenotype of the *wbp1-2* allele and increases in vivo and in vitro oligosaccharyltransferase activity in a *wbp1-2* strain. An analysis of a series of conditional *ost2* mutants demonstrated that defects in the Ost2 protein cause pleiotropic underglycosylation of soluble and membrane-bound glycoproteins. Microsomal membranes isolated from *ost2* 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. Surprisingly, the Ost2 protein was found to be 40% identical to the DAD1 protein (defender against apoptotic cell death), a highly conserved protein initially identified in vertebrate organisms. The protein sequence of *ost2* mutant alleles revealed mutations at highly conserved residues in the Ost2p/DAD1 protein sequence.

Asparagine-linked glycosylation of proteins is a highly conserved protein modification reaction that occurs in the lumen of the rough endoplasmic reticulum in all eukaryotic organisms (Kornfeld and Kornfeld, 1985; Herscovics and Orlean, 1993). The initial stage in the biosynthesis of N-glycosylated proteins, catalyzed by the lumenerally oriented enzyme oligosaccharyltransferase, involves the transfer of a preassembled high-mannose oligosaccharide (Glc3Man9GlcNAc2) from a dolichol-phosphosphate 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). N-linked glycosylation is an obligatory event for the efficient folding and oligomeric assembly of many nascent polypeptides in the endoplasmic reticulum. Inhibition of N-linked glycosylation causes the accumulation of malformed proteins that can become stably associated with endoplasmic reticulum chaperones and are then subjected to degradation as part of a quality control mechanism (Hele-nius, 1994). Oligosaccharides facilitate efficient transport of certain glycoproteins through the secretory pathway (Guan et al., 1985; Riederer and Hinnen, 1991; Winther et al., 1991).

As the donor and acceptor substrates for oligosaccharyltransferase have been extensively conserved during evolution, structurally related enzymes are predicted to catalyze N-linked glycosylation of proteins in diverse eukaryotes. The oligosaccharyltransferase has now been purified from vertebrate (Kelleher et al., 1992; Kumar et al., 1994) and fungal (Kelleher and Gilmore, 1994; Knauer and Lehle, 1994; Pathak et al., 1995a) organisms. The yeast oligosaccharyltransferase was initially purified as a complex of six subunits (α-ζ) with the following molecular weights: α: 62,000/64,000; β: 48,000; γ: 34,000; δ: 30,000; ε: 16,000 and ζ: 9,000 (Kelleher and Gilmore, 1994). However, two subsequent reports describe the purification of catalytically...
active yeast OST as a heterotetramer that lacks both the ε and ζ subunits (Knauer and Lehle, 1994; Pathak et al., 1995a). The α-subunit, designated Ostlp, is 28% identical in sequence to the ribophorin I subunit of the vertebrate oligosaccharyltransferase complex (Pathak et al., 1995b; Silberstein et al., 1995). Expression of the Ostlp protein is essential for vegetative growth of haploid yeast (Silberstein et al., 1995). Analysis of conditional ost1 mutants demonstrated that Ostlp is required for N-linked glycosylation of proteins in vivo and for oligosaccharide transfer to acceptor peptides in vitro (Silberstein et al., 1995). The β-subunit of the yeast OST is the Wbpl protein (Kelleher and Gilmore, 1994; Knauer and Lehle, 1994; Pathak et al., 1995a), a previously identified integral membrane protein of Saccharomyces cerevisiae (te Heesen et al., 1991). Phenotypic analysis of a yeast strain bearing a mutant allele of the essential WBP1 gene has shown that the Wbpl protein is required for in vivo and in vitro N-linked glycosylation of proteins (te Heesen et al., 1992). The Wbpl protein is 25% identical in sequence to the OST48 subunit of the canine oligosaccharyltransferase (Silberstein et al., 1992). The 30-kD β-subunit of the yeast oligosaccharyltransferase corresponds to the Swplp protein (Kelleher and Gilmore, 1994; Knauer and Lehle, 1994; Pathak et al., 1995a). The WBP1 locus was isolated as an allele-specific high-copy suppressor of the wbpl-2 mutant (te Heesen et al., 1993), and Swplp forms complexes with Wbplp in the yeast endoplasmic reticulum (te Heesen et al., 1993). A protein sequence comparison revealed that the Swplp protein is related to the carboxy terminal half of the 62-kD ribophorin II subunit of mammalian oligosaccharyltransferase (Kelleher and Gilmore, 1994). The 34-kD γ-subunit is encoded by the nonessential OST3 gene (Karaoglu et al., 1995). Interestingly, disruption of the OST3 gene causes biased underglycosylation of a subset of yeast glycoproteins (Karaoglu et al., 1995). Mammalian homologues of the Ost3 protein have not been identified.

Now that three of the yeast OST subunits (Ostlp, Wbplp, and Swplp) have been shown to be structurally and functionally related to three subunits from the mammalian oligosaccharyltransferase, the possible function and structural conservation of the remaining yeast subunits must be addressed. Simultaneous overexpression of Ostlp, Wbplp, and Swplp in yeast does not result in increased oligosaccharyltransferase activity (Pathak et al., 1995a), implying that at least one additional polypeptide is required for expression of oligosaccharyltransferase activity. Given that two groups have reported that the S. cerevisiae oligosaccharyltransferase can be purified as an enzymatically active heterotetramer that lacks both the ε and ζ subunits (Knauer and Lehle, 1994; Pathak et al., 1995a), it might be questioned whether the latter proteins are components of the catalytic core of the enzyme. Here, we report the isolation and characterization of a yeast gene (OST2) that encodes the ε-subunit of the yeast oligosaccharyltransferase. The Ost2 protein is shown to be essential for in vivo and in vitro oligosaccharyltransferase activity and was found to be closely related to the vertebrate DAD1 protein.

Materials and Methods

Isolation and Sequencing of an OST2 Genomic Clone

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 Poncova S. NH2-terminal protein sequencing, in situ proteolysis of the 16-kD subunit, peptide purification by narrow-bore reverse phase HPLC, and sequencing of two internal tryptic peptides from the 16-kD subunit were performed by the Worcester Foundation for Experimental Biology Protein Chemistry Facility.

PCR (Saiki et al., 1988) was used to amplify DNA encoding 27 amino acid residues (AKAPKANTPVTSTSSAVLTDFOETK) from the NH2 terminus of the ε-subunit of the oligosaccharyltransferase. Two degenerate oligonucleotide primers (5'GGCAARGCICCNAAARGC and 5'TTAAAGTCTGRAGARTC) were synthesized based on the underlined amino acid sequences. PCR was performed in a 25-μl reaction volume with 50 pmol of each oligonucleotide primer, 0.5 U Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) and 100 ng of S. cerevisiae genomic DNA. Yeast genomic DNA to be used as a PCR template and for Southern analysis was isolated as described (Hoffman and Winston, 1987). To amplify the 80-bp DNA fragment, 30 cycles of denaturation (92°C, 1 min), annealing (40°C, 1 min), and extension (70°C, 2 min), were carried out in an automatic heating/cooling cycler (Programmable Thermal Controller, MJ Research, Watertown, MA). The PCR product was recovered from an 8% polyacrylamide gel and cloned using the TA Cloning System (Invitrogen, San Diego, CA) for DNA sequencing and for preparation of hybridization probes.

Approximately 40,000 colonies bearing recombinant plasmids from a S. cerevisiae genomic library in YEp13 were screened by in situ colony hybridization with a random hexamer 32P-labeled hybridization probe prepared from the PCR product (Sambrook et al., 1989). 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 5 h at ~80°C (Sambrook et al., 1989). A hybridization-positive clone was selected and designated as OST2-1. The nucleotide sequence of the OST2 gene was determined by the dideoxy chain termination method (Sanger et al., 1977). DNA sequence analysis and protein sequence comparisons were performed using the MacVector (IBI) software program.

Disruption of the OST2 Gene

The location of restriction sites used for construction of the plasmid pRS305R2L2 to disrupt the chromosomal OST2 locus are shown in Fig. 1. A 297-bp XhoI–XbaI fragment was generated by standard PCR methods using the following two primers: 5'CGCCCTCGAGCGGGATTCAT-TATTTGTG and 5'CTAATGTTAAATCTGACCC. The sense primer contained the underlined seven nucleotide extension to generate an XhoI site. Digestion of the PCR product with XhoI and HindIII yielded a 266-bp fragment that was ligated to XhoI–PstI digested pRS305 (Sikorski and Hieter, 1989) and the 283-bp HindIII–PstI restriction fragment from pOST2-1 (nucleotides 367 to 650, Fig. 1 B). The resulting construct (pRS305R2L2) was linearized at the unique HindIII site that joins the OST2 derived sequences and used to transform two diploid yeast strains (PRY238 and YPH274) using a modification (Kuo and Campbell, 1983) of the LiOAc transformation procedure (Ito et al., 1983). PRR238 (MATa/ura3-52/ura3-52 leu2-3,112/leu2-3,112 lys2-801/+ his3-A6) was obtained from P. Robbins (Orlean et al., 1988), YPH274 (MATa/ara5-32/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/Δ1 his3-Δ200/Δ200 uRA3-Δ200 leu2-Δ1/leu2-Δ1 [Sikorski and Hieter, 1989]) was obtained from the Amer. Type Culture Collection (Rockville, MD). From each transformation, six independent transformants were sporulated, ascii were dissected and analyzed for spore viability, colony formation and growth on synthetic complete media lacking leucine. Standard laboratory media were used for yeast growth and sporulation (Sherman, 1991). A diploid strain generated by disruption of the OST2 gene in YPH274 (RY202, MATa/ara5-32/ura3-52 ade2-101/ade2-101 trp1-Δ1/Δ1 his3-Δ200/Δ200 ura3-Δ200 leu2-Δ1/leu2-Δ1) was obtained from the lab of J. Sherman.

Abbreviations used in this paper: CPY, carboxypeptidase Y; Endo H, endoglycosidase H; 5-FOA, 5-fluoroorotic acid; HPLC, high pressure liquid chromatography; OST, oligosaccharyltransferase; PVDF, polyvinylidene difluoride.
ly2-801 his3-Δ200/Δ200 leu2-Δ1/Δ1 leu2-Δ1/Δ1 OST2/ΔOST2/LEU2) was selected for the experiments shown in Table I. The resulting gene replacement deletes 40% of the OST2 coding sequence.

A second gene disruption plasmid was constructed to replace the complete coding sequence of the OST2 gene with pRS305. A 657-bp HindIII-XbaI fragment and a 325-bp XbaI-BamHI fragment from pOST2-1 were subcloned into HindIII-BamHI digested pRS305. To remove the XbaI site from the pRS305 polylinker, the resulting plasmid was digested with SpeI and NotI, blunt-ended and religated. The resulting construct (pRS316-OST1) was transformed to the unique XbaI site that joins the OST2 derived sequences and used to transform PRY238 and YPH274. Asci derived from four independent transformants of each diploid were dissected and analyzed for spore viability, colony formation and growth on synthetic complete media lacking leucine. The diploid RGY216 was transformed with pRS316-OST2, and uracil prototrophs were selected by replica plating at 25°C as Leu+Tryp+ prototrophs. Transformants that could lose the plasmid were restreaked and tested for growth on selective media lacking uracil to carboxypeptidase Y (CPY) were performed as described previously (Rothblatt 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 Assay

Microsomal membranes were isolated from mid log phase yeast cultures incubated at 25°C using the procedure described previously (Silberstein et al., 1995). Wild type, ost2 mutants, and wpbl-2 yeast strains were grown in YPD medium. The wpbl-2 strain transformed with pRS426-OST2 was grown in synthetic complete medium lacking uracil. Oligosaccharyltransferase activity in digitonin extracts was assayed as described previously, using an iodinated tripeptide acceptor (N~2-Ac-Asn-[125I]Tyr-Thr-NH2) and bovine lipid-linked oligosaccharide as a donor (Kellerle et al., 1992; Kellerle and Gilmore, 1994). The protein concentration of the microsomal membranes was determined using the Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Isolation of Chitinase and [3H]Mannose-labeled Dolichol-linked Oligosaccharides

Secreted chitinase was isolated from 10 ml of growth medium from saturated yeast cultures in YPD by binding to chitin (Kuranda and Robbins, 1991). Chitinase was eluted from the chitin by boiling in SDS gel sample buffer and was analyzed on a 6% polyacrylamide gel. Secreted chitinase from an isogenic strain (GD-12) carrying a disruption of the GAP1 gene (Abejin et al., 1993) was included as a control for defective extension of O-linked oligosaccharides in the Golgi.

Lipid-linked oligosaccharides were labeled in vivo with [3H]mannose at 25°C as described (Zufferey et al., 1995) with minor modifications. Briefly, 105 yeast cells grown in YPD were pelleted, washed twice with YPD containing 0.1% glucose, and resuspended in 200 µl of YPD containing 0.1% glucose and 250 µCi of [3H]mannose (30 Ci/mmol, New England Nuclear, Boston, MA). The lipid-linked oligosaccharides were isolated by sequential solvent extraction (Zufferey et al., 1995) and subjected to mild acid hydrolysis using the procedure of Zufferey et al. (1995). The oligosaccharides were resolved on a Rainin Dynamax-60A aminopropyl silica HPLC column as described (Mellis and Baenziger, 1981).

Preparation of Antibodies to Ost2p

Ost2p specific antibodies were prepared using an NH2-terminal synthetic peptide (AKAPKANTPKYSTST) coupled to keyhole limpet hemocyanin (KLH) as the antigen. The peptide was coupled to KLH using two different coupling reagents: bi-diazotized benzidine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide using standard procedures (Harlow and Lane, 1988). Peptide synthesis and coupling of the peptide to KLH were performed by the University of Massachusetts Medical Center Peptide Synthesis Core. Rabbits were immunized and immune sera were collected by East Acres Biologicals (Southbridge, MA). To permit affinity selection of antisera, the synthetic peptide was coupled to aminophenyl Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) using 1-ethyl-3-(3-dimethylamino)propyl) carbodiimide as the coupling reagent. Immunoselection of antisera to Ost2p followed standard procedures (Harlow and Lane, 1988), except that Ost2p specific antibodies were observed to elute from the affinity column with 20 mM Tris-Cl, pH 7.5, 1 M NaCl.

Protease Digestions, Endoglycosidase H Digestions, and Protein Immunoblots

Yeast microsomal membranes (120 µg) were digested for 15 min on ice with trypsin (100 µg/ml) in a total volume of 100 µl of 50 mM triethanolamine-OAc, pH 7.5, 100 mM KOAc, 2.5 mM Mg(OAc)2 either in the presence or absence of 0.5% Triton X-100. Protease digestions were terminated by adding phenylmethylsulfonyl fluoride to 2 mM. Endoglycosidase H (Endo H) was purchased from New England Biolabs (Beverly, MA); digestions were performed following the manufacturer’s recommendations. Proteins resolved by polyacrylamide gel electrophoresis in SDS were transferred to PVDF membranes (Bio-Rad Laboratories).
membrane blots were probed with antisera that recognize Ostlp, Wbplp, Swplp, or Ost2p. Peroxidase-labeled second antibodies were visualized using enhanced chemiluminescence (ECL Western blotting detection kit, Amersham Corp., Arlington Heights, IL).

Results

Isolation and Sequencing of the OST2 Gene

The sequences of the mature amino terminus and of two internal tryptic peptides derived from the 16-kD ε-subunit of the oligosaccharyltransferase were determined. The amino terminus of one internal tryptic peptide (VTSTSSAVLTDFQETFK) was found to contain a five residue overlap with the amino-terminal sequence of the mature protein (AKAPKANTPKVTSTS). Two degenerate oligonucleotide primers were synthesized based on the sequence of the contiguous 27-residue sequence (AKAPKANTPKVTSTSSAVLTDFQETFK) and were used to amplify a yeast genomic DNA template using PCR. DNA sequencing confirmed that the PCR product of the predicted size (80 bp) was an authentic amplification product. The gene encoding the 16-kD subunit of the yeast OST, henceforth referred to as Ost2p, was isolated from a yeast genomic library in the YEp13 vector by colony hybridization using the radiolabeled PCR product as a probe. Sequence analysis of a 1.5-kb XbaI restriction fragment from a hybridization-positive plasmid revealed an open reading frame encoding a protein of 130 amino acids (Ost2p) as well as 5' and 3' flanking sequences (Fig. 1 B). A search of DNA sequence databases using the BLASTN DNA sequence comparison algorithm (Altschul et al., 1990) revealed that the nucleotides 1089-1463 of the OST2 sequence shown here are 99.5% identical to nucleotides 855-1229 of the yeast RAS1 sequence (X00527). The two nucleotides that differ fall within the 3' untranslated region separating the two coding sequences. The RAS1 gene has been mapped to the right arm of chromosome XV (Kataoka et al., 1984).

![Diagram](image-url)

**Figure 1.** Restriction endonuclease map, DNA, and protein sequences and gene disruption of the OST2 locus. (A) The OST2 gene is located on the right arm of chromosome XV adjacent to RAS1 (X00527, K01970). The OST2 locus was disrupted by replacement of a 176-bp chromosomal DNA fragment comprising nucleotides 650 (PstI site) to 826 from the XbaI site with the yeast integrating plasmid pRS305 bearing the LEU2 gene. The locations of two PCR primers used to amplify a portion of the OST2 locus for construction of the gene disruption plasmid are designated by the arrows. Restriction sites used for constructions and mapping of the gene disruption are shown. B, BamHI; C, Scal; H, HindIII; P, PstI; S, Sphl; X, XbaI. (B) The nucleotide sequence starting at the 5' XbaI site is shown together with the predicted amino acid sequence of Ost2p. Nucleotide residues are numbered on the right; amino acid residues are numbered on the left. Nucleotide 1416 corresponds to the Scal site in the RAS1 gene. Underlined sequences were determined by gas-phase protein sequencing. Dashed underlining designates hydrophobic sequences detected by hydropathy analysis (Kyte and Doolittle, 1982). These sequence data are available from the EMBL/GenBank/DDBJ under accession number U32307.
The predicted protein sequence contains exact matches for the NH$_2$-terminal and internal tryptic peptide sequence data. Translation initiation at the in frame ATG codon located nine nucleotides upstream from Met 1 is presumably not favored due to a poor match with the consensus sequence for translation initiation (Kozak, 1989). Consistent with the protein sequence data identifying Ala 2 as the amino-terminal residue of mature Ost2p, the enzyme methionine aminopeptidase removes the initiator methionine residue from proteins with a penultimate alanine residue in a cotranslational reaction (Kendall et al., 1990). The Ost2p sequence lacks consensus sites for asparagine-linked glycosylation, consistent with previous data showing that the e-subunit of the yeast OST is not a glycoprotein (Kelleher and Gilmore, 1994). The calculated molecular weight of 14,650 for mature Ost2p is in reasonable agreement with the $M_r$ of 16,000 observed for the e-subunit of the oligosaccharyltransferase on SDS-polyacrylamide gels (Kelleher and Gilmore, 1994).

**Membrane Topology of Ost2p**

Hydropathy analysis using the method of Kyte and Doolittle (1982) revealed three hydrophobic protein segments in the sequence of Ost2p that are denoted by dashed underlines in Fig. 1 B. The Ost2p sequence lacks an amino-terminal hydrophobic segment that could function as a cleavable signal sequence. Although the first two hydrophobic sequences are of sufficient length and hydrophobicity to be membrane-spanning segments (Fig. 2 A, solid bars), the third sequence near the carboxy terminus of Ost2p is somewhat less hydrophobic, and may be of insufficient length to span a membrane without insertion of a charged residue. The orientation of integral membrane proteins can be predicted with reasonable accuracy based upon the distribution of charged residues that flank the first membrane-spanning segment (Hartmann et al., 1989). The positively charged amino-terminal segment and the absence of a signal sequence suggests that the first 50 amino acids of Ost2p are located in the cytosol (Fig. 2 B, models a and b). A recently developed algorithm for predicting the location and orientation of $\alpha$-helical spanning segments in membrane proteins (Jones et al., 1994) indicates that the most probable structure for Ost2p would be similar to that shown in Fig. 2 B, model b. To experimentally address the location of the amino terminus of Ost2p, antisera was raised against an NH$_2$-terminal synthetic peptide (Fig. 2 A). Trypsinization of intact yeast microsomes eliminated Ost2p without producing an immunoreactive polypeptide of greater mobility, consistent with digestion of the epitope recognized by the antisera (Fig. 2 C). The luminal protein Kar2p became accessible to trypsin upon addition of Triton X-100, but was inaccessible in intact membranes. These observations eliminate topology models that locate the NH$_2$ terminus of Ost2p within the lumen (Fig. 2 B, models c and d). However, the proteolysis experiment cannot discriminate between models a and b.

**OST2 Is an Essential Yeast Gene**

To determine whether expression of Ost2p is required for cell viability, one chromosomal copy of the OST2 locus was disrupted in the diploid yeast strain YPH274 by replacing codons 19-78 of the OST2 gene with the yeast integrating plasmid pRS305 bearing the LEU2 gene (Fig. 1 A). Leucine prototrophs were selected and integration of pRS305 into the OST2 locus was confirmed by Southern blot analysis using a combination of restriction sites in pRS305 and the DNA sequences flanking the OST2 gene (data not shown). Diploid strains heterozygous for the OST2 gene disruption (e.g., RGY202) were sporulated and the tetrads dissected. A maximum of two viable colonies were obtained from each tetrad analyzed, both of which were leucine auxotrophs (Table I). Spores bearing the OST2 gene disruption germinated and formed microcolonies of 4–8 cells (data not shown). Identical results were obtained when spores were allowed to germinate at 25°C. Similar results were obtained when the OST2 gene was disrupted in the diploid strain PRY238 (data not shown). The lethal phenotype of an OST2 disruption could be rescued by...
transformation of RGY202 with a centromeric plasmid bearing a copy of the OST2 gene (Table I). Viable Leu<sup>+</sup>, Ura<sup>+</sup> colonies were obtained upon sporulation and dissection of tetrads from RGY208. Sporulation of a yeast strain (RGY203) with an OST2 gene disruption that removes the complete coding sequence as well as 5′ and 3′ flanking sequences also yielded only two viable colonies per tetrad (Table I), unless the strain is complemented with the pRS316-OST2 plasmid (data not shown). From these studies we conclude that the Ost2 protein is essential for the vegetative growth of yeast.

### Isolation of Temperature-sensitive ost2 Mutants

Conditional ost2 mutants were generated to investigate the essential in vivo function of the Ost2 protein. A haploid yeast strain in which the OST2 locus was completely deleted from the chromosome was complemented by a plasmid borne copy of the wild-type OST2 gene and used as a recipient for a plasmid shuffle procedure (Sikorski and Boeke, 1991). The OST2 gene was mutagenized by PCR amplification under conditions that enhance misincorporation of deoxyribonucleotides (Leung et al., 1989), and the resulting DNA fragments were used to repair a gapped plasmid by homologous recombination (Ma et al., 1987). Strains carrying ost2 mutant alleles that were inviable at 37°C were isolated after 5-FOA selection against the pRS316-OST2 gene disruption. The 763-bp BamHI-HindlII fragment containing the entire OST2 coding sequence was cloned into the integrating vector pRS316-OST2 to produce RGY202 and RGY203. Codons 19-78 of Ost2 were deleted in the RGY202 disruption. The 763-bp BamHI-HindIII fragment containing the entire OST2 coding sequence is deleted in the RGY203 strain (data not shown). The diploid RGY202 was transformed with the yeast centromeric vector pRS316 that contained or lacked the OST2 gene to generate RGY207 and RGY208.

### Table I. Tetrad Analysis of OST2 Gene Disruption

| Strain* | Relevant genotype and/or plasmid | Tetrads analyzed | Viable colonies per tetrad |
|---------|---------------------------------|-----------------|---------------------------|
| YPH274  | OST2/OST2                       | 10              | 4                         |
| RGY202  | OST2/ost2::LEU2                 | 12              | 2<sup>2</sup>             |
| RGY203  | OST2/ost2::LEU2                 | 15              | 2<sup>2</sup>             |
| RGY207  | OST2/ost2::LEU2 [pRS316]        | 6               | 2<sup>2</sup>             |
| RGY208  | OST2/ost2::LEU2 [pRS316-OST2]   | 7               | 3-4<sup>4</sup>           |

* Viable colonies were leu<sup>+</sup>. Two microcolonies (<10 cells) were obtained per tetrad.

### Conditional ost2 Mutants Are Defective in Asparagine-linked Glycosylation In Vivo

The biosynthesis of the yeast vacuolar glycoprotein carboxypeptidase Y (CPY) was examined in the temperature-sensitive strains to investigate the role of the Ost2 protein in N-linked glycosylation. CPY was chosen as a model for analyzing in vivo glycosylation because intracellular transport of CPY is not severely disrupted by inhibition of N-linked glycosylation (Stevens et al., 1982; Winther et al., 1991). Upon translocation into the lumen of the ER, proCPY acquires four N-linked oligosaccharides (Stevens et al., 1982). The 67-kD ER form of proCPY (p1 form) 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, proteolytic removal of an 8-kD propeptide generates the mature 61-kD form of CPY. Underglycosylated and nonglycosylated variants of mature CPY accumulate in yeast cells that have reduced amounts of the lipid-linked oligosaccharide donor due to tunicamycin treatment (Stevens et al., 1982), genetic defects in the dolichol pathway (Staglar et al., 1994; te Heesen et al., 1994), and in cells that bear mutations in the oligosaccharide transferase (te Heesen et al., 1992, 1993; Silberstein et al., 1995). CPY was immunoprecipitated from wild-type and ost2 mutant yeast after radiolabeling for 1 h at the permissive temperature (25°C). The predominant form of CPY synthesized by wild-type yeast was fully glycosylated vacuolar CPY, whereas tunicamycin treated yeast synthesized a 51-kD unglycosylated form of vacuolar CPY. The 59-kD polypeptide, designated by the vertical arrow in the sample from tunicamycin treated yeast, is an ER-arrested form of proCPY (Stevens et al., 1982). Underglycosylated variants of mature CPY, containing between 1 and 3 N-linked oligosaccharides migrate between CPY from untreated and tunicamycin-treated wild-type yeast (te Heesen et al., 1992; Silberstein et al., 1995). Multiple glycosyltransferase-deficient mutants were isolated as OST2/Aost2::LEU2 [pRS316-OST2] 6 2*.[H]

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Stevens, J., et al., 1982. Yeast morphological changes involving aggregation or altered cellular shape are often associated with osmotic sensitivity (Stratford, 1994).
The preceding results show that ost2 mutants are defective in asparagine-linked glycosylation at the permissive temperature. Since the ost2 mutants arrest growth after 6–12 h at 37°C, we asked whether the glycosylation defect becomes more severe after prolonged growth at the restrictive temperature. CPY was radiolabeled at various time points after an ost2-1 culture was shifted from 25°C to 37°C, and the immunoprecipitates were analyzed by SDS-gel electrophoresis (Fig. 3 B). After 2 h of incubation at the restrictive temperature, the glycoforms of CPY were essentially identical to those observed at the permissive temperature. However, extended growth at the restrictive temperature was accompanied by more severe underglycosylation of CPY. After 8 h of incubation at 37°C, the majority of the CPY glycoforms lack two or three N-linked oligosaccharides. The distribution of CPY glycoforms synthesized at the final time point were remarkably similar to those synthesized by the most defective mutant at 25°C (Fig. 3 A). As expected, wild-type cells incubated for 10 h at 37°C synthesized fully glycosylated mature CPY (Fig. 3 B). The gradual decline of CPY glycosylation in vivo by the ost2-1 cells at 37°C is likely due to a defect in the assembly and/or stability of the newly synthesized oligosaccharide donor (Fig. 4 A). The oligosaccharyltransferase activity of the three mutant strains was considerably lower than the wild-type strain. A defect in the oligosaccharyltransferase donor assembly pathway should not influence the in vitro oligosaccharyltransferase activity as the assays were supplemented with exogenous bovine dolichol-linked oligosaccharide donor. We conclude that mutations in the OST2 gene cause reductions in the oligosaccharyltransferase activity at both the permissive and restrictive temperature.

\[ ^{3}H \text{Mannose-labeled dolichol-linked oligosaccharides} \]

Microsomal membranes were prepared from 25°C cultures of wild-type and several ost2 mutant strains to determine whether underglycosylation of CPY by the ost2 mutants could be ascribed to a defect in the oligosaccharyltransferase. The microsomes were solubilized with digitonin and assayed using dolichol-linked oligosaccharide isolated from bovine pancreas as the donor and the synthetic tripeptide N\textsuperscript{\textbeta}Ac-Asn-[\textsuperscript{125}I]Tyr-Thr-NH\textsubscript{2} as the oligosaccharide acceptor (Kelleher et al., 1992; Kelleher and Gilmore, 1994). As shown in Fig. 4 A, the oligosaccharyltransferase activity of the three mutant strains was considerably lower than the wild-type strain. A defect in the oligosaccharyl donor assembly pathway should not influence the in vitro oligosaccharyltransferase activity as the assays were supplemented with exogenous bovine dolichol-linked oligosaccharide donor. We conclude that mutations in the OST2 gene cause reductions in the oligosaccharyltransferase activity at both the permissive and restrictive temperature.

\[ ^{3}H \text{Mannose-labeled dolichol-linked oligosaccharides} \]

Underglycosylation by ost2 Mutant Yeast Can be Ascribed to an Oligosaccharyltransferase Defect

Microsomal membranes were prepared from 25°C cultures of wild-type and several ost2 mutant strains to determine whether underglycosylation of CPY by the ost2 mutants could be ascribed to a defect in the oligosaccharyltransferase. The microsomes were solubilized with digitonin and assayed using dolichol-linked oligosaccharide isolated from bovine pancreas as the donor and the synthetic tripeptide N\textsuperscript{\textbeta}Ac-Asn-[\textsuperscript{125}I]Tyr-Thr-NH\textsubscript{2} as the oligosaccharide acceptor (Kelleher et al., 1992; Kelleher and Gilmore, 1994). As shown in Fig. 4 A, the oligosaccharyltransferase activity of the three mutant strains was considerably lower than the wild-type strain. A defect in the oligosaccharyl donor assembly pathway should not influence the in vitro oligosaccharyltransferase activity as the assays were supplemented with exogenous bovine dolichol-linked oligosaccharide donor. We conclude that mutations in the OST2 gene cause reductions in the oligosaccharyltransferase activity at both the permissive and restrictive temperature.

\[ ^{3}H \text{Mannose-labeled dolichol-linked oligosaccharides} \]
Oligosaccharyltransferase activity, but not dolichol-linked oligosaccharide assembly, is effected by mutations in the OST2 gene. (A) Oligosaccharyltransferase activity of the ost2 mutants. Microsomal membranes isolated from the wild-type (RGY216) and ost2 mutant yeast were assayed for in vitro oligosaccharyltransferase activity as described in the Materials and Methods. Activity values, expressed as a percentage of the wild-type strain, are the averages of two determinations. The wild-type strain had a specific activity of 1.8 pmol min⁻¹mg⁻¹.

(B) Composition of dolichol-linked oligosaccharides isolated from ost2 mutants. Wild-type (RGY216), alg5-1 and ost2-1 cells were grown in YPD at 25°C before labeling with [³H]mannose as described in Materials and Methods. Dolichol-linked oligosaccharides were isolated as described in Materials and Methods, and the composition of the [³H]mannose-labeled oligosaccharides released by acid hydrolysis was determined by HPLC as described in Materials and Methods. The chromatograms for alg5-1 and ost2-1 have been displaced vertically by 2,500 and 5,000 cpm, respectively. The labeled arrows designate saccharides or oligosaccharides derived from the following compounds: M, mannose-P-dolichol; M5N2, Man₃GlcNAc₂-PP-dolichol; M9N2, Man₃GlcNAc₂-PP-dolichol; and G3M9N2, Glc₃Man₃GlcNAc₂-PP-dolichol.

**Overexpression of Ost2p Rescues the wbp1-2 Mutant**

Since the oligosaccharyltransferase is a multisubunit enzyme, structural and functional interactions between subunits of the OST can be disclosed by multicopy suppression analysis (te Heesen et al., 1993). Yeast strains bearing the temperature sensitive wbp1-1 or wbp1-2 alleles were transformed with pRS426-OST2, a high-copy number plasmid with an OST2 insert. Growth of transformants at the permissive and restrictive temperatures was analyzed on selective and nonselective media. The wbp1-2 mutant was able to grow at 37°C after transformation with a high-copy plasmid containing an insert (pRS426-OST2), but not when transformed with the vector alone (data not shown). Overexpression of OST2 did not eliminate the restrictive growth phenotype of the wbp1-1 mutant, indicating that the genetic interaction is allele-specific.

The wbp1-2 mutant underglycosylates proteins at both the permissive and restrictive temperatures in vivo, consistent with the observed in vitro defect in oligosaccharyltransferase activity (te Heesen et al., 1992). As observed previously (te Heesen et al., 1992), the wbp1-2 mutant synthesizes glycoforms of CPY at 25°C that lack between one and three N-linked oligosaccharides (Fig. 5 A). Overexpression of Ost2p in the wbp1-2 strain restores glycosylation of CPY to a level that approaches that seen in a wild-type strain (Fig. 5 A). We next asked whether the in vitro oligosaccharyltransferase activity was also increased upon overexpression of Ost2p in the wbp1-2 mutant. Under the conditions of our oligosaccharyltransferase assay, detergent extracts prepared from the wbp1-2 mutant are twofold less active than extracts prepared from wild-type yeast in an oligosaccharyltransferase assay, whereas 90% of wild-type activity was detected in extracts prepared from the wbp1-2 strain overexpressing Ost2p (Fig. 5 B).

**Figure 5.** High-copy suppression of the wbp1-2 mutant by Ost2p. (A) Glycosylation of CPY. Wild-type (RGY216), wbp1-2, and wbp1-2 cells bearing the pRS426-OST2 (wbp1-2[OST2]) were grown in minimal media at 25°C before labeling for 1 h with Tran³⁵S-Label. CPY immunoprecipitates from glass-bead extracts of cells were resolved by PAGE in SDS. Fully glycosylated vacuolar CPY and underglycosylated variants lacking 1 and 2 N-linked oligosaccharides are indicated by labeled arrows on the right side of the panel. The ER form (p₁) and Golgi form (p₂) of proCPY are indicated by labeled arrows on the right side of the panel. (B) Oligosaccharyltransferase activity. Microsomal membranes isolated from the wild-type (RGY216), wbp1-2, and wbp1-2[OST2] cells were assayed for in vitro oligosaccharyltransferase activity as described in the Materials and Methods. Activity values, expressed as a percentage of the wild-type strain, are the averages of two determinations. The wild-type strain had a specific activity of 1.8 pmol min⁻¹mg⁻¹.
Expression and Glycosylation of the Oligosaccharyltransferase Subunits

The membrane preparations that were assayed for oligosaccharyltransferase activity in the preceding experiments (Fig. 4 A and Fig. 5 B) were subjected to protein immunoblot analysis using antibodies to Ost1p, Wbp1p, Swp1p, and Ost2p to determine whether the expression or stability of the oligosaccharyltransferase subunits was altered in strains bearing mutations in Ost2p and Wbp1p. As shown previously (Kelleher and Gilmore, 1994), wild-type yeast express 64-kD and 62-kD glycoforms of Ost1p that contain four and three N-linked oligosaccharides, respectively (Fig. 6 A). Underglycosylated forms of Ost1p, migrating between fully glycosylated and Endo H-digested Ost1p (dOst1p), were detected in membrane preparations from the ost2 mutants. The ost2 mutants that displayed the most severe underglycosylation of CPY (ost2-3 and ost2-4) also showed the most pronounced underglycosylation of Ost1p. Several underglycosylated forms of Ost1p were observed in membranes isolated from the wbp1-2 mutant. Surprisingly, overexpression of Ost2p in the wbp1-2 mutant does not eliminate underglycosylation of Ost1p despite the observed increase in glycosylation of CPY and the synthetic peptide substrate. A comparison of the Endo H–digested membrane samples from the ost2 and wbp1-2 mutants did not reveal a detectable difference in Ost1p content relative to the wild-type membranes (data not shown).

The Wbp1 protein sequence contains two sites for N-linked glycosylation (te Heesen et al., 1991), both of which are glycosylated in vivo by wild-type yeast (Fig. 6 B and Kelleher and Gilmore, 1994). Underglycosylated forms of Wbp1p were detected in membranes isolated from the ost2 yeast strains. As observed for CPY and Ost1p, the extent of Wbp1p underglycosylation correlated with the severity of the growth defect of the ost2 mutants. Densitometric scanning of immunoblots did not reveal a decreased content of Wbp1p in membranes from the ost2 mutants relative to the wild-type strain. In contrast, membranes from the wbp1-2 strain contain 35% of the Wbp1p present in the wild-type strain. Overexpression of Ost2p in the wbp1-2 mutant resulted in a twofold increase in Wbp1p content, strongly suggesting that high-copy suppression of the glycosylation defect is due to a partial stabilization of an assembly-defective form of Wbp1p.

The immunoblots were also probed with an antibody to Swp1p (Fig. 6 C). A reduced amount of this OST subunit was observed in membranes prepared from the ost2-3, wbp1-2, and wbp1-2[OST2] strains (35%, 30%, and 50% of the wild-type amount of Swp1p, respectively). The reduced content of Swp1p in a wbp1-2 strain and the increased content of Swp1p upon overexpression of Ost2p in the wbp1-2 mutant is consistent with previously reported
violence for a physical interaction between these two subunits of the oligosaccharyltransferase complex (te Heesen et al., 1993). This observation is also consistent with the reciprocal instability of Wbp1p and Swp1p in gene product depletion experiments (Knauer and Lehle, 1994).

Protein immunoblots were probed with an affinity-purified antibody to Ost2p (Fig. 6 D). Polypeptides with identical mobility and comparable intensity to the wild type were detected in membranes from the ost2-3 and wbp1-2 mutant strains. As expected, Ost2p was considerably more abundant in the wbp1-2[OST2] strain than in a wild-type strain or in the wbp1-2 strain. More intense immunoreactive proteins of faster mobility were detected in membrane preparations from two of the other mutants (ost2-1 and ost2-4). Densitometric scanning of immunoblots indicates that membranes from the ost2-1 and ost2-4 mutants contain roughly 3- and 2-fold more Ost2p than wild-type yeast, respectively. The explanation for the increased mobility of Ost2p in membranes isolated from the ost2-1 and ost2-4 is not clear. As the anti-peptide antibody to Ost2p was raised against the amino terminus, the increased gel mobility of Ost2p in these strains is probably not caused by extensive amino-terminal proteolysis. Sequencing of the mutant alleles did not reveal nonsense codons within the OST2 coding sequence, hence the altered mobility is not due to a carboxy-terminal truncation. Although COOH-terminal proteolysis of the mutant proteins cannot be entirely discounted, we feel that the most likely cause for the increased mobility is the presence of point mutations that introduce or eliminate charged amino acid residues in the protein sequence. We do not have an explanation for the increased expression levels of Ost2p observed in two of the ost2 mutants.

**Ost2p Is Homologous to DAD1**

Comparison of the Ost2p sequence to the protein sequence databases disclosed an unexpected homology between the yeast Ost2p and the DAD1 protein (Fig. 7). A cDNA encoding the human DAD1 protein (defender against apoptotic cell death) was isolated as a cDNA that complements a temperature-sensitive cell line (tsBN7) derived from BHK cells (Nakashima et al., 1993). The tsBN7 cell line is believed to die by apoptosis at the restrictive temperature (Nakashima et al., 1993), hence the tsBN7 cell line is derived from BHK cells (Nakashima et al., 1993). As shown in Fig. 7, the Ost2 protein sequence is clearly homologous to the vertebrate DAD1 protein. Within the overlapping region of 113 amino acids, the sequence identity between Ost2p and vertebrate DAD1 is 40% and similarity was estimated to be 65%. The region of highest sequence identity extends through the carboxy-terminal half of both proteins; in this region (F72 to N130 of Ost2p) the amino acid sequence identity is 58%. Of the four OST subunits for which both mammalian and yeast sequences are currently available, the sequence identity between the Ost2p and DAD1 homologues is the greatest. The most striking difference between Ost2p and the vertebrate DAD1 protein is the amino-terminal extension of 17 residues in the yeast protein. A comparison of the hydropathy plots of Ost2p and DAD1 reveals an identical arrangement of the three hydrophobic segments (data not shown). Incomplete sequences of DAD1/Ost2p from an invertebrate (C. elegans [T01835]), and two plants (Arabidopsis thaliana [T44943] and Oryza sativa [D24136]) are present in the expressed sequence tags database, and further support the extensive conservation of the DAD1/Ost2 protein during evolution of eukaryotes.

**Point Mutations in the ost2 Temperature-sensitive Alleles**

The conditional ost2 mutants were sequenced to identify amino acid substitutions that might be responsible for defects in Ost2p (Fig 8 A). As expected for mutants produced by error-prone PCR, nucleotide substitutions rather than insertions or deletions were introduced into the coding sequence of OST2. None of the nucleotide changes created a new termination codon or eliminated the normal termination codon. Of the six alleles sequenced, two ost2 alleles contained single amino acid changes, while the remaining alleles contained several substitutions. For ost2-4 and ost2-5, we can conclude that the single amino acid change is responsible for the reduced oligosaccharyltransferase activity. A comparison of the amino acid substitutions in the ost2 alleles with the DAD1/Ost2 sequence alignment (Fig. 7) revealed that each of the mutants had a single nonconservative substitution at a residue that is invariant, or highly conserved between DAD1 (H. sapiens and X. laevis) and Ost2p. Two of the three mutations in the ost2-2 allele increase the homology (L119S) or identity (F123L) between the yeast and vertebrate proteins, whereas the underlined D48V mutation would eliminate a charged residue that is predicted to flank the first membrane-spanning segment (Fig. 8 B). The three hydrophobic segments of Ost2p are depicted as helical wheels with invariant resi-
Figure 8. Point mutations in the OST2 gene. The coding sequence of six ost2 mutant alleles was determined by sequencing both DNA strands. (A) Amino acid changes are shown for each allele using the one letter code for amino acids and the position in the Ost2p sequence. Underlined substitutions from A are wheel representations of the three hydrophobic segments of DNA strands. (B) The sec-Ost2p. Amino acid residues conserved between Ost2p, H. sapiens DAD1, and X. laevis DAD1 are shown in bold-face letters. Underlined amino acid substitutions from A are shown. (B) The first predicted membrane-spanning segment of Ost2p starting at residue D48 and terminating at F128. (C) The second predicted membrane-spanning segment of Ost2p starting at residue F75 and terminating at M92. (D) The third hydrophobic segment of Ost2p starting at residue F111 and terminating at F128.

due shown in bold case (Fig. 8, B–D). Substitution of a charged amino acid for an apolar residue in a membrane-spanning segment is deleterious (Lemmon et al., 1992); the underlined mutations in ost2-1 and ost2-4 substitute charged residues for invariant glycine residues in the second predicted membrane-spanning segment (Fig. 8 C). The tsBN7 mutation replaces an invariant glycine with an arginine in the first hydrophobic segment of the hamster DAD1 protein (Fig. 8 B) (Nakashima et al., 1993). The tsBN7 mutation was shown to cause instability of the DAD1 protein at the restrictive temperature. The Q61R mutation in the ost2-3 allele also introduced a charged residue into the first predicted membrane-spanning segment (Fig. 8 B). Interestingly, two different nonconservative mutations were found at E113 in the ost2-5 and ost2-6. Although glutamate 113 is not an invariant residue, all the Ost2p homologues sequenced to date, including the plant and invertebrate partial sequences cited above, have an aspartate at this position suggesting that an acidic residue is crucial (Fig. 8 D). The significance of the relatively conservative A112S mutation at an invariant residue in the ost2-6 allele is uncertain. It should be noted that we have not tested whether the underlined mutations in alleles with multiple changes uniquely account for the observed conditional phenotype. Conceivably, the observed defects could depend upon multiple substitutions. Since the noncoding sequence of the ost2 mutants was not determined, we cannot discount the presence of additional mutations outside the coding region of the gene, that could contribute to the generation of temperature-sensitive alleles due to an altered expression of the protein.

Discussion

We have described the functional characterization of OST2, a S. cerevisiae gene that encodes a novel subunit of the oligosaccharyltransferase. The molecular genetic evidence we have presented here demonstrates that the 16-kD polypeptide in our oligosaccharyltransferase preparation (Kelleher and Gilmore, 1994) is an essential gene product for growth of yeast. In this respect, Ost2p is similar to three of the previously characterized subunits of the oligosaccharyltransferase; Wbp1p, Swp1p, and Ost1p are all essential gene products (te Heesen et al., 1992, 1993; Silberstein et al., 1995). Analysis of a set of conditional ost2 mutants demonstrated that defects in the Ost2 protein result in the in vivo underglycosylation of soluble and integral membrane glycoproteins at both the permissive and restrictive temperatures. Underglycosylation of newly synthesized proteins by the ost2 mutants can be ascribed to a defect in the oligosaccharyltransferase based upon the dramatic reduction of in vitro oligosaccharyltransferase activity in detergent extracts of microsomal membranes prepared from the ost2 cells. Mutations in the OST2 gene do not appear to cause pleiotropic defects in endoplasmic reticulum function as shown by the normal assembly of the lipid-linked oligosaccharide donor and the normal initiation of O-linked glycosylation. Given these observations, how can one explain the discrepancy between the initial description of a hexameric OST complex (Kelleher and Gilmore, 1994) and two subsequent reports that describe a catalytically active tetrameric OST complex that lacks the 16-kD and 9-kD subunits (Knauer and Lehle, 1994; Pathak et al., 1995a). It would appear that the electrophoretic conditions used by the latter two laboratories may not have been optimal for the resolution and subsequent detection of low molecular weight polypeptides. As loss of the Ost2 protein during purification would likely result in a substantial reduction in the recovery of oligosaccharyltransferase activity, we suggest that the apparent absence of Ost2p in several yeast oligosaccharyltransferase preparations needs to be reevaluated.

Identifying the OST subunit or subunits that contain the substrate-binding sites is a difficult endeavor due to the multiplicity of subunits. Recent biochemical studies indicate that oligosaccharyltransferase activity is selectively inhibited by methyl methanethiosulfonate, a cysteine-directed chemical modification reagent (Pathak et al., 1995a). Interestingly, inhibition of OST by methyl methanethiosulfonate was significantly reduced by inclusion of the lipid-linked oligosaccharide Dol-PP-chitobiose. Modification of the Wbp1 protein correlated with reduced enzyme activity, suggesting that the Wbp1p subunit contains a cysteine residue at or near the oligosaccharide donor binding site (Pathak et al., 1995a). Notably, none of the three cysteine residues in Wbp1p are conserved in the canine (Silberstein et al., 1992), human (D29643), avian (Kumar et
DNA fragmentation in tsBN7 cells after shift to the re-
vertebrates (Nakashima et al., 1993), invertebrates and
cosylation-defective yeast. Allele specific suppression of
cur by a bypass mechanism that permits the survival of gly-
coylation by tunicamycin treatment is reported to induce
apoptosis based upon the observation of chromatin con-
densation, vacuolization of the cytoplasm and subsequent
destruction, vacuolization of the cytoplasm and subsequent
DNA fragmentation in tsBN7 cells after shift to the re-
strictive temperature (Nakashima et al., 1993). Could a
suppression of the mutant by overexpression of the Ost2 protein does not oc-

currents in progress in our laboratory. The DAD1 protein is highly conserved in
vertebrates (Nakashima et al., 1993), invertebrates and
plants, and has been proposed to be a negative regulator of
apoptosis based upon the observation of chromatin condensa-
tion, vacuolization of the cytoplasm and subsequent
DNA fragmentation in tsBN7 cells after shift to the restri-
tive temperature (Nakashima et al., 1993). Could a
previously unanticipated connection between apoptosis and N-linked glycosylation have been revealed by the con-
titional DAD1 mutation in the tsBN7 cells? Inhibition of
an essential cellular function, such as N-linked glycosyla-
tion, does lead to cell death, and may occur by pathways
that share certain features with programmed cell death (for a review see Vaux, 1993). Inhibition of N-linked gly-
cosylation by tunicamycin treatment is reported to induce
apoptosis of HL-60 cells (Pérez-Salva and Mollinedo,
1995).

Allele-specific suppression of the wbpl-2 mutant by overexpression of Ost2p occurs by an enhancement, albeit incomplete, of the in vivo and in vitro glycosylation activ-
ity of the wbpl-2 mutant. Thus, suppression of the wbpl-2 mutant by overexpression of the Ost2 protein does not oc-
cur by a bypass mechanism that permits the survival of gly-
cosylation-defective yeast. Allele specific suppression of
wbpl mutants was previously observed for the high-copy suppression of the wbpl-2 mutant by SWP1 (te Heesen et
al., 1993). Allele-specific high-copy suppression may be indicative of a direct physical interaction between two gene
products (Huffaker et al., 1987). Taken together with the protein immunoblot data showing stabilization of the
Wbp1p and Swp1p subunits upon overexpression of Ost2p, these results suggest that the Ost2p, Wbp1p, and Swp1p
subunits of the oligosaccharyltransferase are in direct
physical contact.

Consistent with a reaction that occurs in the lumen of the endoplasmic reticulum, hydrophathy analysis and HIS4-gene fusion experiments indicate that three of the pre-
viously characterized subunits of the yeast OST (i.e., Wbp1p,
Swp1p, and Ost1p) are integral membrane proteins com-
prised of a large amino-terminal lumenal domain, one to
three membrane-spanning segments, and a short carboxy-
terminal cytoplasmic tail (te Heesen et al., 1992, 1993; Sil-
erberstein et al., 1995). Hydrophathy analysis and proteolysis experiments predict that Ost2p spans the membrane at least twice, with the hydrophilic amino terminus located in
the cytosol. Considering the luminal location of the oli-
gosaccharyltransferase activity, the predicted topology and high degree of sequence conservation of the Ost2 pro-
tein is unexpected. Conceivably, several subunits of the oligosaccharyltransferase complex may not participate di-
rectly in oligosaccharide transfer, but may instead serve
other functions. Support for this view has been provided
by the recent observation that the OST3 gene encoding the 34-kD subunit of the oligosaccharyltransferase is not
essential for viability of yeast, yet loss of this protein re-
duces oligosaccharide transfer to nascent glycoproteins in
vivo (Karaoglu et al., 1995). Possible roles for OST sub-
units not directly engaged in catalysis could include medi-
ating interactions with the components of the transloca-

tion apparatus, the lipid-linked oligosaccharide assembly
pathway or the protein folding and assembly pathways.

The types of mutations observed in the ost2 mutants may provide some insight into the role of the Ost2 protein. Of the six alleles sequenced, four contained mutations that
are likely to perturb the structure of the first or second predicted membrane-spanning segments by substitution of a charged amino acid residue for an apolar residue (ost2-1,
ost2-3, ost2-4) or by elimination of a charged residue that
may flank a membrane-spanning segment (ost2-2). Signifi-
cantly, none of the ost2 alleles characterized here could be
suppressed by overexpression of wild-type Ost1p, Swp1p,
or Wbp1p (data not shown). Interactions between tightly
packed a helices perform a crucial role in the structural in-
tegrity of integral membrane proteins that are composed of helical bundles (Deisenhofer et al., 1985). The more po-
ar face of a membrane-spanning helix is likely involved in
interactions with an adjacent helix, while the more hydro-
phobic face may be exposed to phospholipids (Rees et al.,
1989). When the second predicted membrane-spanning segment of Ost2p is displayed as a helical wheel, the two
point mutations are located on the the more polar, yet less
conserved, face of the helix (Fig. 8 C). Hydrophilic or charged residues in membrane-spanning segments con-
tribute to helical interactions between subunits of oligo-
meric proteins by the formation of salt bridges and hydro-
gen bonds (Manolios et al., 1990; Cosson et al., 1991).
Given the extensive conservation of both the second and
third hydrophobic segments of the Ost2 protein, we specu-
late that these portions of the protein contact the mem-
brane-spanning segments of other oligosaccharyltransferase
subunits.

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