The Saccharomyces cerevisiae Oligosaccharyltransferase
Is a Protein Complex Composed of Wbp1p, Swp1p, and Four Additional Polypeptides*

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Asparagine-linked glycosylation of proteins in the lumen of the endoplasmic reticulum is catalyzed by the oligosaccharyltransferase. Previously, the mammalian oligosaccharyltransferase was shown to copurify with a protein complex consisting of three integral membrane proteins: ribophorin I and ribophorin II and a nonglycosylated 48-kDa polypeptide designated OST48. Here, we describe the purification of the oligosaccharyltransferase from Saccharomyces cerevisiae. The yeast oligosaccharyltransferase complex is composed of six subunits (α, β, γ, δ, ε, and δ). The α subunit of the yeast oligosaccharyltransferase complex is a heterogeneously glycosylated protein with three glycoforms of 64, 62, and 60 kDa that contain, respectively, four, three, and two asparagine-linked oligosaccharides. The β and δ subunits were shown to correspond to the 45-kDa Wbpl glycoprotein and the 30-kDa Swpl protein, respectively. The Wbp1 and Swp1 proteins were previously shown to be essential for asparagine-linked glycosylation in vivo. The nonglycosylated γ, ε, and ζ subunits have apparent molecular masses of 34, 16, and 9 kDa. Homology between the yeast and mammalian oligosaccharyltransferase complexes first became evident when the 48-kDa subunit of the mammalian enzyme was found to be 25% identical in sequence with the Wbp1 protein. Here we present an alignment between the Swp1 protein and the carboxyl-terminal half of human ribophorin II that reveals that these two proteins are related gene products.

Asparagine-linked glycosylation of proteins in the lumen of the rough endoplasmic reticulum (RER) is catalyzed by the enzyme dolichol-diphosphoryl oligosaccharide: protein oligosaccharyltransferase. Acceptor sites within newly translocated polypeptides in the RER lumen contain the sequon Asn-X-Ser/Thr, where X can be any amino acid other than proline (Gavel and Von Heijne, 1990). The lipid-linked oligosaccharide donor (OS-PP-Dol) for asparagine-linked glycosylation is assembled by sequential transfer of monosaccharides to dolichol phosphate in a series of reactions that is initiated on the cytoplasmic face of the RER membrane and is completed within the RER lumen (Hirschberg and Snider, 1987; Kukuruzinska et al., 1987). The preferred lipid-linked oligosaccharide donor for the glycosylation reaction is the glucosylated high mannose oligosaccharide (Glc�Man,GlcnAc₃-PP-Dol) (Turco et al., 1977; Trimble et al., 1980). However, incompletely assembled lipid-linked oligosaccharides can serve as the donor in vitro and in vivo (Sharma et al., 1981; Huffaker and Robbins, 1983; Verostek et al., 1991, 1993). Although a purification of the oligosaccharyltransferase from Saccharomyces cerevisiae has not been reported, biochemical studies of the detergent-solubilized enzyme have revealed considerable similarity between the yeast and mammalian enzymes with respect to substrate specificity and divalent metal ion requirement (Trimble et al., 1980; Sharma et al., 1981). Remarkable progress toward the identification of the yeast oligosaccharyltransferase complex has been achieved by the analysis of a protein designated as wheat germ binding protein 1 (Wbp1) (te Heesen et al., 1991, 1992). The WBP1 gene encodes a 45-kDa integral membrane protein that is essential for vegetative growth of yeast and is localized to the yeast endoplasmic reticulum (te Heesen et al., 1991). Phenotypic analysis of yeast strains bearing a conditional mutation in the WBP1 gene (wbp1-1 and wbp1-2) has shown that the Wbp1 protein is required for asparagine-linked glycosylation of proteins in vivo and for oligosaccharyltransferase activity in vitro (te Heesen et al., 1992). A second gene (SWP1) encoding a 30-kDa polypeptide was identified as an allele-specific high copy suppressor of the wbp1-2 allele (te Heesen et al., 1989). Gene product depletion experiments indicate that the Swp1 protein is also required for expression of oligosaccharyltransferase activity in yeast (te Heesen et al., 1993). Overexpression of the WBP1 protein and the SWP1 protein, either alone or in combination, did not enhance the oligosaccharyltransferase activity of yeast microsomal membrane preparations (te Heesen et al., 1992, 1993) suggesting that an additional protein or proteins are required for oligosaccharyltransferase activity.

Oligosaccharyltransferase activity from canine pancreas co-purifies with a relatively abundant protein complex consisting of ribophorin I (Mᵦ = 66,000), ribophorin II (Mᵦ = 63,000), and OST48 (Mᵦ = 48,000) (Kelleher et al., 1992). The ribophorins are well characterized integral membrane glycoproteins that are restricted to the rough endoplasmic reticulum (Amar-Costesec et al., 1984; Marcantonio et al., 1984). Protein sequence analysis and protease accessibility studies indicate that ribophorin I, ribophorin II, and OST48 are all integral membrane proteins with the bulk of each polypeptide located within the lumen of the endoplasmic reticulum (Crimaudo et al., 1987; Harnik-Ort et al., 1987; Silberstein et al., 1992). Due to the extensive conservation of the donor and acceptor substrates for the oligosaccharyltransferase complex, the yeast enzyme is a good model for understanding the complex mammalian enzyme.

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1 The abbreviations used are: RER, rough endoplasmic reticulum; ConA, concanavalin A; DTT, dithiothreitol; ECL, enhanced chemiluminescence; HPLC, high pressure liquid chromatography; OST, oligosaccharyltransferase; OS-PP-Dol, dolichol-linked oligosaccharide; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; 3'-5'-ADP-agarose, 3'-5'-bis, bis-adenosine diphosphate agarose; PAGE, polyacrylamide gel electrophoresis.
chyltransferase, it would be surprising if the yeast and mammalian enzymes were not related despite the evolutionary distance between mammals and S. cerevisiae. Indeed, a comparison of the protein sequence of OST48 and Wbp1p has revealed that these two polypeptides are 25% identical in sequence (Silberstein et al., 1992). In contrast, the sequence of the 30-kDa Swp1p has been reported to be unrelated to both ribophorins I and II (te Heesen et al., 1993). Here, we report the purification of the yeast oligosaccharyltransferase as a complex composed of six subunits. An initial characterization of the six subunits of the yeast oligosaccharyltransferase shows that two of the subunits correspond to the Wbp1p and Swp1 proteins. A protein sequence comparison indicates that the yeast Swp1 protein is related to the carboxy-terminal half of mammalian ribophorin II with respect to amino acid sequence and organization of hydrophobic segments.

**MATERIALS AND METHODS**

**Isolation of Yeast Microsomal Membranes**—The protease-deficient yeast strain ET101 (MATa his1 prb1-122 pef1-126) grown at 25 °C in 1% yeast extract, 2% peptone, 2% dextrose to a final absorbance at 600 nm of 1.0 was harvested by centrifugation as described above. To avoid overloading the next column, the yeast was diluted with an equal volume of 20 mM Tris-Cl pH 7.4, 1 mM EDTA, protease inhibitor mixture, 0.125% digitonin, 34 μg egg phosphatidylcholine, and adjusted to 110 ml, and then incubated for 30 min on ice after the addition of 90 ml of digitonin solution (Kelleher et al., 1992). Oligosaccharyltransferase-digitonin complex was calculated using the following equation: M = (6/n)Nf, where M is the molecular weight of the oligosaccharyltransferase-digitonin complex, N is Avogadro’s number, n is the stoichiometry of the complex, and Nf is the experimentally determined sedimentation coefficient of the oligosaccharyltransferase-digitonin complex, r is the Stokes radius of the oligosaccharyltransferase-digitonin complex, ε is the partial specific volume of the complex, and ρ is the density of water at 20 °C.

**Protein Isolation and Protein Sequencing**—Approximately 50–100 pmol of the yeast oligosaccharyltransferase complex was resolved into...
subunits by SDS-polyacrylamide gel electrophoresis, electrophoretically transferred onto 0.22-μm polyvinylidene difluoride (PVDF) membranes (Transblot, Bio-Rad) or 0.45-μm nitrocellulose sheets (Schleicher and Schuell), and the subunits were localized by staining with Pronase S, Coomassie Blue, or colloidal gold (Auro Dye Forte; Amersham). The subunits were processed for amino-terminal sequencing using standard procedures (Matsudaira, 1987) and were sequenced by the Worcester Foundation for Experimental Biology Protein Chemistry facility. Internal tryptic peptides from the 62- and 12910 kDa polypeptides were resolved by narrow-bore reverse phase HPLC after in situ trypsinization as described (Aebi et al., 1987). The mass of two peptides from the HPLC elution profiles was determined by matrix-assisted laser desorption mass spectrometry (as reviewed by Chait and Kent (1992)) using a Finnegan Lasermat mass spectrometer. In situ trypsinization, peptide purification, and mass spectrometric analysis of peptides was performed by the Harvard University Microchemistry Facility.

Protein Electrophoresis, Immunoblots, Con A Blots, and Endoglycosidase H Digestions—Proteins resolved by polyacrylamide gel electrophoresis in SDS were stained with Coomassie Blue or with silver (Bio-Rad) or were electrophoretically transferred to a 0.22-μm PVDF membrane. PVDF membranes were probed with a polyclonal rabbit antisera that recognizes the Whp1 protein (te Heesen et al., 1991) or with ConA-peroxidase (Sigma) using standard procedures (Harlow and Lane, 1988). Peroxidase-labeled secondary antibodies or ConA-peroxidase was detected by enhanced chemiluminescence (ECL; Amersham) following the manufacturer’s recommendations. The oligosaccharyltransferase was digested with endoglycosidase H essentially as described (Trimble and Maley, 1984). Endoglycosidase H was kindly provided by Dr. Robert Trimble (NY State Dept. of Health).

RESULTS

Purification of the Yeast Oligosaccharyltransferase—The yeast oligosaccharyltransferase was purified from microsomal membranes by combining the following purification steps: selective removal of peripheral and lumenal membrane proteins, solubilization of integral membrane proteins with digitonin, Con A affinity chromatography, Mono Q ion exchange chromatography, and 3′:5′-ADP-agarose affinity chromatography. Purification of the yeast oligosaccharyltransferase was monitored by analyzing the protein composition of selected fractions by Coomassie Blue staining of SDS-polyacrylamide gels (Fig. 1). Yeast microsomal membranes (lane a) were extracted with 0.5 M NaCl to remove peripheral membrane proteins and then permethylated with 0.05% Nikkol, a nonionic detergent, to selectively extract lumenal content proteins. The salt-stripped, detergent-permethylated membranes (lane b) were solubilized with a combination of 1.5% digitonin and 0.5 M NaCl to obtain a detergent extract (lane c). The majority of the proteins in the digitonin extract do not bind to a Con A-Sepharose column but were instead recovered in a flow-through fraction (lane d) that lacked detectable oligosaccharyltransferase activity. The enzyme activity was eluted from the ConA column with methyl α-n-mannopyranoside (lane e) and further enriched by ion exchange chromatography using a Mono Q ion exchange column. Rechromatography of the active fractions on a second Mono Q column to concentrate the sample and reduce the divalent metal ion concentration yields the Mono Q pool shown in lane f. Based upon the supposition that the oligosaccharyltransferase contains a binding site for Mn2+, as enzyme activity is strictly dependent upon Mn2+ (Trimble et al., 1980; Sharma et al., 1981), we applied the eluate from the Mono Q column to a 3′:5′-ADP-agarose column that had been equilibrated with MnCl2. Enzyme activity bound to the affinity column and was subsequently eluted with a linear gradient of NaCl and MnCl2 (lane g). Selective binding of oligosaccharyltransferase to the column was evident, as the majority of proteins with molecular weights greater than 70,000 did not bind and were recovered in a flow-through fraction as shown by a comparison of the load (lane f) and eluate (lane g) fractions. Additional experiments indicate that the enzyme will bind to an ATP-agarose column that was equilibrated in an identical manner (data not shown).

Furthermore, the concentration of MnCl2 required to elute the activity from the 3′:5′-ADP-agarose column was not significantly altered when the NaCl concentration was held constant during elution (data not shown), suggesting that immobilized Mn2+ rather than the adenine nucleotide is the affinity ligand.

The Coomassie Blue staining intensity of a number of polypeptides in lanes e–g was proportional to the amount of enzyme activity loaded on the gel. These proteins, which are the presumed subunits of the oligosaccharyltransferase complex, include a triplet of polypeptides migrating between 60 and 64 kDa (α), a 45-kDa (β), a 34-kDa (γ), a 30-kDa (δ), a 16-kDa (ε), and a 9-kDa (ζ) polypeptide. An additional 7-kDa polypeptide was visible when large aliquots of the preparation were analyzed by polyacrylamide gel electrophoresis in SDS. The 7-kDa polypeptide co-migrated with the bovine lung aprotinin that was included in all purification buffers as a protease inhibitor. For this reason, we tentatively identify the 7-kDa polypeptide as aprotinin. Although the final preparation contains traces of several other polypeptides (e.g., 75 and 90 kDa), we present evidence below showing that the latter polypeptides do not correlate with enzyme activity.

The recovery and enrichment of the oligosaccharyltransferase were monitored by activity assays using the tripeptide Nε-Ac-Asn [125I]Tyr-Thr-NH2 as the acceptor and dolichol-linked oligosaccharide isolated from bovine pancreas as the donor (Table I). Oligosaccharyltransferase assays of intact membranes cannot be compared with assays of detergent extracts due to the low quantity of endogenous oligosaccharide donor (OS-PP-Dol). Instead, rough microsomal membranes and the salt-stripped, Nikkol-permeabilized membranes were as-
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### Table I

| Fraction          | Protein mg | Total activity<sup>b</sup> pmol min<sup>-1</sup> | Specific activity<sup>b</sup> pmol min<sup>-1</sup> mg<sup>-1</sup> | Yield<sup>c</sup> % | Enrichment<sup>d</sup> |
|-------------------|------------|---------------------------------|---------------------------------|----------------|---------------------|
| Rough microsomes (RM) (a) | 1455.0     | (1165)                          | (0.8)                           | (100)          | 1                   |
| Salt-stripped, detergent-permeabilized RM (b) | 467.2      | (1454)                          | (3.1)                           | (100)          | 3.2                 |
| Digitonin extract (c) | 219.0      | 2511                            | 11.4                            | 94             | 6.2                 |
| Concanavalin A eluate (e) | 11.0       | 2168                            | 198                             | 86             | 110                 |
| Mono Q eluate (f) | 1.0        | 1596                            | 1572                            | 64             | 874                 |
| 3'5'-ADP-agarose (g) | 0.18       | 1304                            | 7526                            | 52             | 4070                |

<sup>a</sup> The letter in parentheses following each fraction designates the corresponding gel lane in Fig. 1.

<sup>b</sup> Total activity and specific activity values for membrane fractions are enclosed in parentheses due to the large apparent increase in activity that occurs upon preparation of the digitonin extract (see text). The oligosaccharyltransferase activity in the ConA-Sepharose eluate was assayed after gel filtration chromatography to remove the α-methyl mannanosidase.

<sup>c</sup> The yield of 94% for the oligosaccharyltransferase activity in the digitonin extract assumes that 6% of the oligosaccharyltransferase activity was trapped within the detergent-insoluble residue (see Fig. 6).

<sup>d</sup> The 6.2-fold enrichment of activity in the digitonin extract is calculated from the recovery of protein in the digitonin extract relative to the RM fraction corrected for the assumed 94% yield of activity (i.e. 6.2 = (1455 + 219) x 0.94).

Co-purification of Oligosaccharyltransferase Activity with a Protein Complex—Experimental evidence for the existence of an oligosaccharyltransferase complex was obtained by determining which polypeptides co-purify with enzyme activity. Yeast oligosaccharyltransferase purified as described above can be partially resolved from several less rapidly sedimenting proteins by glycerol gradient centrifugation (Fig. 2). In the experiment shown here, an additional 1.3-fold enrichment in enzyme activity was obtained. Nonspecific hydrophobic or ionic interactions that could result in artifactual co-sedimentation of proteins were minimized by including digitonin and 0.5 M NaCl in the glycerol gradient. The enzyme activity, which was recovered in fractions 8–10 of a glycerol gradient (Fig. 2B), co-sedimented precisely with the putative α-ζ subunits of the oligosaccharyltransferase complex (Fig. 2A). Co-sedimentation of the α-ζ polypeptides on a glycerol gradient suggests that these proteins are subunits of a complex, as monomeric proteins ranging between 9 and 64 kDa would sediment less rapidly. We noted that the relative sedimenting intensity of the subunits varied depending upon whether the polyacrylamide gels were stained with Coomassie Blue (Fig. 1) or with silver (Fig. 2A). For example, the β subunit stained most intensely with Coomassie Blue, while the δ subunit stained most intensely with silver. Therefore, the lower staining intensity of the γ subunit relative to the δ subunit in Fig. 2 should not be equated with a substoichiometric yield of the γ subunit in this experiment.

A co-sedimentation coefficient ($s_{20,w}$) of 14 can be estimated for the oligosaccharyltransferase complex in digitonin solution by comparison with the sedimentation rates of protein standards (Fig. 2B). Gel filtration chromatography of the yeast oligosaccharyltransferase complex on a Superose 12 column (Pharma-
cia) yielded a Stokes radius of 50 Å relative to protein standards (data not shown). The molecular weight of the oligosaccharyltransferase-digitonin complex can be calculated from these data provided that the partial specific volume of the protein-detergent complex is known. Although the latter value was not experimentally determined, the partial specific volume ($\bar{\rho}$) of the digitonin micelle is 0.73 cm$^3$/g (Steele et al., 1978); a value that falls within the range of partial specific volumes for typical proteins (0.71–0.74 cm$^3$/g). Consequently, 0.73 cm$^3$/g is a reasonable estimate for the $\bar{\rho}$ of the oligosaccharyltransferase-digitonin complex. From the sedimentation velocity and gel filtration data, we can estimate a molecular weight of 287,000 for the oligosaccharyltransferase-digitonin complex. The combined molecular weight of the $\alpha$-$\zeta$ subunits of the oligosaccharyltransferase complex was estimated to be 196,000 by denaturing gel electrophoresis (Fig. 1), suggesting that the protein-detergent complex contains roughly 90 kDa of digitonin. The latter value is consistent with the reported molecular mass of 70 kDa for a digitonin micelle in 0.1 M NaCl (Smith and Pickels, 1940). The size of detergent micelles increases as the ionic strength is raised, so the precise size of the digitonin micelle under the experimental conditions used here is not known. Nonetheless, the hydrodynamic data obtained here are consistent with the proposed subunit composition of the oligosaccharyltransferase complex.

Additional information concerning the subunit composition of the oligosaccharyltransferase complex was obtained by examining the elution profile of the enzyme on a Mono Q anion exchange column (Fig. 3). Two partially resolved peaks of oligosaccharyltransferase activity were obtained when enriched samples of the enzyme were eluted from a Mono Q column with a shallow NaCl gradient (Fig. 3A). For the experiment shown here, the fractions from the Mono Q column were analyzed on a 9% polyacrylamide gel to maximize the resolution of the 60–64-kDa polypeptides (Fig. 3B). As expected, the active fractions from the anion exchange column contain the presumed subunits of the oligosaccharyltransferase complex, while the inactive fractions lack these proteins. The 75- and 90-kDa polypeptides designated by diamonds (Fig. 3B) do not co-elute with either peak of enzyme activity and are therefore not subunits of the oligosaccharyltransferase. The most remarkable feature of the elution profile is the partial resolution of the 60–64-kDa polypeptides. The 64-kDa polypeptide is enriched in the first peak of enzymatic activity (fractions 13–15), while the 60- and 62-kDa polypeptides were enriched in the second peak of activity (fractions 16–20). While one interpretation of this result is that the co-purification of the 60–64-kDa polypeptides with oligosaccharyltransferase activity is fortuitous, we favor a different explanation. Instead, we suggest that the 60–64-kDa polypeptides correspond to alternate forms of a single polypeptide, and that differences in the ion exchange properties of these variants are responsible for the unanticipated separation of the enzyme into two partially resolved activity peaks.

The relative amount of the $\alpha$-$\delta$ subunits in each fraction from the Mono Q column was determined by densitometric scanning of the Coomassie Blue-stained gel. The densitometric units for a given subunit were then normalized to the amount of that subunit in fraction 14 so that the relative staining intensity of each subunit could be compared with the normalized oligosaccharyltransferase activity (Fig. 3C). In the case of the 60–64-kDa polypeptides, the densitometric units for the three presumed variants of the $\alpha$ subunit were combined before normalization. The correlation between enzymatic activity and the staining intensity of the $\alpha$-$\delta$ subunits was within the experimental error expected for quantification of proteins by densitometry. When Mono Q elution profiles were analyzed on higher percentage polyacrylamide gels, the $\epsilon$ and $\zeta$ subunits also co-eluted with enzyme activity (data not shown). However, densitometric quantification of these subunits was not possible due to the low staining intensity of these polypeptides.

The Glycosylated Subunits of the Oligosaccharyltransferase Complex—The subunits of the yeast oligosaccharyltransferase complex were transferred to a PVDF membrane after denaturing gel electrophoresis, and the membrane was probed with ConA to determine which polypeptides contain oligosaccharides. The major glycoproteins in the preparation co-migrate precisely with the $\alpha$ and $\beta$ subunits (Fig. 4A). The two more
abundant variants of the α subunit (i.e. the 64- and 62-kDa forms) were readily detected (lanes a and b) while the less abundant 60-kDa variant was detected when greater amounts of protein were loaded (lane d). The remaining subunits of the complex do not bind ConA, so they lack N-linked and O-linked oligosaccharides. Several less abundant proteins were not quantitatively removed by the 3′:5′-ADP-agarose column (Fig. 1, compare lanes f and g), and these glycoproteins (α and η) persist as minor contaminants in the preparation (Fig. 4A, lanes d and e). The oligosaccharyltransferase complex was digested with endoglycosidase H to determine whether the α and β subunits contain N-linked or O-linked oligosaccharide (Fig. 4B). After an extensive digestion with endoglycosidase H, the mobility of the β subunit was reduced by approximately 3 kDa consistent with the presence of either one or two N-linked oligosaccharides (compare lanes b and c). The presence of a single digestion intermediate in addition to the final product (βp) after a brief incubation with endoglycosidase H indicates that the β subunit contains two N-linked carbohydrates (lane d). A larger reduction in molecular weight was seen after digestion of the α subunit (lanes b and c). More importantly, a single major product (α′) was derived from the three variants of the α subunit by extensive digestion with endoglycosidase H (lane e). The ladder of evenly spaced digestion intermediates (lane d), strongly suggests that the 64-, 62-, and 60-kDa forms of the α subunit contain, respectively, four, three, and two N-linked oligosaccharides.

The formation of a single major endoglycosidase H digestion product from the 60- to 64-kDa polypeptides supports the hypothesis that these three polypeptides are glycosylation variants of a single protein. To solidify this conclusion, the 62- and 64-kDa polypeptides were resolved by PAGE in SDS, transferred to nitrocellulose, and localized by staining with Ponceau S. Peptides derived by in situ trypsin digestion of the immobilized 62- and 64-kDa polypeptides were eluted and resolved on a reverse phase high pressure liquid chromatography (HPLC) column using a gradient of acetonitrile in 1% trifluoroacetic acid (Fig. 5). A comparison of the HPLC elution profiles for tryptic peptides derived from the 64-kDa (panel A) and 62-kDa (panel B) polypeptides revealed a common set of major absorbance peaks. Two well-resolved peptides from each elution profile were analyzed by matrix-assisted laser desorption mass spectrometry. The molecular mass values obtained for the two peptides derived from the 64-kDa polypeptide were identical, within experimental error, with the mass values obtained for the corresponding peptides derived from the 62-kDa subunit. Given that the elution position as well as the molecular mass of these peptides is identical, we can conclude that the 62- and the 64-kDa polypeptides contain identical protein segments. Amino-terminal sequencing of the 62- and 64-kDa polypeptides also yielded identical amino acid sequences (data not shown). As the latter sequence is not present in the current releases of the protein sequence databases (SWISS-PROT, NBRF PIR, and translated GenBank), we conclude that the α subunit of the yeast oligosaccharyltransferase complex is a previously undescribed yeast protein. Although sufficient quantities of the 60-kDa polypeptide were not available for protein sequence analysis, the endoglycosidase H digestion experiment suggests that the latter polypeptide is also a variant of the α subunit.

Identification of the β and δ Subunits of the Oligosaccharyltransferase Complex—Having isolated a protein complex with demonstrable oligosaccharyltransferase activity, we asked whether any of the subunits corresponded to the WBP1 or SWP1 gene products. After signal sequence cleavage, the Wbp1 protein has a calculated molecular weight of 46,900 and has two consensus sites for N-linked glycosylation (te Heesen et al.,...
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FIG. 5. Reverse phase HPLC and mass spectrometry analyses of tryptic peptides derived from the 64- and 62-kDa polypeptides. Tryptic peptides derived from the 64-kDa (panel A) and 62-kDa (panel B) polypeptides were resolved by narrow-bore reverse phase HPLC (see "Materials and Methods"). The absorbance (mAU) of the eluate was monitored at 210 nm. The UV-absorbing material eluting after 80 min (panel B) is contaminants from the buffer used to elute peptides from the nitrocellulose membrane. The mass of two peptides (a and b) derived from the 62- and 64-kDa polypeptides is shown in the chart adjacent to each elution profile.

FIG. 6. Identification of the β subunit as the Wbp1 protein. Selected fractions from the oligosaccharyltransferase preparation were resolved on a 7.5–15% polyacrylamide gel in SDS, transferred to a PVDF membrane, and probed with a rabbit polyclonal antibody to the Wbp1 protein. The fractions analyzed were: lane a, yeast rough microsomal membranes (10 eq., 73 μg of protein); lane b, salt-stripped detergent-permeabilized microsomal membranes (10 eq., 23 μg of protein); lane c, supernatant from digitonin extraction (10 eq., 12 μg of protein); lane d, insoluble residue from the digitonin extraction (10 eq., 12 μg of protein); lanes e–h, 15, 30, 60, and 180 ng of the glycerol gradient-purified oligosaccharyltransferase complex. The arrow on the right designates the migration position of the β subunit of the oligosaccharyltransferase complex as detected by colloidal gold staining of the PVDF membrane. The asterisk designates a protein in lane a that is not recognized by affinity-purified antibody to the Wbp1 protein (Heesen et al., 1992). No immunoreactive bands other than Wbp1 were detected in the oligosaccharyltransferase preparation.

(1991). Protein immunoblot experiments were conducted to determine whether the 48-kDa glycosylated β subunit of the yeast oligosaccharyltransferase complex was identical with the Wbp1 protein (Fig. 6). Antibody to the Wbp1 protein recognizes a 48-kDa polypeptide in the purified oligosaccharyltransferase preparation (lanes e–h). More importantly, the Wbp1 protein co-migrates precisely with the β subunit of the yeast oligosaccharyltransferase complex. Early steps in the purification procedure that were designed to selectively remove peripheral and luminal RER proteins do not reduce the membrane content of the integral membrane protein Wbp1p (compare lanes a and b). Likewise, more than 90% of the Wbp1 protein was recovered in the supernatant fraction (lane c) after digitonin solubilization of the membranes. The presence of low amounts of the Wbp1p in the digitonin pellet (lane d) is likely due to trapping, as the pellet volume accounted for 6% of the solution volume prior to centrifugation. The estimated yield (94%) and enrichment (6.2-fold) of the oligosaccharyltransferase at this point in the purification are based upon these data (see Table I).

The Wbp1 protein has a calculated molecular weight of 31,700 and lacks consensus sites for N-linked glycosylation (Heesen et al., 1993). Both the 34-kDa γ subunit and the 30-kDa δ subunit of the oligosaccharyltransferase complex lack oligosaccharides and have apparent molecular weights that are similar to the calculated molecular weight of the Wbp1 protein. The γ and δ subunits were resolved by PAGE in SDS and were electrophoretically transferred to a PVDF membrane for amino-terminal sequence analysis. The amino-terminal sequence of the γ subunit does not correspond to any portion of the Swpl protein (data not shown), nor could this sequence be aligned with any sequence in the current releases of the protein sequence databases (SWISS-PROT, NBRF PIR, and translated GenBank). However, the amino-terminal 16 residues of the δ subunit could be aligned with the Swpl protein beginning at residue 20 of the published protein sequence (Fig. 7). Consistent with this alignment, hydropathy analysis suggests that the Swpl protein is an integral membrane protein with a cleavable amino-terminal signal sequence (Heesen et al., 1993). The most probable signal sequence cleavage site for the Swpl protein was calculated using the weight matrix method of von Heijne (von Heijne, 1986) and was found to agree with that determined by N-terminal sequencing of the mature protein (Fig. 7). Thus, the β and δ subunits of the yeast oligosaccharyltransferase complex correspond to previously identified yeast proteins that had been shown to be required for N-linked glycosylation (Heesen et al., 1992, 1993).

Homology between Swplp and Ribophorin II—The observation that the yeast oligosaccharyltransferase is a protein complex was not unexpected, based upon analogy to the canine enzymes (Kelleher et al., 1992). When the mammalian and yeast enzymes are electrophoresed in adjacent lanes, the Wbp1 protein co-migrates with the homologous mammalian subunit OST48 (data not shown). Otherwise, only the α subunit (Mₚ = 62,000–64,000) in the yeast complex has a relative molecular mass similar to either ribophorin I (Mₚ = 66,000) or ribophorin II (Mₚ = 63,000). Antibodies raised against the mammalian ribophorins recognize avian and amphibian ribophorins I and II, but do not cross-react with any yeast microsomal membrane proteins (Crimaudo et al., 1987). The difference in subunit size and the lack of antibody cross-reactivity raises the question of whether the yeast oligosaccharyltransferase complex contains homologues for either ribophorin I or ribophorin II. A partial answer to this question is provided by a comparison of the protein sequences of ribophorin II and Swplp (Fig. 8). Both ribophorin II and Swplp have a cleavable amino-terminal hydrophobic signal sequence, as suggested by hydropathy analysis (Crimaudo et al., 1987; Heesen et al., 1993) and confirmed by amino-terminal sequencing of ribophorin II (Crimaudo et al., 1987) and Swplp (Fig. 7). More importantly, hydropathy analysis also reveals the presence in each protein of three hydrophobic segments near the carboxyl terminus that resemble
ferase based upon the finding that their expression is essential. This proposal as subunits of the yeast oligosaccharyltransferase was supported by studies on the N-linked glycosylation of proteins, which showed that two subunits of the yeast oligosaccharyltransferase complex, Wbplp and Swplp, were co-purified and co-eluted with enzyme activity on a glycerol gradient and to co-elute with enzyme activity. Six polypeptides were observed to co-sediment with enzyme activity and to stain more intensely than the corresponding yeast subunits. The sequence similarity was estimated to be 49%.

Interestingly, the spacing and relative hydrophobicity of the carboxyl-terminal segments in Swplp and ribophorin II are remarkably similar as shown by a comparison of the published hydrophathy plots (Fig. 8A). Together, the N- and C-terminal hydrophobic segments specify membrane integration of these proteins into the endoplasmic reticulum with the bulk of the protein located within the RER lumen. Proteinase of canine microsomal membranes does not reduce the apparent molecular weight of ribophorin II, suggesting that all three carboxyl-terminal hydrophobic segments may be membrane-associated.

The BLASTP protein sequence comparison algorithm (Altchul et al., 1990) was used to search the protein sequence databases (SWISS-PROT, NBRF PIR, and translated GenBank) for proteins homologous to the Swplp protein. The most closely related proteins to Swplp were rat and human ribophorin II. Optimal sequence alignment between ribophorin II and Swplp was obtained by comparing the mature sequence of Swplp with the carboxyl-terminal half of human ribophorin II (Fig. 8B). The sequence similarity between the two proteins extends throughout the entire mature region of Swplp. The sequence identity within this region was 22%, whereas sequence similarity was estimated to be 49%.

**FIG. 7. Identification of δ subunit as the Swplp protein.** After polyacrylamide gel electrophoresis in SDS and transfer to a PVDF membrane, the amino-terminal sequence of the δ subunit was determined by sequential Edman degradation. An alignment between the amino terminus of the δ subunit and the sequence of the Swplp protein derived from a yeast genomic SWPlp clone is shown (te Heesen et al., 1993). The most probable signal sequence cleavage site (arrow) for the Swplp protein was calculated using the weight matrix method of von Heijne (1986).

**DISCUSSION**

The yeast oligosaccharyltransferase preparation contains six major polypeptides that correlate with enzyme activity. These six polypeptides were observed to co-sediment with enzyme activity on a glycerol gradient and to co-elute with enzyme activity on an ion exchange column. We conclude that these six polypeptides correspond to the subunits of an oligosaccharyltransferase complex. Adventitious co-purification of these six polypeptides is unlikely as the relative staining intensity of the six proteins remained constant during fractionation procedures that rely on different biochemical and biophysical properties. Two subunits of the yeast oligosaccharyltransferase were shown to be Wbplp and Swplp. These two proteins were initially proposed as subunits of the yeast oligosaccharyltransferase based upon the finding that their expression is essential for N-linked glycosylation of proteins in vivo and for glycosylation of acceptor peptides in vitro (te Heesen et al., 1992, 1993). Furthermore, the Wbpl protein can be cross-linked to the Swpl protein in detergent extracts of yeast microsomal membranes, suggesting that the two proteins form a complex in vivo (te Heesen et al., 1993). However, alternate mechanisms could be invoked to explain how expression of the Wbpl and Swpl proteins could be indirectly required for oligosaccharyltransferase activity. Thus, co-purification of these two proteins with the oligosaccharyltransferase activity provides direct biochemical evidence that Wbpl and Swpl are subunits of the yeast oligosaccharyltransferase complex. Sequence alignment has disclosed that two of the yeast oligosaccharyltransferase subunits (Wbplp and Swplp) are homologous to mammalian oligosaccharyltransferase subunits (OST48 and ribophorin I). We anticipate that further characterization of the yeast subunits will reveal a homologue for ribophorin I.

An unexpected difference between the yeast and mammalian oligosaccharyltransferase complexes was first noted when the amount of yeast and canine enzyme loaded on adjacent gel lanes was equalized with respect to total activity units. The subunits of the canine oligosaccharyltransferase complex stained more intensely than the corresponding yeast subunits (data not shown). Subsequent experiments suggest that the turnover number for the yeast enzyme complex may be 5-10-fold higher than that estimated for the canine enzyme (Keller et al., 1992). However, before we can conclude that the yeast enzyme is catalytically more active, we must explore several alternative explanations for this difference in apparent enzyme activity. The oligosaccharyl donor that we have isolated from bovine pancreas for the oligosaccharyltransferase assay is not a chemically homogeneous compound with respect to lipid content or oligosaccharide structure. OS-PP-Dol preparations contain other lipophilic compounds that could conceivably interfere with enzyme activity. The nonglucosylated compounds (Man$_{p}$-GlcNAc$_{p}$-PP-Dol) have been reported to be severalfold more abundant than the glucosylated lipid-linked oligosaccharides (Glc$_{p}$Man$_{p}$GlcNAc$_{p}$-PP-Dol) in OS-PP-Dol preparations isolated from calf pancreas (Badet and Jeanloz, 1988). Incompletely assembled lipid-linked oligosaccharides lacking the terminal glucose residues are utilized at 10-35-fold reduced rates by the mammalian (Turco et al., 1977; Spiro et al., 1979) and yeast (Trimble et al., 1980) enzymes, when tested using solubilized membranes as the source for the oligosaccharyltransferase. The less restrictive utilization of incompletely assembled oligosaccharides by the yeast enzyme may account for the greater apparent turnover number of the latter enzyme when mixed populations of lipid-linked oligosaccharides are used as the donor substrate, primarily because the effective concentration of the donor substrate may be higher. A more accurate comparison between the purified yeast and mammalian enzymes will require the isolation and characterization of more highly purified donor substrates. Although the iodinated tripeptide substrate is chemically homogeneous, we have not determined whether similar differences in specific activity are observed when larger peptides are used as the oligosaccharyl acceptor. Finally, the observed differences in subunit composition between the yeast and canine enzymes may be indicative of the selective loss of several low molecular weight subunits from the mammalian oligosaccharyltransferase complex during purification. If this were the case, the previously purified heterotrimeric canine oligosaccharyltransferase complex may be a low specific activity core of a more active oligosaccharyltransferase complex that may exist in intact membranes. The latter possibility is currently being explored.

Limited information is currently available concerning the α, γ, ε, and ζ subunits of the yeast oligosaccharyltransferase complex. The artificial proteolytic derivation of the γ, ε, and ζ polypeptides from the α, β, and δ subunits is considered unlikely because protease inhibitors were present throughout the purification, and the microsomal membranes were isolated.
from a yeast strain that is deficient in two of the major vacuolar proteases (Jones, 1991). In addition, extensive amino-terminal and internal tryptic peptide sequence data have been obtained for the α, γ, and ε subunits. Based upon these data, as well as the protein immunoblot and the CoA blot data presented here, we are confident that the γ and ε subunits are not derived from the Wbp1 or Swp1 proteins by proteolysis or protein modification. Furthermore, there is no indication that these polypeptides are derived from the α subunit. However, until the complete sequences of the α, γ, ε, and ζ subunits can be compared, it will remain a formal possibility that one of the low molecular weight subunits is derived from a larger subunit by proteolysis.

To date, Wbp1 and Swp1 are the only subunits of the oligosaccharyltransferase complex that have been shown to be essential for expression of enzyme activity in vivo and in vitro (Perez et al., 1993). Analogous experiments will likewise be required to determine whether the novel subunits described here are required for expression of oligosaccharyltransferase activity. All six subunits of the oligosaccharyltransferase complex may not participate directly in oligosaccharide transfer, but may instead interact with the proteins that are components of the protein translocation apparatus (Deshaies et al., 1991).

Alternatively, some subunits may interact with the mannose- and glucosyltransferases that assemble the lipid-linked oligosaccharide donor (Kukuruzinska et al., 1987). In either case, the generation of partially defective forms of the oligosaccharyltransferase may provide insight into which subunits participate directly in substrate recognition and oligosaccharyltransfer.

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