# 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 nonidentical 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 lumenally oriented enzyme oligosaccharyltransferase, involves the transfer of a preassembled high-mannose oligosaccharide ($Glc_3Man_9GlcNAc_2$) from a dolichol-pyrophosphate 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 malfolded proteins that can become stably associated with endoplasmic reticulum chaperones and are then subjected to degradation as part of a quality control mechanism (Helenius, 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., 1995*a*) 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

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active yeast OST as a heterotetramer that lacks both the ε and ζ subunits (Knauer and Lehle, 1994; Pathak et al., 1995*a*). The α-subunit, designated Ost1p, is 28% identical in sequence to the ribophorin I subunit of the vertebrate oligosaccharyltransferase complex (Pathak et al., 1995*b*; Silberstein et al., 1995). Expression of the Ost1 protein is essential for vegetative growth of haploid yeast (Silberstein et al., 1995). Analysis of conditional *ost1* mutants demonstrated that Ost1p 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 Wbp1 protein (Kelleher and Gilmore, 1994; Knauer and Lehle, 1994; Pathak et al., 1995*a*), 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 Wbp1 protein is required for in vivo and in vitro N-linked glycosylation of proteins (te Heesen et al., 1992). The Wbp1 protein is 25% identical in sequence to the OST48 subunit of the canine oligosaccharyltransferase (Silberstein et al., 1992). The 30-kD δ-subunit of the yeast oligosaccharyltransferase corresponds to the Swp1 protein (Kelleher and Gilmore, 1994; Knauer and Lehle, 1994; Pathak et al., 1995*a*). The *SWP1* locus was isolated as an allele-specific high-copy suppressor of the *wbp1-2* mutant (te Heesen et al., 1993), and Swp1p forms complexes with Wbp1p in the yeast endoplasmic reticulum (te Heesen et al., 1993). Gene product depletion experiments indicate that Swp1p is also required for N-linked glycosylation in yeast (te Heesen et al., 1993). A protein sequence comparison revealed that the Swp1p 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 (Ost1p, Wbp1p, and Swp1p) 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 Ost1p, Wbp1p, and Swp1p in yeast does not result in increased oligosaccharyltransferase activity (Pathak et al., 1995*a*), 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., 1995*a*), 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)[1] membrane or onto a nitrocellulose sheet and stained with *Ponceau S.* $NH_2$-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 (AKAPKANTPKVTSTSSAVLTDFQETFK) from the mature $NH_2$ terminus of the ε-subunit of the oligosaccharyltransferase. Two degenerate oligonucleotide primers (5′GCIAARGCICCNAARGC and 5′TTRAAIGTYTCYTGRAARTC) 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 $^{32}$P-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 pOST2-1. 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 pRS305RL2 to disrupt the chromosomal *OST2* locus are shown in Fig. 1 *A*. A 297-bp XhoI–XbaI fragment was generated by standard PCR methods using the following two primers: 5′CGCCTCGAGCGGGATTCATTATTTGTG and 5′CTAATGGTTAATCTGCCACC. 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 (pRS305RL2) 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). PRY238 (*MATa/α ura3-52/ura3-52 leu2-3,112/leu2-3,112 lys2-801/+ +/his4-619*) was obtained from P. Robbins (Orlean et al., 1988), YPH274 (*MATa/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-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, asci 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 (RGY202; *MATa/α ura3-52/ura3-52 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 lys2-801/*

1. *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.

*lys2-801 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-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 (pRS305RL3) was linearized at 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 RGY203 (*MATa/α ura3-52/ura3-52 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 lys2-801/lys2-801 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 OST2/Δost2::LEU2*), derived from YPH274, was used to generate an haploid recipient for the plasmid shuffle procedure.

## Isolation of Temperature-sensitive S. cerevisiae ost2 Mutants

A 722-bp HindIII fragment derived from pOST2-1 was subcloned into the HindIII site of the yeast centromeric plasmid pRS316 (Sikorski and Hieter, 1989) and the 2-μm plasmid pRS426 (Christianson et al., 1992), to generate pRS316-OST2 and pRS426-OST2, respectively. The diploid RGY203 was transformed with pRS316-OST2, and uracil prototrophs were selected, sporulated, and tetrads were dissected. A haploid segregant with the genotype *MATα ura3-52 ade2-101 leu2-Δ1 trp1-Δ1 lys2-801 his3-Δ200 Δost2::LEU2 [pRS316-OST2]* was selected and designated as RGY216. The plasmid pRS316-OST2 was used as a template for PCR performed under conditions that favor misincorporation of deoxynucleotides by Taq polymerase (Leung et al., 1989). The oligonucleotide primers complementary to vector sequences flanking the gene and the conditions for PCR were as described previously (Silberstein et al., 1995), except that the error-prone PCR contained 1 mM $Mg^{++}$ and 0.25 mM $Mn^{++}$. The PCR product obtained consists of the 722-bp HindIII segment of the *OST2* gene flanked by 165 bp of 5′ and 256 bp of 3′ vector derived sequence. RGY216 was cotransformed with the 1,134-bp PCR product and EcoRI digested pRS314 (Sikorski and Hieter, 1989) 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 $Leu^+Trp^+$ prototrophs. Transformants that could lose the plasmid pRS316-OST2 bearing the wild-type gene were selected by replica plating onto synthetic complete media containing 5-fluoroorotic acid (5-FOA) (Sikorski and Boeke, 1991) while simultaneously selecting for temperature sensitivity by incubation of replica plates at 25°C and 37°C. From approximately 600 transformants, ten $Leu^+Trp^+Ura^-$ colonies were isolated that could grow at 25°C but not at 37°C. Of these ten colonies, six 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 *E. coli* for amplification. DNA sequencing of both strands of the *ost2* alleles in pRS314 was performed by the University of Georgia Molecular Genetics Facility.

## Multicopy Suppression of the wbp1 Temperature-sensitive Strains

The yeast strains MA7-B (*MATa wbp1-1 ade2-101 his3Δ200 ura3-52 lys2-801*) and MA9-D (*MATa wbp1-2 ade2-101 his3Δ200 ura3-52 lys2-801*) (te Heesen et al., 1993) were transformed with pRS426-OST2 or with pRS426. Transformants selected at 25°C on synthetic complete media lacking uracil were restreaked and tested for growth on selective media lacking uracil and on YPD plates at 25°C and 37°C. Growth phenotypes at the permissive and restrictive temperatures were scored after 4 d of incubation at the corresponding temperatures. Temperature-sensitive colonies were recovered when the transformants were cured of pRS426-OST2 by growth on synthetic complete media containing 5-FOA, confirming that suppression of the growth defect at 37°C was dependent upon the presence of pRS426-OST2.

## Radiolabeling and Immunoprecipitation of Glycoproteins

In vivo radiolabeling of yeast glycoproteins was performed as described previously (Silberstein et al., 1995) with one minor modification. Yeast cells resuspended at a density of 5 $A_{600}$/ml in prewarmed minimal medium were labeled for 60 min with 100 μCi of Tran-$^{35}$S-label (ICN Biomedicals Inc., Costa Mesa, CA) per $A_{600}$ unit of cells. Rapid lysis of cells with glass beads and immunoprecipitation of radiolabeled proteins with antibodies 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 *wbp1-2* yeast strains were grown in YPD medium. The *wbp1-2* 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 ($N^{\alpha}$-Ac-Asn-[$^{125}$I]Tyr-Thr-$NH_2$) and bovine lipid-linked oligosaccharide as a donor (Kelleher et al., 1992; Kelleher 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 [$^3$H]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 *GDA1* gene (Abeijon 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 [$^3$H]mannose at 25°C as described (Zufferey et al., 1995) with minor modifications. Briefly, $10^9$ 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 [2-$^3$H]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 $NH_2$-terminal synthetic peptide (AKAPKANTPKVTSTY) coupled to keyhole limpet hemocyanin (KLH) as the antigen. The peptide was coupled to KLH using two different coupling reagents: bis-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 aminohexyl Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) using 1-ethyl-3-(3-dimethylaminopropyl) 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). The

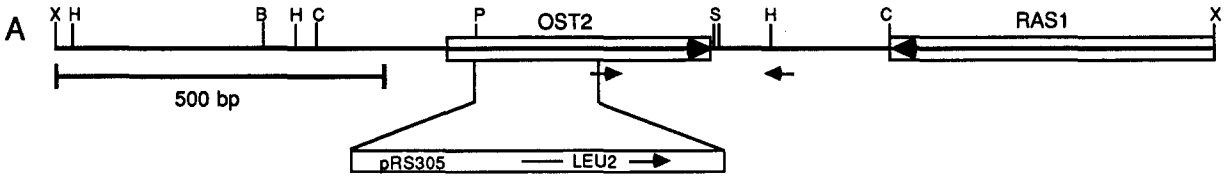
membrane blots were probed with antiserum that recognize Ost1p, Wbp1p, Swp1p, or Ost2p. Peroxidase-labeled second antibodies were visualized using enhanced chemiluminescence (ECL Western blotting detection kit, Amersham Corp., Arlington Heights, IL).

## 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).




B

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TCTAGAGTAGAAGTTGCGGTTCCAGTAGATCTTGAAGCTTTTTCGAGATGCATCATTTATATTTTTCCCCATTTGGTTATAAGAGAAGAG      90
GCATTTTCCTACAACGAAAAATAAGCATAAAAAGGGCCACGTTCGTAGCAAAAAAAAGCTAAGACTGATACTTTGTAGCAAGAAACTGAA     180
TGAAAAACTGGGATTTTCATTTTAATTATTTACAGTAAAAGAATTTATCAAAGCTGTTTCATTTGGGCAGCGCAACGGGGACTTTTTTAC     270
CAGCCATTAAGACTTAGATGACGAAAAAAACACGCGTATACGCCAGCCAATTGAGGATCCTAACCAATTCATCACACTGGAAATAACGAG     360
TCGATAGAAGCTTGTTGGTTCTATTTGGCGAGTACTTGGTCCTCTGCTATTTGCACTTGTGTCGTAAGTGCAATGATTGGGTGTATATCG     450
CTATTAGAAAAAATCCCGGTGAGGGCAAAAATAGAGAATAACGTGTTTTAAAGGATGAAAAAGATACAATAGAATCTGCGTGAACGCTAT     540
ACTTCATAACGCTATCACACAACAAAAAGAATTGTAACGAGGAATACATGACTAATATGGCAAAAGCACCAAAGGCAAACACACCAAAAG     630
                                                  M  A  K  A  P  K  A  N  T  P  K

TAACCAGCACGTCATCTGCAGTCTTAACAGATTTTCAAGAAACTTTCAAGACTTCTAAGAGGGCATATTTTGCCCAAATTGAAAAATATC     720
12 V  T  S  T  S  S  A  V  L  T  D  F  Q  E  T  F  K  T  S  K  R  A  Y  F  A  Q  I  E  K  Y

CTAAACTGAAACTAATTGATACATTCTGCTTCTTTTTGGTATTATTAGGGGTGATCCAGTGTACATTCATTATCTTGATCCGTGATAATT     810
42 P  K  L  K  L  I  D  T  F  C  F  F  L  V  L  L  G  V  I  Q  C  T  F  I  I  L  I  R  D  N

TTCCATTCAATGCGTTTTTAGCGGGATTCATTATTTGTGTGGGACAATTCGTCCTATTGATGAGTTTGCGGTTACAATTGTGTAATAGTT     900
72 F  P  F  N  A  F  L  A  G  F  I  I  C  V  G  Q  F  V  L  L  M  S  L  R  L  Q  L  C  N  S

TTCCAGGCATTTCTAAAAACAGAGCTTTTGCCGAGTTTATTGTTGCTAGTTTGATTTTACATTTCGTTTGTTTACATTTTATAAATTGAG     990
102 F  P  G  I  S  K  N  R  A  F  A  E  F  I  V  A  S  L  I  L  H  F  V  C  L  H  F  I  N  Ter

GAAGGGTTTGCCGGCGTGCTTGCATGCATGCTTGTATGTGTATAGTATATATACTTTGTACGAGAATAAAAGAACATATTACAATTGTAC    1080
ATTATAGAAAGCTTACAAAAGGTGGCAGATTAACCATTAGTATTAATGTTTGGTGGCAAGCAACTCTTTTACTAGATACACAGCATAGGA    1170
AAATGGATACAAAAAGATGCTGGTGAAACCAATTGAACAAGTAAGAATAGACTTGCAATCAAAACCATGTCATATCAAGAGAGCAGGATC    1260
ATTTTCAACAAATTATACAACAACCACCACTAGATTCTTTTCTAGCGTTGGCGCTTGAATTTTTTTGTGGCTCAGCAGACTGTTTACTTC    1350
TCGAATTTAAGGCATTATTTTGATTACCACGACTAATATCACTTCGATTTCCGTTGCCGTTACTGGAGTACTTGGTATTTCCTTCATTGT    1440
TATTGTTTTGATTAACGTAATTC                                                                        1463
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*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, ScaI; H, HindIII; P, PstI; S, SphI; 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 ScaI 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 ϵ-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 ϵ-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 α-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 lumenal 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

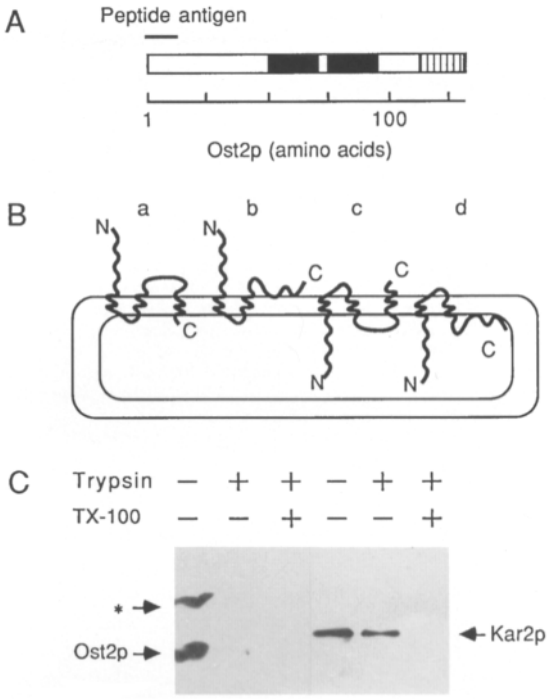


*Figure 2.* Topology analysis of Ost2p. (*A*) The locations of hydrophobic sequences predicted to be membrane-spanning segments are shown as solid bars. A shorter hydrophobic segment near the carboxy terminus is striped. A synthetic peptide based upon the amino-terminal sequence was used as an antigen to obtain Ost2p specific antisera. (*B*) Four possible topologies for Ost2p with two or three membrane-spanning segments are depicted. (*C*) Three aliquots of yeast microsomes (120 μg in 100 μl) were incubated on ice for 15 min either in the absence or presence of trypsin (100 μg/ml) and Triton X-100 (0.5%) as described in the Materials and Methods. After PMSF treatment, each digestion sample was divided for subsequent PAGE in SDS using either a 14% polyacrylamide gel (Ost2p, 60 μg of membrane/lane) or a 10% polyacrylamide gel (for Kar2p, 30 μg of membrane/lane). Electrophoretic transfer to PVDF membranes, probing with antibodies specific for Ost2p and Kar2p, and ECL detection of bound antibodies are described in Materials and Methods. The polypeptide indicated by the asterisk is a nonspecific immunoreaction product. Cell fractionation experiments indicate that the latter polypeptide is not localized to the endoplasmic reticulum (data not shown).

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

*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/Δost2::LEU2* | 12 | 2‡ |
| RGY203 | *OST2/Δost2::LEU2* | 15 | 2‡ |
| RGY207 | *OST2/Δost2::LEU2* [pRS316] | 6 | 2‡ |
| RGY208 | *OST2/Δ0st2::LEU2* [pRS316-*OST2*] | 7 | 3–4§ |

*Diploid strains were sporulated, tetrads dissected on YPD plates, and incubated for 4 d at 30°C. Colonies obtained were replica plated on selective medium to determine nutritional markers. YPH274 was the recipient for the *OST2* disruption to produce RGY202 and RGY203. Codons 19–78 of Ost2p were deleted in the RGY202 disruption. The 763-bp BamHI–HindIII fragment containing the entire *OST2* coding sequence is deleted in the RGY203 strain. The diploid RGY202 was transformed with the yeast centromeric vector pRS316 that contained or lacked the *OST2* gene to generate RGY207 and RGY208.

‡Viable colonies were leu⁻. Two microcolonies (<10 cells) were obtained per tetrad.

§Viable leu⁺ colonies were also ura⁺.

transformation of RGY202 with a centromeric plasmid bearing a copy of the *OST2* gene (Table I). Viable Leu⁺, Ura⁺ 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 *URA3*-marked plasmid bearing the wild-type *OST2* gene. Six independent colonies were isolated that were viable at 25°C but not at 37°C. In liquid media at 37°C, the *ost2* mutants arrested growth after 6–12 h of incubation (data not shown). At the permissive temperature, doubling times comparable to the wild-type strain were observed for the *ost2-1*, *ost2-2* , *ost2-5,* and *ost2-6* mutants, whereas *ost2-3* and *ost2-4* showed reduced growth rates. The *ost2* mutants display a marked tendency to form aggregates in liquid media presumably due to a failure of buds to separate from the mother cell. This growth characteristic becomes more evident after incubation at the restrictive temperature where clusters of enlarged cells can be observed in liquid media at 37°C. Yeast strains that are defective in the assembly (*alg* mutants), transfer (*ost1* mutants), and elongation (*mnn* mutants) of N-linked oligosaccharides are prone to extensive aggregation in liquid culture (Ballou et al., 1980; Kukuruzinska and Lennon, 1995; Silberstein et al., 1995). Yeast morphological changes involving aggregation or altered cellular shape are often associated with osmotic sensitivity (Stratford, 1994).

### *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, pro CPY 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 donor assembly pathway (Stagljar et al., 1994; te Heesen et al., 1994), and in cells that bear mutations in the oligosaccharyltransferase (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 glycoforms of CPY were synthesized by each of the *ost2* mutants at the permissive temperature (Fig. 3 *A*). As reported previously for yeast strains bearing conditional mutations in the Ost1p subunit of the yeast oligosaccharyltransferase (Silberstein et al., 1995), the severity of the in vivo glycosylation defect at 25°C for an *ost2* allele correlated with the growth defect at the permissive temperature. The mutants that showed the more severe pattern of underglycosylation of CPY (*ost2-3* and *ost2-4*) grew at roughly half the wild-type rate at the permissive temperature (data not shown). Endoglycosidase H digestion of the CPY immunoprecipitates from the *ost2* mutants yielded a single form of CPY which comigrated with unglycosylated vacuolar CPY (data not shown) indicating that intracellular transport of CPY is not grossly impaired at the permissive temperature. Two additional *ost2* mutants that were not analyzed in this experiment (*ost2-5* and *ost2-6*) were shown to underglycosylate CPY by protein immunoblotting using antisera to CPY (data not shown).

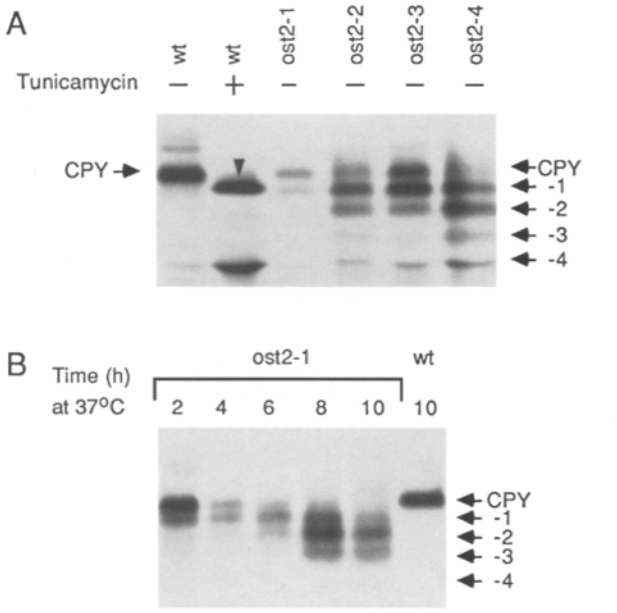


*Figure 3.* The *ost2* mutants are defective in core oligosaccharide transfer to CPY in vivo. CPY immunoprecipitates from glass-bead extracts of cells labeled for 1 h with Tran-$^{35}$S-Label were resolved by PAGE in SDS. Fully glycosylated vacuolar CPY and underglycosylated variants of CPY lacking between 1 and 4 N-linked oligosaccharides are indicated by labeled arrows. (*A*) The *ost2* mutants underglycosylate CPY at the permissive temperature. Wild-type (RGY216) and *ost2* mutant cells were grown in minimal media at 25°C before labeling. As indicated, wild-type cells were incubated for 15 min with tunicamycin before labeling. A vertical arrowhead in the tunicamycin-treated sample designates an ER-arrested form of proCPY (Stevens et al., 1982). (*B*) Extended incubation of *ost2-1* cells at 37°C results in reduced glycosylation of CPY. The *ost2-1* cells were grown for 2–10 h after shift to 37°C before a 1-h labeling period. CPY immunoprecipitates from wild-type cells incubated for 10 h at 37°C before labeling are included as a control.

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 oligosaccharyltransferase complexes at the restrictive temperature. Consistent with this hypothesis, protein immunoblot experiments revealed a reduced cellular content of several oligosaccharyltransferase subunits (Wbp1p, Swp1p, and Ost2p) upon extended incubation of *ost2-1* cells at the restrictive temperature (data not shown).

Both mannose donors for the synthesis of the lipid-linked oligosaccharide (i.e., GDP-mannose and dolichol-P-mannose) also serve as the donors for the biosynthesis of O-linked oligosaccharides in yeast (Kukuruzinska et al., 1987). The secreted O-linked glycoprotein chitinase was isolated from 25°C cultures of wild-type (RGY216), four *ost2* mutants, and a *Δgda1* mutant (G2-12) as described (Kuranda and Robbins, 1991; Abeijon et al., 1993) to determine whether O-linked glycosylation was affected by mutations in the *OST2* gene. Chitinase isolated from the *ost2* mutants comigrated with chitinase isolated from the wild-type strain, but was well resolved from chitinase isolated from the *Δgda1* strain (data not shown) which bears truncated O-linked oligosaccharides due to reduced transport of GDP-mannose into the Golgi (Abeijon et al., 1993). We conclude that O-linked glycosylation is normal in the *ost2* mutants.

### *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 $N^{\alpha}$ -Ac-Asn-[$^{125}$I]Tyr-Thr-$NH_2$ 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 oligosaccharide donor assembly pathway should not influence the in vitro oligosaccharyltransferase activity as the assays were supplemented with exogenous bovine dolichol-linked oligosaccharide. We conclude that mutations in the *OST2* gene cause reductions in the oligosaccharyltransferase activity at both the permissive and restrictive temperature.

[$^3$H]Mannose-labeled dolichol-linked oligosaccharides were isolated from *ost2-1* and *ost2-3* yeast cells grown at 25°C and were compared to the dolichol-oligosaccharides isolated from wild-type and *alg5-1* yeast to determine whether mutations in the *OST2* gene block lipid-linked oligosaccharide assembly. Unlike the *alg5-1* mutant, which synthesizes $Man_9GlcNAc_2$-PP-dolichol as the predominant oligosaccharide donor due to a defect in dolichol-P-glucose synthesis (Runge et al., 1984), the *ost2-1* mutant synthesizes $Glc_3Man_9GlcNAc_2$-PP-dolichol as the largest oligosaccharide donor (Fig. 4 *B*). Essentially identical results were obtained for *ost2-3* (data not shown). Consistent with the observation that transfer of oligosaccharides is reduced in an *ost2* mutant relative to a wild-type cell, more than 90% of the radiolabeled lipid-linked oligosaccharide from the *ost2* mutant was fully assembled (i.e., $Glc_3Man_9GlcNAc_2$-PP-dolichol). In contrast, ~30% of the labeled

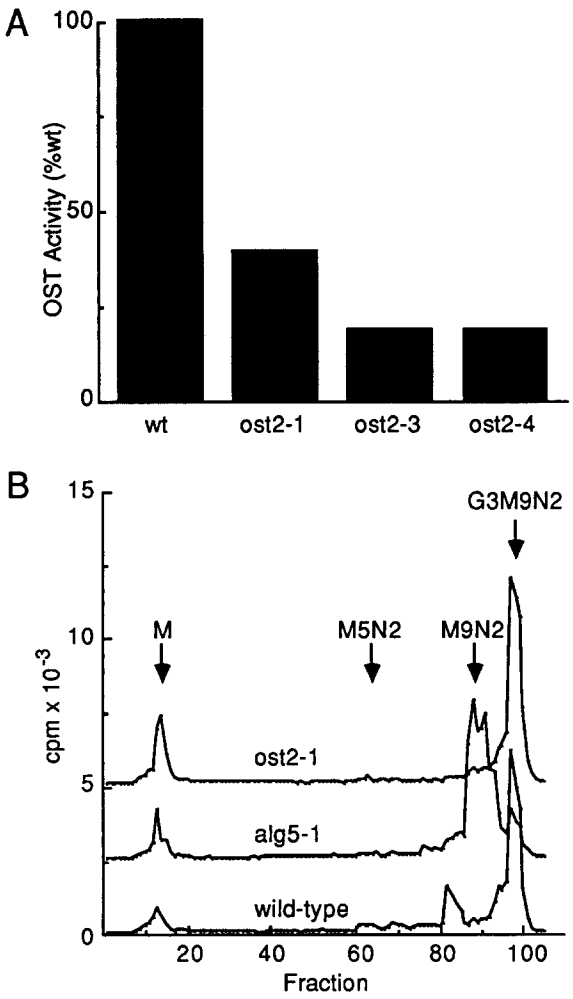


*Figure 4.* Oligosaccharyltransferase activity, but not dolichol-linked oligosaccharide assembly, is effected by mutations in the *OST2* gene. (*A*) Oligosaccharyltransferase activity of the *ost2* mutants. Microsomal membranes isolated from the wild-type (RGY216) and *ost2* mutant yeast were assayed 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 $min^{-1}mg^{-1}$. (*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 [$^3$H]mannose as described in Materials and Methods. Dolichol-linked oligosaccharides were isolated as described in Materials and Methods, and the composition of the [$^3$H]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, $Man_5GlcNAc_2$-PP-dolichol; M9N2, $Man_9GlcNAc_2$-PP-dolichol; and G3M9N2, $Glc_3Man_9GlcNAc_2$-PP-dolichol.

dolichol-oligosaccharides from the wild-type cells are assembly intermediates.

### *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 two-fold 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*).

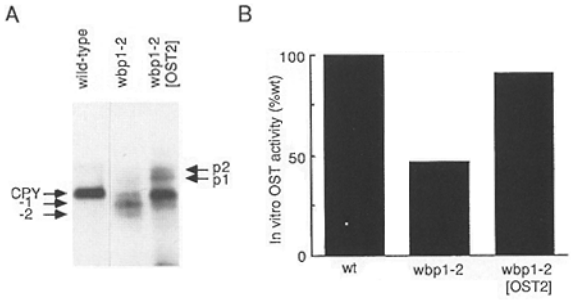


*Figure 5.* High-copy suppression of the *wbp1-2* mutant by Ost2p. (*A*) Glycosylation of CPY. Wild-type (RGY216), *wbp1-2*, and *wbp1-2* cells bearing the pRS426-OST2 (*wbp1-2*[OST2]) were grown in minimal media at 25°C before labeling for 1 h with Tran-$^{35}$S-Label. CPY immunoprecipitates from glass-bead 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 left 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 $min^{-1}mg^{-1}$.

### *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

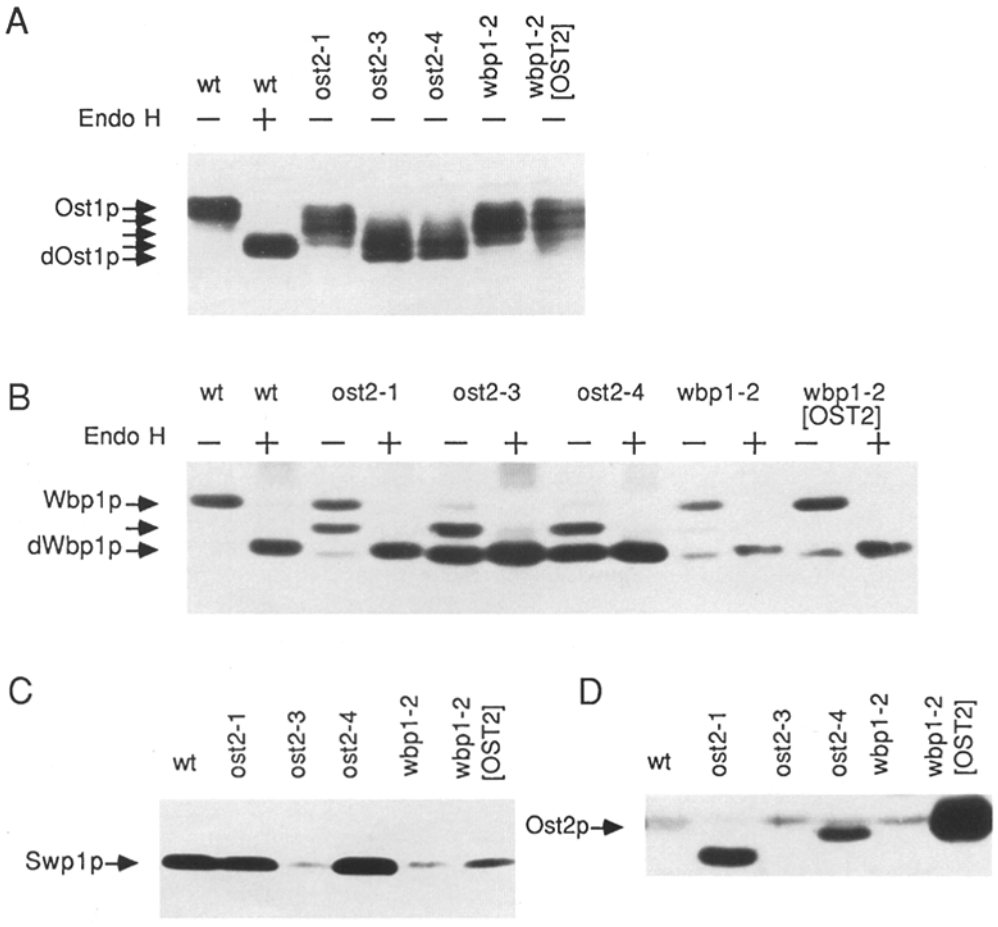


*Figure 6.* Expression of Ost1p, Wbp1p, Swp1p, and Ost2p. Membranes that were isolated from wild-type (RGY216), several *ost2* mutants, *wbp1-2*, and *wbp1-2*[OST2] were resolved by PAGE in SDS, transferred to PVDF membranes and probed with antiserum specific for Ost1p (*A*), Wbp1p (*B*), Swp1p (*C*) or Ost2p (*D*). As indicated, samples were incubated overnight in the presence of Endo H before gel electrophoresis. Immunoreactive proteins were detected as described in the Materials and Methods. Fully glycosylated (Ost1p), underglycosylated, and Endo H–digested (dOst1p) forms of Ost1p are indicated by arrows. Glycoforms of Wbp1p are indicated by the arrow labeled Wbp1p, while the unglycosylated and Endo H–digested forms of Wbp1p are indicated by the arrow labeled dWbp1p. Each gel lane contained the following amounts of membrane protein: *A*, 30 μg; *B*, 10 μg; *C*, 60 μg; *D*, 60 μg with the exception of the *wbp1-2*[OST2] which contained 3 μg.

evidence 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 Ost2 protein 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 DAD1 protein is hypothesized to be a negative regulator of apoptosis (Nakashima et al., 1993). The human and hamster DAD1 sequences are identical, whereas the *Xenopus laevis* DAD1 protein is 91% identical to the mammalian DAD1 protein (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-

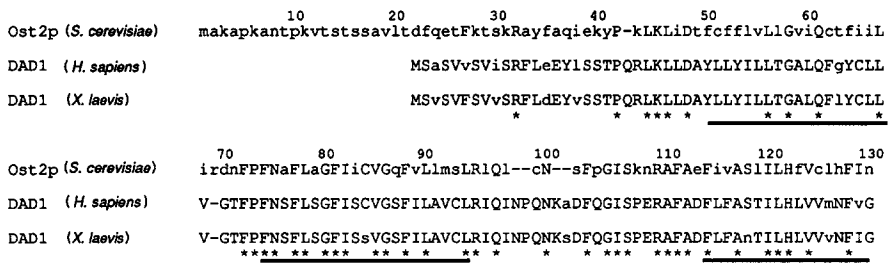


*Figure 7.* Sequence alignment between yeast Ost2p and vertebrate DAD1. The amino acid sequence of Ost2p was aligned with the sequences of the human (A54437) and *X. laevis* (B54437) DAD1 protein. Amino acid identities between any of the two sequences are shown in upper case letters. Identical residues in all three sequences are indicated by asterisks below the *X. laevis* sequence. The three conserved hydrophobic segments are underlined.

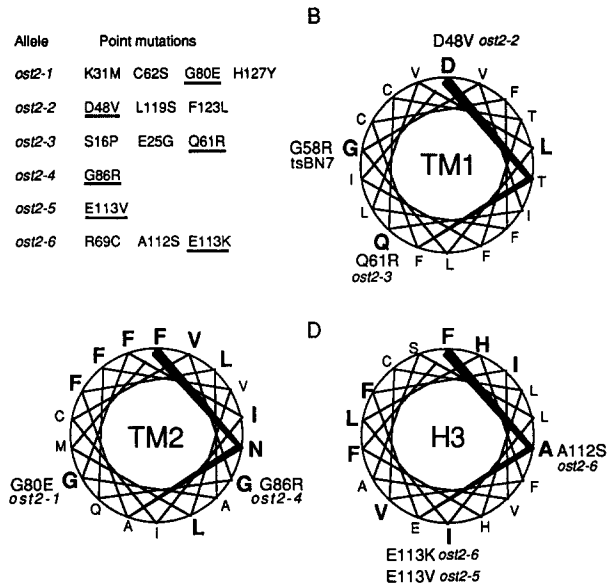


*Figure 8.* Point mutations in the *OST2* gene. The coding sequence of six *ost2* mutant alleles was determined by sequencing both DNA strands. (*A*) Amino acid changes are shown for each allele using the one letter code for amino acids and the position in the Ost2p sequence. Underlined substitutions are at invariant, or highly conserved, sites in the Ost2p sequence. (*B–D*) Helical wheel representations of the three hydrophobic segments of Ost2p. Amino acid residues conserved between *S. cerevisiae* Ost2p, *H. sapiens* DAD1, and *X. laevis* DAD1 are shown in boldface 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 I65. (*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.

dues 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., 1995*a*). 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., 1995*a*). 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., 1995α). Notably, none of the three cysteine residues in Wbp1p are conserved in the canine (Silberstein et al., 1992), human (D29643), avian (Kumar et

al., 1994), or nematode (Z36753) OST48 sequences, suggesting that a direct role for an active site cysteine in catalysis is unlikely. Although the membrane topology of Ost2p appears to be inconsistent with a direct role for Ost2p in catalysis, it is worth noting that the Ost2p sequence contains five cysteine residues, one of which is conserved between mammals and yeast.

Upon purification of the yeast hexameric OST complex, we speculated that mammalian homologues of the yeast 34- and 16-kD subunits may exist, but that these polypeptides might have dissociated during purification of the canine enzyme (Kelleher and Gilmore, 1994). Nonetheless, a homology between the Ost2p subunit of the oligosaccharyltransferase and the previously characterized DAD1 protein was not anticipated. Experiments designed to determine whether the DAD1 protein is a subunit of the canine oligosaccharyltransferase are currently 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 condensation, vacuolization of the cytoplasm and subsequent DNA fragmentation in tsBN7 cells after shift to the restrictive temperature (Nakashima et al., 1993). Could a previously unanticipated connection between apoptosis and N-linked glycosylation have been revealed by the conditional DAD1 mutation in the tsBN7 cells? Inhibition of an essential cellular function, such as N-linked glycosylation, 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 glycosylation by tunicamycin treatment is reported to induce apoptosis of HL-60 cells (Pérez-Salva and Mollinedo, 1995).

Allele-specific suppression of the *wbp1-2* mutant by overexpression of Ost2p occurs by an enhancement, albeit incomplete, of the in vivo and in vitro glycosylation activity of the *wbp1-2* mutant. Thus, suppression of the *wbp1-2* mutant by overexpression of the Ost2 protein does not occur by a bypass mechanism that permits the survival of glycosylation-defective yeast. Allele specific suppression of *wbp1* mutants was previously observed for the high-copy suppression of the *wbp1-2* 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, hydropathy analysis and HIS4-gene fusion experiments indicate that three of the previously characterized subunits of the yeast OST (i.e., Wbp1p, Swp1p, and Ost1p) are integral membrane proteins comprised 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; Silberstein et al., 1995). Hydropathy analysis and proteolysis experiments predict that Ost2p spans the membrane at least twice, with the hydrophilic amino terminus located in the cytosol. Considering the lumenal location of the oligosaccharyltransferase activity, the predicted topology and high degree of sequence conservation of the Ost2 protein is unexpected. Conceivably, several subunits of the oligosaccharyltransferase complex may not participate directly 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 reduces oligosaccharide transfer to nascent glycoproteins in vivo (Karaoglu et al., 1995). Possible roles for OST subunits not directly engaged in catalysis could include mediating interactions with the components of the translocation 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*). Significantly, 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 $\alpha$ helices perform a crucial role in the structural integrity of integral membrane proteins that are composed of helical bundles (Deisenhofer et al., 1985). The more polar face of a membrane-spanning helix is likely involved in interactions with an adjacent helix, while the more hydrophobic 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 contribute to helical interactions between subunits of oligomeric proteins by the formation of salt bridges and hydrogen 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 speculate that these portions of the protein contact the membrane-spanning segments of other oligosaccharyltransferase subunits.

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