The highly conserved Stt3 protein is a subunit of the yeast oligosaccharyltransferase and forms a subcomplex with Ost3p and Ost4p

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The oligosaccharyltransferase has been purified from Saccharomyces cerevisiae as an hetero-oligomeric complex composed of four or six subunits. Here, the in vivo subunit composition and stoichiometry of the oligosaccharyltransferase were investigated by attaching an epitope coding sequence to a previously characterized subunit gene, OST3. Five (Ost1p, Wbp1p, Swp1p, Ost2p, and Ost5p) of the seven polypeptides that were immuno-noprecipitated with the epitope-tagged Ost3p were identical to those obtained by the conventional purification procedure. Two additional coprecipitating polypeptides with apparent molecular masses of 60 and 36.6 kDa were identified as the 78-kDa Stt3 protein and the 36-residue Ost4 protein, respectively. Stt3p and Ost4p were previously identified in screens for gene products involved in N-linked glycosylation. Quantification of the in vivo radiolabeled subunits and the radiiodinated purified enzyme shows that the yeast oligosaccharyltransferase is composed of equimolar amounts of eight subunits. Exposure of the immunoprecipitated oligosaccharyltransferase to mild protein denaturants yielded a subcomplex comprised of Stt3p, Ost3p, and Ost4p. These experiments, taken together with genetic and biochemical evidence for subunit interactions, suggest that the enzyme is composed of the following three subcomplexes: (a) Stt3p-Ost4p-Ost3p, (b) Swp1p-Wbp1p-Ost2p, and (c) Ost1p-Ost5p.

N-Glycosylation of proteins is an essential, highly conserved protein modification reaction that occurs in all eukaryotic organisms. The oligosaccharyltransferase (OST) catalyzes the transfer of a preassembled high mannose oligosaccharide (Glc3Man9GlcNAc2) onto Asn-X-Ser/Thr acceptor sites on nascent polypeptides as they are translocated into the lumen of the rough endoplasmic reticulum (for reviews see Refs. 1 and 2). Biochemical, molecular biological, and genetic studies have led to the identification of a surprisingly large number of proteins that are required for the expression of wild-type OST activity.

The yeast OST was initially purified as an oligomeric complex composed of six subunits that are designated as Ost1p (62/64 kDa), Wbp1p (45 kDa), Ost3p (34 kDa), Swp1p (30 kDa), Ost2p (16 kDa), and Ost5p (9 kDa) (3). However, catalytically active tetrameric OST complexes that appear to lack Ost2p and Ost5p have also been described (4, 5). In addition, the 34-kDa Ost3 protein appears to be present in reduced amounts relative to the other three subunits in the purified OST tetramer (4), raising the possibility that oligomeric forms of the OST may exist that differ with respect to the presence of regulatory or accessory subunits.

Prior to purification of the yeast enzyme, genetic and biochemical studies had established that Wbp1p and Swp1p were essential for in vivo and in vitro expression of OST activity (6, 7). Mutations in genes encoding Ost1p, Ost2p, Ost3p, and Ost5p also cause substantial reductions in both the N-linked glycosylation of proteins in vivo and the transfer of dolichol-linked oligosaccharides to acceptor peptide substrates in vitro (8–11). Defects in the transfer and modification of N-linked oligosaccharides cause morphological, biochemical, and structural alterations in the yeast cell wall (12). Yeast mutants that are defective in the synthesis of the fully assembled dolichol oligosaccharide donor (alg mutants, i.e. asparagine-linked glycosylation) or in the elongation of the N-linked oligosaccharide display an enhanced resistance to sodium vanadate (13) and are hypersensitive to aminoglycoside antibiotics (14). The ost4 mutants, which were isolated based upon enhanced resistance to sodium vanadate, express greatly diminished OST activity in vivo and in vitro (15). The OST4 gene encodes a 36-residue hydrophobic protein that was not detected when the OST was purified.

The glycosylation defect caused by the wbp1-2 mutation is exaggerated when assembled of the optimal oligosaccharide donor (Dol-PP-GlcNAc2-ManαGlcα) for the OST is prevented by a mutation in the ALG5 gene, resulting in a synthetic lethal phenotype (16). Based upon this observation, two genetic screens were devised, one of which was selective for mutations that affect assembly of the oligosaccharide donor (17), and a second that was selective for genes encoding the OST subunits (11). In addition to mutant alleles of genes that encode six of the known OST subunits (Wbp1p, Swp1p, Ost1p, Ost2p, Ost3p, and Ost5p) the latter screen yielded mutants in the STT3 locus (11). Because the 78-kDa Stt3 protein had not been detected as a subunit in the purified OST complex (3, 4, 5), several alternative roles were proposed for the Stt3p (17).

To determine the composition and subunit stoichiometry of the native yeast OST, we appended an epitope recognized by an antibody raised against the influenza virus hemagglutinin (HA) to either the C terminus of Ost3p or Stt3p and expressed the epitope-tagged proteins in Saccharomyces cerevisiae. Non-denaturing immunoprecipitation of the OST from radiolabeled...
cultures of a yeast strain expressing the epitope-tagged Ost3 protein showed that the yeast enzyme is composed of eight subunits (Stt3p, Ost1p, Wbp1p, Ost3p, Swp1p, Ost2p, Ost5p, and Ost4p) in approximately equimolar amounts. After exposure to mild denaturants Stt3p, Ost3p, and Ost4p remain bound to the immunoaffinity reagent suggesting that these subunits form a stable subcomplex within the oligosaccharyltransferase. These results are discussed in the context of a model for the structural organization of the OST complex.

EXPERIMENTAL PROCEDURES

Construction of Yeast Strains

Epitope-tagged Ost3p Strains—Standard yeast genetics (18) and molecular biology (19) techniques were used for all strain constructions. The termination codon (nt 1370–1372) of the OST3 gene was replaced with an MscI restriction site (underlined) by PCR-based site-directed mutagenesis (20) to obtain the sequence 5' AAATGGCCAG 3' using the plasmid pOST3-1 (10) as the template. The resulting PCR fragment (nt 1098–1545) was subcloned into EcoRI-XbaI-digested pRS306OST3 (nt 1–1654 of the OST3 gene) to obtain pRS306OST3-M. A 90-bp DNA fragment encoding three tandem copies of the influenza virus hemagglutinin epitope (21) was obtained by PCR amplification of plasmid pTEP1 (20) using 5' TACCCATACGATGTTCCTGAC 3' and 5' TCAGTGCCATGTAATCAATCGGTTCCAGCATC 3' (underlined) as primers. The PCR product encoding the HA epitope was fused to a 269-bp PCR fragment corresponding to nucleotides 1098–1367 of the OST3 gene and amplified using a PCR ligation-PCR mutagenesis procedure (23). The final DNA fragment was digested with EcoRI and MscI and cloned into the EcoRI-MscI-digested pRS306OST3-M to obtain plasmid pRS306OST3-HA3 encoding Ost3p bearing a C-terminal triple HA epitope tag. After digestion with MluI and EcoRI to delete nucleotides 287–1098 in the OST3 gene, plasmid pRS306OST3-HA3 was used to transform strain RGY323 (MATa ure3-52 leu2-1 lys2-201 ade2-101 trp1-1 his3-Δ200) (10), MA9-D (MAT a wpb1-2 ura3-52 lys2-801 ade2-101 his3-Δ200), and MA7-B (MATa wpbl-1 ura3-52 lys2-801 ade2-101 his3-Δ200) (7) to uracil prototrophy. The resulting strains are designated RGY329, RGY331, and RGY332, respectively.

MscI (22) using an internal deletion fused to the triple HA tag followed by 175 bp of the 3' untranslated sequence from the OST3 gene (24) were amplified using S. cerevisiae genomic DNA as a template. The PCR products were ligated and further amplified as described above. The resulting DNA fragment was digested with EcoRI and MscI and cloned into the EcoRI-MscI-digested pRS306OST3-M to obtain plasmid pRS306OST3-HA3 encoding Ost3p bearing a C-terminal triple HA epitope tag. After digestion with MluI and EcoRI to delete nucleotides 287–1098 in the OST3 gene, plasmid pRS306OST3-HA3 was used to transform strain RGY323 (MATa ure3-52 leu2-1 lys2-801 ade2-101 trp1-1 his3-Δ200) (10), MA9-D (MAT a wpb1-2 ura3-52 lys2-801 ade2-101 his3-Δ200), and MA7-B (MATa wpbl-1 ura3-52 lys2-801 ade2-101 his3-Δ200) (7) to uracil prototrophy. The resulting strains are designated RGY329, RGY331, and RGY332, respectively.

wbp1-2 Strains—A two-step PCR-based gene disruption method described above was used to amplify a DNA fragment containing a heterologous HIS3 marker flanked by 5' (218 to 143) and 3' (1633 to 2514) regions of the STT3 gene (24) were amplified using S. cerevisiae genomic DNA as a template. The PCR products were ligated and further amplified as described above. The resulting DNA fragment was digested with HindIII and subcloned into HindIII-MscI-digested pRS306OST3-HA3 encoding Ost3p bearing a C-terminal triple HA epitope tag. After digestion with MluI and EcoRI to delete nucleotides 287–1098 in the OST3 gene, plasmid pRS306OST3-HA3 was used to transform strain RGY323 (MATa ure3-52 leu2-1 lys2-801 ade2-101 trp1-1 his3-Δ200) (10), MA9-D (MAT a wpb1-2 ura3-52 lys2-801 ade2-101 his3-Δ200), and MA7-B (MATa wpbl-1 ura3-52 lys2-801 ade2-101 his3-Δ200) (7) to uracil prototrophy. The resulting strains are designated RGY329, RGY331, and RGY332, respectively.

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STT3 Strains—A 1655-bp HindIII-Styl fragment derived from pOST3-1 (10) was subcloned into the HindIII-Styl-digested 2-μm plasmid pRS426 (26). The resulting plasmid, pRS426OST3, or the vector pRS426 was transformed into the strains MA7-B and MA9-D (7). Transformants selected at 25 °C on synthetic complete medium lacking uracil were tested for growth at 25 and 37 °C. A 30-amino acid sequence corresponding to three tandem repeats of the HA epitope was appended to the C terminus of Ost3p to serve as an affinity tag for immunopurification of the yeast OST. A one-step allele replacement method was used to substitute the epitope-tagged OST3 allele for the chromosomal copy of OST3 in a haploid yeast strain. Replacement of the wild-type Ost3 protein with the epitope-tagged Ost3 protein had no detectable effect on cell growth or in vivo OST activity indicating that HA-tagged Ost3p is fully functional (not shown).

Protein immunoblot and denaturing immunoprecipitation experiments showed that the monoclonal anti-HA antibody recognized a protein of about 36 kDa in total cell extracts prepared from a strain that expresses the HA-tagged Ost3 protein but not from a strain that expresses untagged Ost3p (not shown). To identify proteins that were specifically associ-
Immunopurification of the yeast OST using epitope-tagged Ost3p. Nondenaturing immunoprecipitates from [35S]methionine-labeled cell extracts prepared from yeast strains that express either untagged Ost3p (−) or HA-tagged Ost3p (+) were resolved in SDS using Tris-Tricine-buffered 10% polyacrylamide gels. The gels were either (A) dried and exposed to an x-ray film or (B) transferred to a PVDF membrane and probed with antibodies to Ost1p, Wbp1p, HA-Ost3p, Swp1p, Ost2p, and Ost5p. The asterisk indicates the IgG heavy chain. A, light and dark exposures of the gel are shown to permit the detection of Ost2p and Ost4p.

To calculate the relative molar amounts of the OST subunits in the immunoprecipitates, polyacrylamide gels (Fig. 1A) containing [35S]methionine-labeled samples were quantified. The relative intensity of each radiolabeled polypeptide should be directly proportional to the methionine content of each subunit provided that the subunits are present in equimolar amounts. The calculated amounts of the OST subunits relative to the tagged Ost3p protein were as follows: Ost1p (3.2), Wbp1p (2.4), Ost3p (1.0), Swp1p (0.9), Ost2p (1.5), and Ost4p (0.4). The apparent excess of Ost1p and Wbp1p relative to the other subunits prompted further examination of this region of the autoradiogram. Protein immunoblots of the native immunoprecipitate revealed a glycoform doublet for Ost1p (Fig. 1B) rather than the broad band that was visible in the native immunoprecipitate (Fig. 1A). A successive immunoprecipitation experiment was performed to determine whether the excess radiolabel in the Ost1p region of the gel could be explained by a fortuitous comigration of Ost1p with another polypeptide. After the Ost was immunoprecipitated under nondenaturing conditions from the HA-tagged Ost3p strain (Fig. 2A, lane b), the immune complexes were denatured in SDS and subjected to a second immunoprecipitation using an antibody to Ost1p. The Ost1p glycoform doublet that was recovered after denaturing immunoprecipitation (Fig. 2A, lane c) migrated slightly slower than an intensely labeled diffuse band that was recovered in the unbound fraction with the other OST subunits (Fig. 2A, lane d). Given the genetic evidence supporting a role for the Stt3 protein in N-linked glycosylation (11), the 78-kDa Stt3 protein (17) was the most likely candidate for the diffusely migrating polypeptide.

Subunit Composition and Stoichiometry of the Yeast OST—To obtain more direct evidence that Stt3p is a subunit of the OST, total cell extracts were prepared from [35S]methionine-labeled cultures of a yeast strain that expresses HA-tagged Stt3p (Fig. 2B). At least four of the polypeptides (Wbp1p, Swp1p, Ost2p, and Ost4p) that copurified with HA-tagged Ost3p (Fig. 2B, lane b) comigrated precisely with proteins that copurified with HA-tagged Stt3p (Fig. 2B, lane c). The identity of the HA-tagged Stt3 protein was confirmed by immunoprecipitating the tagged protein from SDS-denatured cell extracts (Fig. 2B, lane d). The addition of the 3-kDa affinity tag to the C terminus of Stt3p is responsible for the decreased mobility of HA-tagged Stt3p relative to untagged Stt3p (Fig. 2B, compare lanes b and c). Endoglycosidase H digestion of the HA-tagged Stt3p (Fig. 2B, lane b) increased the mobility of the protein consistent with a previous report that the luminal domain of Stt3p contains at least one N-linked oligosaccharide (17). Deglycosylated Stt3p migrated as a broad band indicating that heterogeneous glycosylation is not responsible for the diffuse electrophoretic migration of the polypeptide. As expected, the untagged Ost3 protein migrated slightly slower than Swp1p (Fig. 2B, lane c).

Although the preceding experiments demonstrated that the immunopurified OST complex contains Stt3p, a protein of this size had not been detected when the hexameric or tetrameric OST complexes were resolved by SDS-PAGE and stained with either Coomassie Blue or with Silver (3–5). To resolve this discrepancy, the OST was purified as described previously (3), denatured in SDS, and radiolabeled with 125I. Resolution of the radioiodinated OST subunits by PAGE in SDS revealed an intensely labeled polypeptide that migrated in the vicinity of Ost1p (Fig. 2B) rather than the well resolved Ost1p glycoform doublet that we detect by staining with Coomassie Blue. Radiolabeled bands corresponding to the other OST subunits (Wbp1p, Ost3p, Swp1p, Ost2p, and Ost5p) were also detected. Radiolabeled aprotinin (~6.5 kDa), a protease inhibitor that is included in the buffer used for the OST purification (Fig. 2B), was not effectively separated from 9.5-kDa Ost5p on this gel but was resolved on a 7.5–17.5% polyacrylamide gel (see Fig. 3). We were not able to unambiguously assign a radiolabeled band to Ost4p due, in part, to the presence of a single tyrosine residue in this protein.

The subunit stoichiometry of the yeast OST was recalculated...
in light of the discovery that Stt3p was not resolved from Ost1p in Fig. 1. The value presented in Table I for the molar ratio of Ost1p and Stt3p relative to Ost3p assumes that equal amounts of Ost1p and Stt3p are present. The results of this analysis suggest that the OST subunits are present in equimolar amounts in the immunopurified complex. Wbp1p and Ost2p each contain a single methionine residue; consequently, the values we obtain for these subunits are subject to the greatest error. The subunit stoichiometry was also calculated after quantification of the radioiodinated OST subunits (Fig. 2B and

Table I

| Subunit    | Molecular mass | Number of methionines | Molar ratio | Number of tyrosines | Molar ratio |
|------------|----------------|-----------------------|-------------|---------------------|-------------|
| Stt3p + Ost1p | 78 + 64/62 | 21 + 6 | 0.7 | 38 + 21 | 1.0 |
| Wbp1p     | 45            | 1             | 2.4 | 20             | 1.0 |
| Ost3p     | 34            | 8             | 1.0 | 9               | 1.0 |
| Swp1p     | 30            | 3             | 0.9 | 7               | 1.3 |
| Ost2p     | 16            | 1             | 1.5 | 2               | 1.0 |
| Ost5p     | 9.5           | 0             | N.D. | 5               | 1.3 |
| Ost4p     | 3.6           | 4             | 0.4 | 1               | N.D. |

a The relative molar amounts of OST subunits, labeled either with [35S]methionine (Fig. 1A) or with 125I (Fig. 2B and Fig. 3), were estimated by scanning polyacrylamide gels using a PhosphorImager. The signal produced by each of the subunits was normalized to the number of methionine or tyrosine residues predicted by the amino acid sequence of the mature protein as indicated.

b As Ost1p and Stt3p were not resolved on the gels, they were treated as a group, and the signal produced together by both of the subunits was normalized to the total number of methionine or tyrosine residues in these two proteins.

Fig. 3. If the OST complex contains equimolar amounts of the subunits, the incorporation of 125I should be proportional to the tyrosine content of the subunits provided that the radiodination efficiency of individual tyrosine residues is comparable after denaturation with SDS. Quantification of the 125I-labeled OST subunits also indicated that the seven larger OST subunits are present in roughly equimolar amounts in the purified yeast OST complex (Table 1).

The epitope-tagged Ost3p strain provided a means to determine whether Ost3p is present in reduced stoichiometry relative to the other subunits of the OST complex as suggested by Pathak et al. (4). If that were the case, yeast may contain a population of OST complexes that lack Ost3p in vivo. To address this question, the supernatants from nondenaturing immunoprecipitates of the untagged and epitope-tagged Ost3 mutant strains that express either untagged (−) or HA-tagged Ost3p or Stt3p (+) were resolved by PAGE in SDS using a 7.5%–17% gradient gel. The radiolabeled proteins corresponding to OST subunits are labeled. The supernatants after immunodepletion (a and b) or the total cell extracts (c) were precipitated with trichloroacetic acid and resolved by SDS-PAGE. After transfer to a PVDF membrane, the blots were probed with antibodies to Wbp1p, the HA epitope, or Swp1p. The IgG heavy chain and several cross-reactive bands are designated by an asterisk and triangles.
Deletion of Ost3p Does Not Disrupt the Oligosaccharyltransferase Complex—Disruption of the OST3 gene is not lethal in a haploid yeast strain but instead causes hypoglycosylation of proteins in vivo and a 2-fold reduction in the in vitro OST activity (10). Protein immunoblot analysis of the Δost3 mutant did not disclose an obvious reduction in the membrane content of Ost1p, Wbp1p, Swp1p, and Ost2p. Although this suggests that Ost3p is not critical for the assembly or stability of the OST complex, we sought more direct evidence concerning the composition of the oligosaccharyltransferase in the Δost3 mutant. The OST complex was immunopurified from radiolabeled cultures of the wild-type or Δost3 mutant strains expressing epitope-tagged OST subunits (Fig. 3). With the exception of Ost3p, comparable amounts of the radiolabeled OST subunits were present in the nondenaturing immunoprecipitates from the two wild-type strains and the Δost3 strain. These results strongly suggest that Ost3p is not required for the assembly or stability of the OST.

A radiolabeled band of 31 kDa (denoted by an arrowhead) was resolved on the 7.5–17% polyacrylamide gradient gel shown in Fig. 3. The 31-kDa protein was present in the immunoprecipitates from strains expressing HA-tagged Stt3p but not in the untagged control strain or the HA-tagged Ost3p strain. A search of the yeast protein sequence database revealed a hypothetical protein designated YML019W that is 21% identical in sequence to Ost3p. Moreover, Ost3p and YML019W have a similar predicted arrangement of an N-terminal signal sequence and four membrane spanning segments. The predicted molecular weight of the YML019W (34 kDa) is in reasonable agreement with the estimated size of the 31-kDa polypeptide. Further studies will be required to determine whether the 31-kDa polypeptide is YML019W.

Ost3p, Stt3p, and Ost4p Form a Subcomplex within the Oligosaccharyltransferase—We sought additional evidence to support the hypothesis that the 3.6-kDa polypeptide in the immunopurified OST complex is Ost4p. Because expression of Ost4p is not essential for viability of yeast at 25 °C, the OST4 gene was disrupted in yeast strains that express the HA-tagged OST subunits. The compositions of the OST complexes that were immunopurified from the wild-type and Δost4 mutant strains were compared (Fig. 4). With the exception of HA-tagged Ost3p, only trace amounts of the OST subunits were recovered in nondenaturing immunoprecipitates from the Δost4 mutant that expresses HA-tagged Ost3p (Fig. 4, lane b). The 3.6-kDa polypeptide was clearly absent when the OST was immunopurified from a Δost4 mutant that expresses HA-tagged Stt3p (Fig. 4, lane e). Since the absence of the 3.6-kDa polypeptide in the immunopurified OST complex correlates with disruption of the OST4 gene, this experiment supports the identification of the 3.6-kDa protein as Ost4p. Untagged Ost3p migrated slightly slower than Swp1p on this gel (Fig. 4, lane d); a comparison of lanes d and e reveals that lane e also lacks Ost3p. The absence of untagged Ost3p in complexes isolated from the Δost4 mutant (Fig. 4, lane e) is consistent with the inability to immunoprecipitate the OST from the Δost4 mutant that expresses HA-tagged Ost3p (Fig. 4, lane b).

As in the preceding experiments, the immunopurified complexes were washed several times at 4 °C with a nonionic detergent/high salt buffer (Nikkol buffer) to maintain the integrity of the complex and to prevent nonspecific interactions (Fig. 4, lanes a, b, d, and e). Two sequential washes of the immunopurified complex with a Triton X-100/SDS-mixed micelle buffer followed by the mixed micelle buffer supplemented with 2 m urea eluted Wbp1p, Swp1p, Ost2p, and Ost4p (Fig. 4, lanes c and f). Elution of Ost1p from the immunoprecipitates was confirmed by protein immunohotting using the antibody to Ost1p (not shown). Surprisingly, Stt3p, Ost3p, and Ost4p were retained after successive washes with mixed micelle buffer supplemented with 2 m urea eluted Wbp1p, Swp1p, Ost2p, and Ost4p (Fig. 4, lanes c and f) or 500 mM NaCl (data not shown). The recovery of the 36-residue Ost4 protein in the denaturant-washed immunoprecipitates from the strain that expresses HA-tagged Stt3p was lower (Fig. 4, lane f), due, in part, to the reduced recovery of both Stt3p and Ost3p in the immunoprecipitate. Whereas only one of the three OST subunits contained the epitope tag in each strain, retention of all three subunits is diagnostic of an Ost3p-Ost4p-Ost3p subcomplex that is resistant to a short (5–10 min) exposure to mild denaturants. As expected, Ost3p was not present in the subcomplex isolated from the Δost4 mutant (Fig. 4, lane g).

Interaction of Wbp1p with Ost3p—We next asked whether the HA-tagged Stt3 and Ost3 proteins could be used to rapidly isolate the OST from the strains that express mutant OST subunits. The well-characterized wbp1-1 and wbp1-2 mutants were selected for this analysis (6, 7). Nondenaturing immuno-
precipitates from the epitope-tagged \textit{wbp1} mutants were resolved by PAGE in SDS (Fig. 5A). Regardless of whether the cells were radiolabeled for 20 min (not shown) or 1 h at the permissive temperature (Fig. 5A), the yield of all of the OST subunits, including epitope-tagged Ost3p, was greatly reduced when the HA-tagged Ost3p was the affinity ligand for isolation of the complex from the \textit{wbp1-1} or \textit{wbp1-2} mutants. With the exception of the tagged Ost3 protein, the radiolabeled OST subunits were scarcely more intense than protein contaminants that precipitate from an untagged wild-type strain. When the HA-tagged Ost3 protein was used as the affinity ligand for isolation of the complex from the \textit{wbp1} mutants, we observed reduced yields of Wbp1p, Swp1p, Ost3p, and Ost2p (Fig. 5A) and slightly reduced yields of the HA-tagged Stt3p.

Microsomes isolated from the \textit{wbp1-2} mutant contain reduced amounts of Wbp1p and Swp1p relative to microsomes isolated from wild-type cells (9). The results presented in Fig. 5A suggest that the Ost3 protein may also be unstable in the \textit{wbp1} mutants. To address this possibility, microsomes were isolated from the wild-type, \textit{wbp1-1}, and \textit{wbp1-2} mutant strains that express HA-tagged Ost3p or Stt3p. Protein immunoblotting that were probed with the anti-HA antibody showed a substantial reduction in the membrane content of Ost3p in both the \textit{wbp1-1} and \textit{wbp1-2} mutants (Fig. 5B). The steady state levels of HA-tagged Ost3p were extremely low in the \textit{wbp1-2} mutant but were detectable when greater amounts of membrane protein (80 \(\mu\)g) were loaded on the gel (not shown). In contrast, the Stt3p content of microsomes isolated from the \textit{wbp1} mutants and wild-type cells were quite similar (Fig. 5C).

The OST3 gene was disrupted in the \textit{wbp1-2} strain to explore the possible biological interaction between these gene products. The \textit{wbp1-2} \textit{ost3} double mutant is viable at 30 °C, yet grows significantly slower than the \textit{wbp1-2} mutant. Protein immunoblots of microsomes from wild-type yeast and from the \textit{wbp1-1}, \textit{wbp1-2}, \textit{ost3}, and \textit{wbp1-2 \textit{ost3}} mutants were probed with an antibody to Ost1p as a simple method to evaluate \textit{in vivo} activity (Fig. 5D). Wild-type yeast express two glycoforms of Ost1p that contain four and three N-linked oligosaccharides, respectively (3). As observed previously, hypoglycosylated forms of Ost1p are synthesized by the \textit{wbp1-2} mutant and the \textit{ost3} mutant (9, 10). When the two mutations are combined in a single strain, a more severe glycosylation defect is observed. Notably, the membrane content of Ost1p is not reduced in the \textit{wbp1} mutants, the \textit{ost3} mutant, or the \textit{wbp1-2 \textit{ost3}} double mutant. High copy expression of Ost3p in the \textit{wbp1-1} and the \textit{wbp1-2} mutants does not suppress the temperature-sensitive growth phenotype of either strain (not shown). However, overexpression of Ost3p partially corrects the glycosylation defect of the \textit{wbp1-1} mutant (Fig. 5E). Overexpression of Ost3p in the \textit{wbp1-2} mutant did not noticeably alter the average number of oligosaccharides transferred to Ost1p.

**DISCUSSION**

**In Vivo Analysis of the Subunit Composition of the OST—**Previous biochemical studies of the yeast oligosaccharyltransferase suggested that the enzyme contains four, five, or six subunits (3–5, 31). Here we have utilized yeast strains that express epitope-tagged OST subunits to define the \textit{in vivo} subunit composition and stoichiometry of the enzyme. The results we have obtained demonstrate that the native enzyme has a composition that is consistent with the subunit composition that has been suggested by genetic studies (11) and is remarkably similar to the OST complex that was purified by Kelleher and Gilmore (3). In addition to the six previously characterized subunits (Ost1p, Wbp1p, Swp1p, Ost3p, Ost2p, and Ost5p) two additional polypeptides routinely copurified with the epitope-tagged Ost3 protein. The radiolabeled 3.6-kDa polypeptide was identified as Ost4p based upon its migration on SDS-polyacrylamide gels and its absence from the immunopurified OST complex in a \textit{\textit{ost4}} mutant. The \textit{\textit{ost4}} gene was identified by Chi et al. (15) in a genetic screen for mutations that cause defects in the biosynthesis of N-linked oligosaccharides. Although the 3.6-kDa Ost4 protein is essential for viability of yeast at 37 °C, loss of this subunit causes a severe defect in the \textit{in vivo} transfer of oligosaccharides to nascent glycoproteins at 30 °C, suggesting that Ost4p is either a structural component of the enzyme or an accessory component that is important for OST assembly (15). Here we present biochemical evidence that the Ost4 protein is a structural subunit of the mature OST complex. Although the quantitative data suggest that Ost4p is not as abundant as the other subunits, a reduced recovery of proteins of this size is not unexpected when gels are fixed and dried. Notably, open reading frames of this size were not cataloged in the yeast genome project nor are proteins of this size re-
solved and detected by commonly used gel electrophoresis methods.

The 78-kDa Stt3p protein has an N-terminal hydrophobic domain with 12 predicted membrane-spanning segments and a C-terminal hydrophilic domain that is located in the endoplasmic reticulum lumen (17, 24). The apparent absence of Stt3p in both the tetrameric and hexameric OST preparations (3–5) led to the hypothesis that Stt3p might be a substoichiometric component of the OST that is important for assembly of the enzyme (17). Here, we have presented several lines of evidence conclusively demonstrating that the Stt3 protein is a subunit of the yeast OST. By using a yeast strain that expresses HA-tagged Ost3p, we were able to determine the subunit stoichiometry of the OST by rapidly isolating the enzyme from radiolabeled yeast cultures. Sequential immunoprecipitation experiments indicated that the bulk of the radiolabeled protein in the Ost1p–yeast cultures. Quantification of the [35S]methionine-labeled OST subunits strongly supports the conclusion that Stt3p is present in equimolar amounts relative to the six previously characterized subunits. Given that there was no indication of a labile association between Stt3p and the other OST subunits, how can we account for the apparent lack of Stt3p in the purified OST preparations? As presented in this study, the 78-kDa Stt3 protein has an anomalous gel mobility, migrating instead as a diffuse 60-kDa band that is not resolved from Ost1p under most electrophoresis conditions. Radiodination of the purified OST preparation revealed an intensely labeled diffuse polypeptide that comigrates with Ost1p. Apparently, the Stt3 protein stains poorly with Coomassie Blue and with silver, hence the diffuse migrating Stt3 protein was simply not detected in previous studies. The extreme hydrophobicity of the N-terminal region of Stt3p is presumably responsible for both the aberrant gel mobility and the poor staining characteristics.

The epitope-tagged strains were useful for evaluating the stability and the subunit composition of the OST complexes in mutants that express reduced OST activity. Notably, the composition and integrity of the OST was not detectably altered in the ost3 mutant, indicating that Ost3p is not required for the assembly or stability of the enzyme. We speculate that Ost3p is a peripherally located subunit within the OST complex. A possible role for Ost3p as an accessory subunit was suggested by the observation that loss of Ost3p causes substantial reductions in oligosaccharide transfer to some but not all acceptors (10). Here, we observed that most of the cellular pool of Wbp1p, Swp1p, or Ost1p copurified with the epitope-tagged Ost3 protein, indicating that the majority of the OST complexes in wild-type yeast contain Ost3p as a subunit.

In the absence of Ost4p, the association between Ost3p and the other OST subunits was labile, suggesting that the interaction between Stt3p and Ost3p is dependent upon Ost4p. In contrast to what was observed in the wbp1 mutants, the recovery of the HA-tagged Ost3p was comparable in the wild-type and the Δost4 mutant. One interpretation of this difference is that the association between Ost3p and the other OST subunits in the Δost4 mutant is destabilized by the detergents used for immunoprecipitation of the complex.

Evidence for Discrete Subcomplexes within the OST—A physical interaction between the Wbp1p, Swp1p, and Ost2p subunits (Fig. 6) is supported by complementary genetic and biochemical observations. The SWP1 gene and the OST2 gene are allele-specific high copy suppressors of the wbp1-2 mutant (6, 9). Microsomes isolated from the wbp1-2 mutant contain reduced amounts of Wbp1p and Swp1p at the permissive temperature (9). Suppression of the wbp1-2 growth defect by overexpression of Swp1p or Ost2p is explained by an enhanced stability of Wbp1p, which results in increased OST activity (6, 9). In vivo depletion of either Wbp1p or Swp1p leads to a reciprocal reduction in the membrane content of the other subunit (5). Wbp1p can be cross-linked to Swp1p in yeast microsomes suggesting a direct interaction between these two subunits (6).

Recent studies reveal that the purified canine OST contains DAD1 in addition to ribophorin I, ribophorin II, and OST48 (30). The four mammalian subunits are homologous to Ost2p, Ost1p, Swp1p, and Wbp1p, respectively (2). Cross-linking experiments have provided evidence for a direct physical interaction between the subunits of the canine enzyme (30). Readily identified cross-linked products included DAD1-OST48 heterodimers, ribophorin II-OST48 heterodimers, and ribophorin II-OST48-DAD1 heterotrimers (30). These biochemical findings strongly suggest that the Swp1p-Wbp1p-Ost2p subcomplex is a structural unit in the OST that is conserved between vertebrates and fungi. Several groups have obtained evidence suggesting that the active site of the OST is associated with Wbp1p or its vertebrate homologue OST48 (4, 34). Most recently, an epoxymethylglycine derivative of the tripeptide acceptor was shown to react with both OST48 and ribophorin I, suggesting that the active site may reside at an interface between OST48 and ribophorin I (35).

During the course of these experiments we obtained evidence for a direct interaction between Ost3p, Ost4p, and Ost4p. The Ost3p-Ost4p-Ost3p subcomplex was not disrupted by an exposure to Triton X-100/SDS-mixed micelle buffer that was supplemented with 2M urea or 0.5M NaCl. In contrast, at least four (Ost1p, Swp1p, Wbp1p, and Ost2p) of the five other OST subunits dissociated from the Ost3p-Ost4p-Ost3p subcomplex upon exposure to the mixed micelle buffer. As the mixed micelle buffer lacked reducing agents, we cannot rule out the possible stabilization of the Ost3p-Ost4p-Ost3p complex by disulfide bonds. Genetic support for an interaction between Ost3p and Ost3p was provided by the observation that immunopurified OST complexes lack Ost3p when expression of Stt3p was reduced in vivo by placing the STT3 gene under control of the
addition, overexpression of Ost3p suppresses the restrictive allele-specific high copy suppressors of stt3 sequence data base have revealed that the yeast Stt3 protein is lytic core of a larger complex that contains homologues of Stt3p, speculated that the purified canine enzyme may be the catalytic core of a larger complex that contains homologues of Stt3p, Ost3p, and perhaps Ost4p (30). Indeed, searches of the protein sequence data base have revealed that the yeast Stt3 protein is 60% identical in sequence to Caenorhabditis elegans, murine, and human proteins of unknown function (17). Northern blots have shown that the mRNA encoding the human homologue of Stt3p is expressed in all human tissues that were tested (39). A putative tumor suppressor gene encoding a human homologue of the Ost3p, which is called N33, was localized to chromosome band 8p22 (40). Although the sequence identity between Ost3p and N33 is only 20%, the two proteins share an identical arrangement of four predicted membrane-spanning segments. The expressed sequence tag data base contains mouse and human cDNAs that encode 37-residue proteins that are 36% identical in sequence to Ost3p. Given that yeast Stt3p and human cDNAs that encode 37-residue proteins are 36% identical in sequence to Ost3p, is drastically reduced in the strains that contain a mutant allele of an OST subunit. Here, we have obtained information about the structural organization of this large integral membrane protein prior to the elucidation of its three-dimensional structure.

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**Imunoaffinity Isolation of the Oligosaccharyltransferase**

Now that the composition of the yeast OST has been defined, the epitope-tagged strains can be used for the rapid and efficient isolation of the enzyme from wild-type and mutant strains for enzymatic studies. The results we have obtained concerning the Stt3p-Ost4p-Ost3p subcomplex may lead to the isolation of the homologous mammalian proteins once immunological probes for the putative homologues have been produced. Finally, by defining interactions between several OST subunits, we have obtained information about the structural organization of this large integral membrane protein prior to the elucidation of its three-dimensional structure.