The *Saccharomyces cerevisiae* Oligosaccharyl Transferase

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for my mother

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Abstract

Carbohydrate modification of secreted, luminal, and integral membrane proteins is an essential eukaryotic cellular process. Folding, stability, localization, and recognition are some of the many properties contributed to glycoproteins by oligosaccharides. Oligosaccharyl transferase catalyzes the covalent attachment of oligosaccharides to asparagine residues within selected Asn-Xaa-Ser/Thr sequences of polypeptides translocated into the endoplasmic reticulum.

Oligosaccharyl transferase was purified from a solubilized fraction of *Saccharomyces cerevisiae* membrane proteins by a sequence of chromatography on concanavalin A-agarose, heparin-agarose, and Q-sepharose. Enzymatic activity copurified with an apparent complex of four polypeptides with molecular weights of 64 kDa, 45 kDa, 34 kDa, and 30 kDa. The 45 kDa glycoprotein and the 30 kDa protein were identified as the previously described proteins Wbp1p and Swp1p.

The essential *NLT1* gene encoding the 64 kDa glycoprotein subunit of the enzyme was cloned using obtained amino acid sequences. The *NLT1* gene encodes a 476 amino acid polypeptide with a signal sequence, a carboxy-terminal hydrophobic domain, and four potential glycosylation sites. Antiserum prepared from a six-histidine tagged version of the *NLT1* gene product expressed in *E. coli* recognized the two glycoforms of the 64 kDa protein in a crude yeast lysate and in the purified native enzyme complex.

To further the characterization of the multisubunit enzyme, a version of Nlt1p with carboxy-terminal FLAG epitope and six-histidine tags was expressed from the *CUP1* promoter. The construct expressing the affinity-tagged Nlt1p complemented a deletion of the *NLT1* locus yielding

oligosaccharyl transferase activity levels indistinguishable from strains expressing the native enzyme. Affinity purification of the tagged enzyme yielded a multimeric complex with polypeptide subunits of 70 kDa, 45 kDa, 34 kDa, 30 kDa, and 16 kDa; these subunits have been identified as the products of the tagged *NLT1* gene and the native *WBP1*, *OST3*, *SWP1*, and *OST2* genes. The purified affinity-tagged oligosaccharyl transferase has been used to examine the minimum polypeptide composition necessary for catalytic activity and to probe the enzyme's peptide binding site with a series of photoaffinity labels.

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Abbreviations Used

Standard one and three letter codes are used for the naturally occurring amino acids. Standard one letter codes are used for nucleic acid bases.

α-MeMan	:	α-methylmannopyranoside
AEBSF	:	4-(2-aminoethyl)-benzenesulfonylfluoride
Aloc	:	Allyloxycarbonyl
Amb	:	Aminobutyric acid
BCA	:	Bicinchoninic acid
bp	:	Base pair
Boc	:	*tert*-butoxycarbonyl
BOP	:	Benzotriazole-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate
Bz	:	Benzoyl
DIPCDI	:	Diisopropylcarbodiimide
DMF	:	Dimethylformamide
DMSO	:	Dimethylsulfoxide
Dol	:	Dolichol
EDTA	:	Ethylenediamine-*N,N,N′,N′*-tetraacetic acid
EGS	:	Ethylene glycobis(succinimidylsuccinate)
ENZ	:	Enzyme
ER	:	Endoplasmic reticulum
ESI-MS	:	Electrospray ionization mass spectrometry
FAB-MS	:	Fast atom bombardment mass spectrometry
Fmoc	:	Fluorenylmethyloxycarbonyl
Glc	:	Glucose

GlcNAc	:	*N*-acetyl glucosamine
HEPES	:	*N*-[2-hydroxyethyl]-piperazine-*N'*-2-ethanesulfonic acid
HOBt	:	Hydroxybenzotriazole
HPLC	:	High performance liquid chromatography
IgG	:	Immunoglobulin
kb	:	Kilobase
kDa	:	Kilodalton
krpm	:	One thousand revolutions per minute
MALDI-MS	:	Matrix assisted laser desorption ionization mass spectrometry
Man	:	Mannose
Ni-NTA	:	Nickel - nitrilotriacetic acid
OTtag	:	Affinity-tagged oligosaccharyl transferase
PC	:	Phosphatidylcholine
PCR	:	Polymerase chain reaction
pfu	:	Plaque forming units
PNGaseF	:	Peptide *N*-glycanase F
PP	:	Pyrophosphoryl
PVDF	:	Polyvinylidene difluoride
SDS-PAGE	:	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
tBu	:	*tert*-butyl
TEA	:	Triethylamine
Tris	:	Tris(hydroxymethyl) aminoethane
YPD	:	1 % yeast extract / 2 % peptone / 2 % dextrose

Chapter 1

Introduction

The glycosylation of proteins is an essential process to all eukaryotic cells. The addition of oligosaccharides to proteins expands the molecular diversity available to them by several orders of magnitude, and the glycosylated proteins seem to span nearly as great a range of functions. The central dogma of biology yields a polypeptide sequence built up from twenty different amino acids; the post-translational addition of oligosaccharides adds the orthogonal dimension of over ten monosaccharide units and the potential of elaborate branched structures to the molecular alphabet available to glycoproteins. The diversity of the oligosaccharides is evident in the changing patterns of glycosylation during development, the antigenic variation of the cell surface glycoproteins of pathogens, tissue-specific variation of glycoproteins, and interspecies variations in the glycosylation of specific proteins (1). In addition to the diversity imparted to glycoproteins by glycosylation, oligosaccharides perform specific biochemical functions. They can initiate polypeptide folding, protect proteins from proteolysis, improve the solubility of proteins, act as receptors or ligands, and target proteins to their correct cellular location (1).

Three types of carbohydrate modifications occur in the cell. Glycosylphosphatidylinositol anchors are attached to the carboxy-terminus of some proteins to localize them to the surface of the various cellular membranes (2). Short oligosaccharides are also transferred to the side chains of serine and threonine residues of some proteins (*O*-linked glycosylation), and branched oligosaccharides can be transferred to the side chain of asparagine residues (*N*-linked glycosylation) (2). Glycosylation begins in the rough endoplasmic reticulum (ER) where carbohydrates are covalently linked to polypeptides co- or post-translationally (2). The oligosaccharides undergo further processing in the ER and in the Golgi apparatus (3).

The enzyme oligosaccharyl transferase catalyzes the *N*-linked glycosylation of polypeptides (figure 1-1). The carbohydrate donor in the reaction is biosynthesized by the sequential construction of a complex, fourteen residue oligosaccharide upon a dolichol pyrophosphate carrier by the enzymes of the rough endoplasmic reticulum (2). The carbohydrate acceptor is the asparagine residue in selected Asn-Xaa-Thr/Ser sequences, where Xaa is any amino acid except proline (4), of nascent polypeptides as they are translocated into the lumen of the rough ER or soon thereafter.

The transferase reaction is unusual in that the relatively unreactive amide side chain appears to act as a nucleophile in displacing the oligosaccharide from the lipid pyrophosphate donor. Studies with constrained peptide substrates have led to a structural and functional model that explains the enhanced nucleophilicity of the asparagine residue (5). The peptide adopts an Asx-turn conformation with the side chain amide oxygen of the asparagine residue hydrogen bonded to the backbone amide of the threonine or serine of the acceptor sequence (figure 1-2); abstraction of an amide proton by an enzyme active site base causes the formation of the side chain imidol tautomer yielding a nucelophilic nitrogen atom for the transferase reaction (5, 6).

Several efforts have been made to purify and identify the proteins responsible for the oligosaccharyl transferase reaction. The enzyme was first solubilized and partially purified from hen oviduct microsomal membranes with the detergent Nonidet P40 (7). The enzyme was also solubilized from yeast microsomal membranes with the detergent Nonidet P40 (8). This solubilized enzyme preparation was used to kinetically characterize the donor and acceptor substrates of the reaction, to describe the broad pH optimum for oligosaccharide transfer, and to define the divalent cation requirement of the

enzyme (8). However, little progress was made for several years in the purification of the enzyme, presumably due to the instability of the solubilized transferase. In fact, the word "labile" became inseparable from many descriptions of the oligosaccharyl transferase.

To circumvent the instability of the solubilized enzyme, a photoaffinity label based on a peptide substrate of the transferase was designed to cross-link and identify polypeptides responsible for the reaction (9). The photoaffinity label inhibited enzymatic activity and specifically cross-linked a 60 kDa polypeptide from hen oviduct microsomal membranes (10). Antibodies raised against the 60 kDa protein were used to immunoprecipitate the polypeptide from extracts of a number of eukaryotic cells; the precipitated protein bound the photoaffinity peptide but did not catalyze oligosaccharide transfer (11). The 60 kDa polypeptide was identified as protein disulfide isomerase, an abundant protein resident in the lumen of the ER, from the sequence of the corresponding cDNA (12). Subsequent depletion experiments showed conclusively that protein disulfide isomerase is not required for the oligosaccharyl transferase reaction (13). Recognition that the photolabeled polypeptide was unrelated to the oligosaccharyl transferase reopened the question of the molecular identity of the enzyme.

A major breakthrough in the purification of the transferase occurred with the observation that the phospholipid phosphatidylcholine enhanced the activity and stability of the enzyme (14). Inclusion of the phospholipid in solubilization and chromatography buffers dramatically increased the stability of the enzyme and made its purification possible (15). The enzyme was first purified from dog pancreas microsomal proteins solubilized with the detergent digitonin (16). To date, the enzyme has also been purified from

avian (17), pig liver (18), human liver (19), and yeast microsomal membranes (20-22).

The purified canine oligosaccharyl transferase contains three subunits with molecular weights of 66 kDa, 63 kDa, and 48 kDa (16). The 66 kDa subunit was identified as ribophorin I, and the 63 kDa subunit was identified as ribophorin II (16). The ribophorins were initially described in rat liver microsomes as potential components of a ribosome binding site in the rough ER membrane (23, 24). The third component was identified as a novel protein by cDNA sequencing and named OST48 (25). The purified complexes from avian, pig liver, and human liver microsomes all contain the ribophorins and homologues of the OST48 protein (17-19); the pig liver enzyme contains an additional 40 kDa subunit which has yet to be characterized (18).

The purification of the oligosaccharyl transferase from the yeast *Saccharomyces cerevisiae* has brought the techniques of molecular biology and genetics to the study of the enzyme. The transferase was independently purified by three different chromatography sequences as similar multimeric complexes. The transferase was purified as a six subunit complex by a sequence of chromatography on concanavalin A - sepharose, Mono Q media, and 3':5'-bis, bis-adenosine diphosphate agarose (20). The complex had subunits with molecular weights of 64 kDa, 45 kDa, 34 kDa, 30 kDa, 16 kDa, and 9 kDa (20). A similar active complex with all but the two smallest subunits was isolated by immunoaffinity chromatography (21). And a third purification of an active transferase complex followed a sequence of chromatography on concanavalin A - agarose, heparin - agarose, Q - sepharose, and hydroxyapatite media (chapter 2) (22); this purification yielded

a heterotetrameric complex with 64 kDa, 45 kDa, 34 kDa, and 30 kDa subunits and has since been revised to include the 16 kDa subunit (chapter 4) (26).

To date, nearly all of the genes encoding the subunits of the yeast oligosaccharyl transferase have been cloned and sequenced (table 1-1) (15). Amino acid sequences from the 64 kDa glycoprotein subunit were used to clone the *OST1 / NLT1* gene (chapter 3) (27, 28). The essential gene encodes a 476 residue polypeptide with a 22 residue amino-terminal signal sequence and one hydrophobic membrane anchor at the carboxy-terminus (20, 26-28). The predicted protein sequence includes four potential glycosylation sites in the mature protein (27, 28); the protein is actually expressed as a pair of glycoforms with three or four oligosaccharides (20, 28). Sequence comparison shows that Ost1p / Nlt1p is the yeast homologue of the ribophorin I protein of the oligosaccharyl transferase from higher eukaryotes (27, 28). Temperature sensitive point mutations in the *OST1* gene yield cells strains with underglycosylated proteins and reduced oligosaccharyl transferase activity (27).

The 45 kDa subunit was identified as the product of the essential *WBP1* gene by amino-terminal amino acid sequencing (22) and by immunoblotting (20). The *WBP1* gene had previously been cloned as a potential nuclear pore protein (29) and later identified as a candidate oligosaccharyl transferase gene (30). Depletion of the *WBP1* gene product under the control of the *GAL* promoter resulted in cell growth arrest and a significant reduction of oligosaccharyl transferase activity, and temperature sensitive mutations in the gene resulted in *in vivo* protein underglycosylation (30). The *WBP1* gene encodes a 420 residue polypeptide with a 20 residue signal sequence, a carboxy-terminal hydrophobic domain, and two glycosylation sites which are both utilized *in vivo* (22, 29). Wbp1p is the yeast homologue of the mammalian

OST48 protein (25). OST48 homologues have also been identified and sequenced from *Drosophila melanogaster* and *Caenorhabditis elegans* (15, 31). In a biochemical experiment, it was shown that the oligosaccharyl transferase could be inhibited with a biotinylated analog of the sulfhydryl-modifying reagent methyl methanethiosulfonate (22). This reagent specifically labeled the Wbp1p subunit of the enzyme, and protection from inhibition by dolicholpyrophosphoryl chitobiose suggested that a cysteine residue of Wbp1p is proximal to the substrate binding site (22).

The *OST3* gene was cloned using amino acid sequences from the 34 kDa subunit of the oligosaccharyl transferase (32). *OST3* is unusual in that it is the only transferase gene cloned to date which is not essential for the vegetative growth of the yeast cell; in fact, a cell strain lacking the *OST3* gene is viable even at 37° C (32). However, deletion of the *OST3* gene does lead to underglycosylation of glycoproteins *in vivo* and a reduction in oligosaccharyl transferase activity (32); this phenotype is similar to the phenotypes of temperature sensitive mutations of the essential transferase genes. The gene encodes a 350 residue protein with a 22 residue signal sequence (32). The protein is significantly hydrophobic with four potential membrane spanning domains at its carboxy-terminus. The protein has a homologue from the *C. elegans* genome (32); however, no homologue has been identified genetically or biochemically from higher eukaryotes (16-19, 32).

Amino-terminal amino acid sequencing was used to identify the 30 kDa oligosaccharyl transferase subunit as the product of the *SWP1* gene (16, 22). The essential *SWP1* gene had been cloned as a high copy suppressor of a temperature sensitive mutation of the *WBP1* gene (33). Depletion of the *SWP1* gene product under the control of the *GAL* promoter led to cell growth arrest and a significant loss of oligosaccharyl transferase activity (33). The

SWP1 gene encodes a 286 residue polypeptide with a 19 residue signal sequence and three carboxy-terminal hydrophobic domains (16, 22, 33). Wbp1p and Swp1p were directly cross-linked in a membrane fraction of yeast cells indicating that they are in direct contact in a multimeric protein complex (33). The 30 kDa Swp1p is conserved as it shows significant homology to the carboxy-terminal half of the 63 kDa ribophorin II from the higher eukaryotic oligosaccharyl transferase complex (25).

Amino acid sequences were also used to clone the essential *OST2* gene encoding the 16 kDa subunit of the oligosaccharyl transferase (34). The *OST2* gene encodes a hydrophobic polypeptide with no amino-terminal signal sequence (34). Unlike the other transferase subunits which have carboxy-terminal membrane anchors and extensive luminal domains, Ost2p potentially has its soluble domain oriented on the cytoplasmic side of the ER membrane (34). Like similar mutations in the *OST1* and *WBP1* genes, temperature sensitive mutations in *OST2* yield cell strains with underglycosylated proteins and reduced oligosaccharyl transferase activity (34). In addition, overexpression of Ost2p suppressed the phenotype of the same mutation in the *WBP1* gene that was suppressed by multiple copies of *SWP1* (34). Ost2p is homologous to the vertebrate DAD1 protein, a protein potentially involved in apoptotic cell death (34); however, DAD1 did not copurify with any of the vertebrate oligosaccharyl transferase complexes (16, 18, 19). Ost2p was observed in one purification of the yeast transferase (20), but not in the other two (21, 22); one of the purifications has been revised and clearly shows that Ost2p is a member of the transferase complex (chapter 4) (26).

Several other yeast genes have also been associated with the oligosaccharyl transferase. The *OST5* gene encodes the 9 kDa subunit of the

enzyme, but it has not yet been fully characterized (15). The *OST4* gene encodes a 36 residue polypeptide; deletion of the gene yields a cell strain with reduced transferase activity and an underglycosylated phenotype (35). *OST4* might encode a very small subunit of the transferase that is not essential like Ost3p, or it might encode a protein that influences the structure or function of the enzyme. Finally, the 78 kDa membrane protein product of the *STT3* gene influences the function of the oligosaccharyl transferase even though it does not copurify with enzymatic activity *in vitro* (36). Mutation of the *STT3* gene affects oligosaccharyl transferase substrate specificity, and genetic depletion of Stt3p causes a reduction of transferase activity and loss of the transferase complex (36). Stt3p seems to be required for the *in vivo* assembly or stability of the oligosaccharyl transferase.

Nature has created a complex enzyme to catalyze the oligosaccharyl transferase reaction. To date, a number of the polypeptides that contribute to the enzyme complex have been characterized. A conclusive description of the molecular composition of the complex would help define the limits of any experiments that attempt to describe the function of the enzyme. It is likely that the bulk of the oligosaccharyl transferase genes have been identified and sequenced, but the possibility of low molecular weight or substoichiometric polypeptide components of the enzyme remains. Obtaining greater quantities and higher concentrations of the enzyme – perhaps by overexpression – would facilitate any experiment aimed at the biochemical function of the enzyme. To date, the contribution of the enzyme, unlike that of the peptide substrate, to the mechanism of the oligosaccharyl transferase reaction is an unexplored subject.

Here, I describe my efforts at furthering the understanding of the structure and function of the yeast oligosaccharyl transferase. The wild type

enzyme was purified as a heterotetrameric complex of membrane proteins, and the polypeptide subunits of the complex were identified by amino acid sequencing (chapter 2). The sequences of the 64 kDa subunit were used to clone the *NLT1* gene which was sequenced and characterized (chapter 3). Nlt1p was tagged with an affinity sequence to facilitate the purification of the enzyme and to enable the rapid analysis of mutated transferase proteins (chapter 4). Efforts were then made to overexpress the cloned transferase genes and to define the minimal polypeptide complex necessary for the catalysis of the transferase reaction (chapter 5). Finally, a series of photoaffinity probes based on oligosaccharyl transferase peptide substrates were designed and synthesized to map the enzyme's peptide binding site (chapter 6).

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Figure Legends

Figure 1-1. The oligosaccharyl transferase reaction. The oligosaccharyl transferase catalyzes the nucleophilic displacement of an elaborate oligosaccharide from a lipid pyrophosphate donor to the acceptor side chain amide of the asparagine residue in Asn-Xaa-Thr/Ser sequences.

Figure 1-2. Proposed oligosaccharyl transferase reaction mechanism. The peptide substrate of the oligosaccharyl transferase has been shown to adopt an Asx-turn conformation with the amide oxygen of the asparagine side chain hydrogen bonded to the backbone amide of the threonine or serine residue in the Asn-Xaa-Thr/Ser sequence. Abstraction of an amide proton by an enzyme base causes the formation of the imidol tautomer yielding a nucleophilic nitrogen for the displacement of the oligosaccharide from the lipid pyrophosphate carrier (5).

Table 1-1 The subunits of the yeast oligosaccharyl transferase.

Subunit Size	Yeast Gene	Essential	Homology
64 kDa	*OST1 / NLT1*	yes	Ribophorin I
45 kDa	*WBP1*	yes	OST48
34 kDa	*OST3*	no	
30 kDa	*SWP1*	yes	Ribophorin II
16 kDa	*OST2*	yes	DAD1
9 kDa	*OST5*		

Note: *OST5* encodes the 9 kDa subunit of the oligosaccharyl transferase, but has not yet been fully characterized (15).

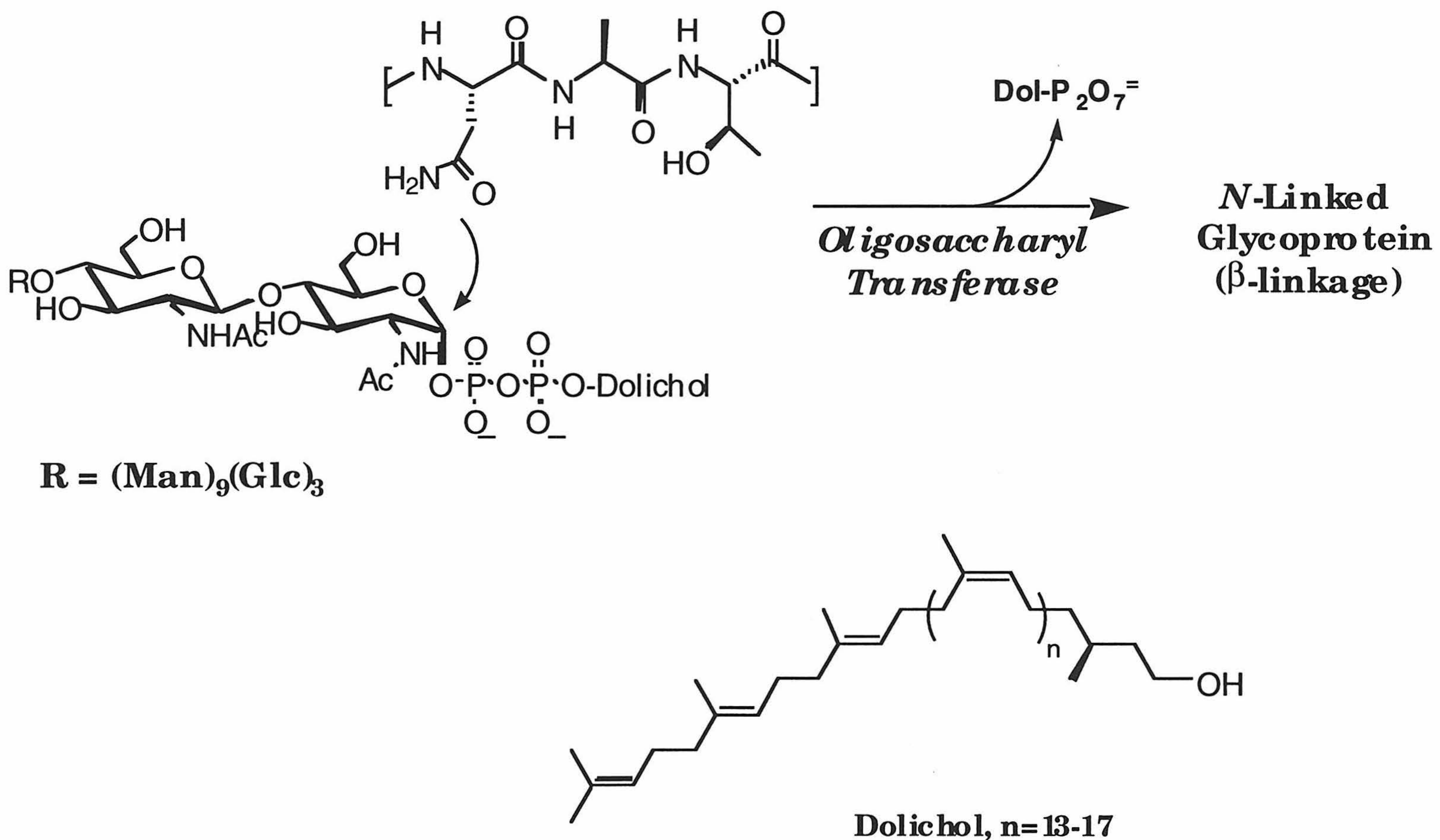

Figure 1-1 The oligosaccharyl transferase reaction.

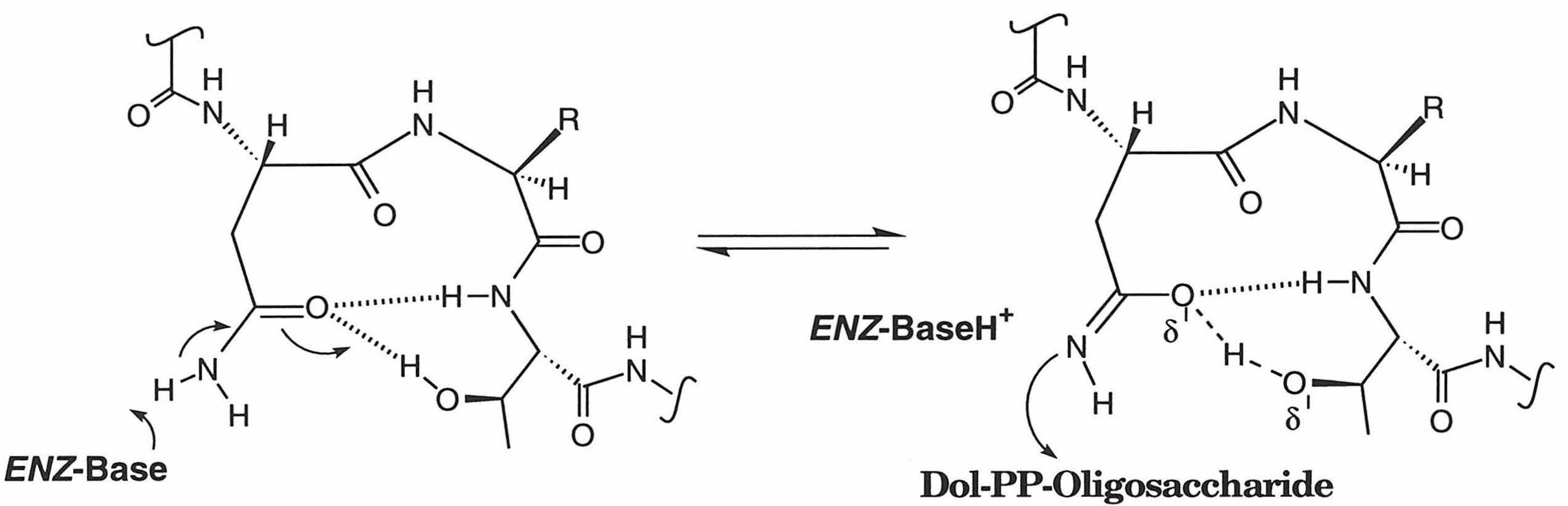

Figure 1-2. Proposed oligosaccharyl transferase mechanism.

Chapter 2

Purification of the *Saccharomyces cerevisiae* Oligosaccharyl Transferase

In eukaryotic cells, most secreted, vesicular, and integral membrane proteins bear covalently linked carbohydrate groups on asparagine, serine, or threonine residues. The oligosaccharide chains of glycoproteins contribute to their conformation and stability and also participate in cellular targeting and recognition processes (1). In catalyzing the *N*-linked glycosylation of proteins, oligosaccharyl transferase acts at the nexus of two essential biochemical pathways. The enzyme brings together a complex, lipid-linked oligosaccharide donor, dolichylpyrophosphoryl$GlcNAc_2Man_9Glc_3$, which is synthesized by a series of glycosyl transferases in the membrane of the rough endoplasmic reticulum and an acceptor peptide sequence which is part of a nascent polypeptide chain being translocated into the lumen of the rough endoplasmic reticulum (1-3). Specifically, the peptide acceptor reacts at the side chain of asparagine residues in selected Asn-X-Thr/Ser sequences where X is any residue other than proline (4). Since the features of both acceptor and donor in the oligosaccharyl transferase reaction are identical in yeast and in higher eukaryotes including mammalian species, the accessible genetics of *Saccharomyces cerevisiae* provides an ideal system for the study of the enzyme.

The molecular characterization of the yeast oligosaccharyl transferase has associated a heteromeric membrane complex with enzymatic activity. The yeast proteins Wbp1p and Swp1p are both necessary for *in vivo* transferase activity; when the *WBP1* gene or the *SWP1* gene was placed under the control of the repressible *GAL* promoter, the depletion of either gene product correlated with a loss of enzyme activity (5, 6). However, simultaneous overexpression of both gene products did not yield a significant increase in transferase activity (6). Two independent purifications of the enzyme from yeast membranes have yielded complexes of four and six

polypeptides (7, 8); both complexes include the Wbp1p and Swp1p proteins as well as several novel proteins. Based on the amino acid sequences of the two characterized subunits, the yeast enzyme complex shows significant homology to the oligosaccharyl transferase complexes purified from higher eukaryotes (9, 10). The canine pancreas enzyme was purified as a trimeric membrane protein complex including ribophorin I, ribophorin II, and OST48 (9, 11); Wbp1p is homologous to the OST48 subunit (11), and Swp1p is homologous to the carboxy-terminal half of the ribophorin II subunit (8) Comprehensive and accurate study of the enzyme requires the identification of all the subunits essential for oligosaccharyl transferase activity.

To begin the examination of the chemical roles of each subunit of the yeast enzyme complex we have developed an independent purification of the oligosaccharyl transferase. Enzymatic activity copurified with a tetrameric complex. Two of the polypeptide subunits were identified by amino-terminal residue sequencing; one is the 48 kDa protein Wbp1p (5), and the other is the 32 kDa protein Swp1p (6). In addition, a novel 64 kDa glycoprotein, Nlt1p (chapter 3) (12), and a nonglycosylated 34 kDa polypeptide, Ost3p (13), also cofractionated with activity.

Materials and methods

Oligosaccharyl transferase assay

Oligosaccharyl transferase activity was measured as described (14) with the following modifications. For each assay, 440 pmol [^{3}H]-dolichylpyrophosphoryl-*N,N'*-diacetylchitobiose (0.22 nCi/pmol) (15) in 3:2 $CHCl_3$:MeOH was evaporated under nitrogen and redissolved in 10 µl DMSO containing 2.5 mM Bz-Asn-Leu-Thr-NMe. At the start of each assay, an

aliquot of the experimental sample, typically 10 - 20 μl, was mixed with the peptide and lipid-disaccharide substrates and incubated for ten minutes in a final volume of 200 μl of 2.2 μM Dol-PP-GlcNAc-[^{3}H]GlcNAc, 125 μM Bz-Asn-Leu-Thr-NMe, 140 mM sucrose, 50 mM HEPES pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1.0 % Nonidet P-40, and 0.5 mg/mL phosphatidylcholine (PC). Both substrates were present in the assays near their experimentally determined K_M concentrations. At each of four different time points spanning the ten minute incubation, a 40 μl aliquot was removed from the reaction mixture and quenched into 1.2 mL of 3:2:1 $CHCl_3$:MeOH:4 mM $MgCl_2$. Aliquots of each assay reaction were measured at four different time points to ensure accurate and linear kinetic measurement of the amount of enzyme present in each sample. The aqueous layer was separated, and the organic layer was extracted twice with 0.6 mL 192:186:12:2.69 MeOH:H_2O: $CHCl_3$:250 mM $MgCl_2$. The combined aqueous extracts were counted in EcoLite(+) (ICN) on a Beckman LS-5000TD scintillation counter. After the organic layer had evaporated, the proteinaceous residue was dissolved in 200 μl Solvable (New England Nuclear) and counted in Formula 989 (New England Nuclear). The amount of radioactivity transferred into the aqueous extracts was normalized against the total radioactivity present in the aqueous extracts and the organic layer. Oligosaccharyl transferase activity was measured as the rate of transfer of the labeled disaccharide from the dolichylpyrophosphoryl carrier to the acceptor peptide; activity was expressed as pmol peptide glycosylated per minute.

Buffers

Buffer A contained 50 mM Tris-Cl pH 7.5 and 2.5 mM $MgCl_2$. Buffer B contained 140 mM sucrose, 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0.1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF, CalBiochem), 0.5 μg/mL pepstatin A, and 0.5 μg/mL leupeptin. Buffer C contained 140 mM sucrose, 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, 0.5 mg/mL PC, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. Buffer D contained 140 mM sucrose, 50 mM HEPES pH 7.5, 25 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, and 0.5 mg/mL PC. Buffer E contained 140 mM sucrose, 20 mM MES pH 6.4, 25 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, and 0.1 mg/mL PC. Buffer F contained 140 mM sucrose, 50 mM HEPES pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, and 0.5 mg/mL PC.

Purification

Yeast strain PRY46 (*MATα his- pep4-3 prc1-1126 prb1-1122*; P. Robbins, personal communication) was grown to an optical density of approximately 1.5 in 240 L of 1 % yeast extract / 2 % peptone / 2 % dextrose (YPD) media. The cells were frozen at -80° C in 100 g batches. Cells were thawed in 100 g batches overnight to 4° C and then washed twice with buffer A. All further purification steps were carried out at 4° C. The cells were suspended to a total volume of 150 mL in buffer A plus protease inhibitors (0.1 mM AEBSF, 0.5 μg/mL pepstatin A, 0.5 μg/mL leupeptin; CalBiochem) and beaten for sixteen 20 second intervals with 40 second cooling intervals in a glass bead beater (BioSpec) with 100 mL of 425 - 600 μm glass beads (Sigma). The suspension was harvested and the beads were washed with a total of 50 mL buffer A; the suspension and the washes were combined and spun at 8 krpm for 10

minutes. The supernatant was saved and the pellets were resuspended in 100 mL buffer A and again spun at 8 krpm for 10 minutes; the supernatants were combined and spun at 8 krpm for 10 minutes. Microsomal membranes were pelleted from this supernatant by centrifugation at 40 krpm for 60 minutes in a Beckman 45Ti rotor. The membranes were suspended at an A_{280} of 50 in 60 mL of buffer B. This suspension was brought to 0.5 M NaCl and incubated for 15 minutes (16); the membranes were recovered after centrifugation at 40 krpm for 60 minutes. The pellet was suspended in 60 mL of buffer B with 0.1 M NaCl and 0.05% Nonidet P-40 (Sigma) and incubated for 15 minutes; the membranes were recovered by centrifugation at 40 krpm for 60 minutes. Membrane proteins were partially solubilized in 68 mL buffer B with 0.6% Nonidet P-40 and 0.5 M NaCl, incubated 15 minutes, and clarified at 40 krpm for 60 minutes.

Concanavalin A agarose (10 mL; Vector) was equilibrated by washing twice with 30 mL buffer C. The solubilized membrane fraction (68 mL) was incubated with the media overnight at 4° C. The media was recovered by centrifugation at 1 krpm and washed three times with 30 mL buffer C. Bound proteins were eluted by three successive four hour incubations with 70 mL of 750 mM α-methylmannopyranoside (α-MeMan) in buffer C. The eluate (200mL) was dialyzed overnight against 140 mM sucrose, 17 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0.4% Nonidet P-40, and 0.1 mg/mL PC to dilute the α-MeMan.

A 2.5 mL heparin-agarose (Pierce) column was equilibrated in buffer D. The pooled concanavalin A eluate was applied to the column at a flow rate of 1.0 mL/min. Activity was eluted sharply with a 50 mL gradient of 25 to 500 mM NaCl in buffer D at a flow rate of 0.5 mL/min. The active fractions (20 mL) were dialyzed overnight with one change against 500 mL of buffer E.

A 2.5 mL Q-sepharose (Pharmacia) column was equilibrated in buffer E plus 0.5 mg/mL PC. Twenty milliliters of the heparin eluate was applied at a flow rate of 0.5 mL/min. Activity was eluted with a gradient of 25 to 200 mM NaCl in buffer E plus 0.5 mg/mL PC. The active fractions (20 mL) were applied directly to a 1.0 mL BioGel HTP hydroxyapatite (BioRad) column. The column was washed with 1.0 M NaCl in buffer E plus 0.5 mg/mL PC, and the enzyme was eluted with a gradient of 0 to 0.8 M NaF in buffer E plus 0.5 mg/mL PC. A total of 12 mL of the active fractions was dialyzed overnight with one change against 500 mL of buffer F.

Analytical techniques

Purified protein was precipitated with chloroform and methanol (17) prior to separation by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (18). Separated proteins were stained with Coomassie brilliant blue R250. For immunological detection of Wbp1p, separated proteins were transferred to nitrocellulose (19) and then incubated in 1:1000 diluted antiserum raised against Wbp1p (20) followed by a 1:1000 diluted anti-rabbit IgG alkaline phosphatase conjugate (ICN). For the detection of glycoproteins, nitrocellulose-bound polypeptides were incubated in 10 μg/mL biotinylated concanavalin A (Sigma) followed by an avidin-biotinylated alkaline phosphatase complex (Pierce). Glycoproteins were deglycosylated with peptide *N*-glycanase F (PNGaseF) (New England Biolabs) in 1.0 % β-mercaptoethanol, 1.0 % Nonidet P-40, 0.5 % SDS, and 50 mM sodium phosphate pH 7.5. For amino acid sequencing, protein bands were transferred to ProBlott (Applied Biosystems) and sequenced by the California Institute of Technology Protein and Peptide Micro Analytical Facility. The

total protein in each purification fraction was determined by the Micro BCA assay (Pierce) after precipitation with chloroform and methanol.

Results

Purification

The yeast oligosaccharyl transferase was partially purified from a crude microsomal membrane extract (table 2-1). The purification was followed by the assay described above which monitors the transfer of a labeled disaccharide from a dolichylpyrophosphate donor to an acceptor Bz-Asn-Leu-Thr-NMe tripeptide; both donor and acceptor substrates were present in the assays at concentrations comparable to their experimentally determined K_M concentrations. Yeast cells were lysed and the microsomal membrane fraction was isolated and suspended in buffer; the activity of this initial microsomal fraction was estimated because of the instability of the enzyme in membrane suspensions (table 2-1). The membranes were washed with NaCl to remove peripherally associated proteins and then with detergent at a low NaCl concentration to deplete luminal proteins and to partially solubilize the integral membrane protein fraction. The enzyme was solubilized with the detergent Nonidet P-40 at up to a 90 % yield of the estimated activity in the membrane fraction; the solubilized enzyme remained active for weeks at 4° C. Enzyme activity was enhanced with phosphatidylcholine (21); in addition, the phospholipid increased the stability of the detergent-solubilized enzyme, and chromatography experiments did not yield active enzyme when it was omitted from the medium.

The solubilized extract was applied to a concanavalin A affinity column to which the enzyme bound tightly. Efficient elution of the activity

required a high concentration of the lectin inhibitor α-methylmanno-pyranoside and up to twenty media volumes of elution buffer over twelve hours. After dilution of the lectin inhibitor by dialysis, the enzyme was bound to heparin-agarose and eluted with an NaCl gradient. Enzyme activity was then bound to Q-sepharose at a slightly lowered pH and eluted with a gentle NaCl gradient. The enzyme could be further purified with hydroxyapatite media with a NaF gradient elution; an active enzyme could not be eluted from this media with sodium phosphate, and activity remained bound to the media in the presence of high NaCl concentrations. However, after the previous three steps of purification, chromatography on hydroxyapatite yielded a modest two-fold purification and little change in the composition of the protein fraction (figure 2 -1).

Oligosaccharyl transferase activity copurified with a tetrameric complex of polypeptides (figure 2 -1). The largest polypeptides were near 64 kDa. Up to three bands could be seen at this size; two of them were intensely labeled by concanavalin A blotting, and a third was faintly labeled (figure 2 -2); treatment with PNGase F yielded one polypeptide band of 55 kDa (figure 2 -1). The amino-terminal residue sequences of the glycosylated 64 kDa bands and deglycosylated 55 kDa band were identical (chapter 3) (12). The next polypeptide at 48 kDa was identified as Wbp1p by amino-terminal sequencing (figure 2 -3a) and immunoblotting with an anti-Wbp1p antiserum (20) (figure 2 -4); this band was also stained intensely by concanavalin A and shifted to a molecular weight of 43 kDa upon treatment with PNGase F (figure 2 -1). The first twenty residues of the genomic amino acid sequence from the *WBP1* gene (20) were not detected by amino-terminal sequencing and were probably cleaved by the endoplasmic reticulum signal peptidase (figure 2 -4). The sequence of the amino terminus of the band at 32 kDa identified the protein

as Swp1p (figure 2 -3b); this protein was not glycosylated (figure 2 -2), and amino-terminal sequencing indicated that a nineteen residue signal peptide was cleaved by the signal peptidase. A nonglycosylated band at 34 kDa consistently appeared in the purification of the oligosaccharyl transferase (figure 2-1). However, its staining intensity varied in different purifications, and it often stained with significantly less intensity than the other three polypeptides in the complex; this protein has recently been identified as the nonessential Ost3p (13).

Discussion

The yeast oligosaccharyl transferase has been purified as a tetrameric complex from an extract of microsomal membranes. The mammalian enzyme was also purified as a similar multimeric complex from canine pancreas cells (9). The largest polypeptide in the yeast complex has a deglycosylated molecular weight of 55 kDa and is glycosylated in at least two positions; the amino-terminal amino acid sequence of this polypeptide, named Nlt1p, shows similarity to ribophorin I (chapter 3) (12), the largest polypeptide of the mammalian complex. The next component of the complex, the Wbp1p protein, has a deglycosylated molecular weight of 43 kDa and is also glycosylated *in vivo*. When the *WBP1* gene product was expressed under the control of the *GAL* promoter, depletion of the gene product correlated with a reduction in oligosaccharyl transferase activity (5). Wbp1p shows significant amino acid similarity and identity to the mammalian OST48 protein, a component of the mammalian transferase complex (22). The Swp1p protein is the third component of the complex, and it has a molecular weight of 32 kDa. The depletion of Swp1p from yeast cells also

yielded a corresponding reduction in oligosaccharyl transferase activity (6). Swp1p shows significant amino acid similarity and identity to the carboxy-terminal half of the mammalian ribophorin II, the third component of the trimeric mammalian complex (8). The final component of the yeast complex has a molecular weight of 34 kDa, and has recently been identified as the product of the nonessential *OST3* gene (13). Overexpression of the cloned *WBP1* and *SWP1* genes did not increase oligosaccharyl transferase activity in yeast cells (6); the cloned genes encoding the 55 kDa Nlt1p and 34 kDa Ost3p subunits should enable the complete expression of the enzyme.

Acknowledgment

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Table 2-1. Purification of the yeast oligosaccharyl transferase.

Fraction	Total Activity pmol/min	Total Protein mg	Specific Activity pmol/min/mg	Enrichment fold
Yeast microsomes	(2960)	756	3.9	1.0
Nonidet P-40 Solubilization	2960	150	19.7	5.1
Concanavalin A eluate	1020	5.6	182	46.7
Heparin agarose eluate	490	1.6	306	78.5
Q-Sepharose eluate	350	0.22	1590	408

Table 2-1. Purification of the yeast oligosaccharyl transferase. Fractions from the purification of the enzyme were assayed for total transferase activity and for total protein according to the experimental. The total activity of the microsomal fraction is approximate due to the instability of the enzyme in a microsomal suspension. The enzyme could be further purified on hydroxyapatite with a two-fold increase in enrichment.

Figure Legends

Figure 2-1. Purification of the yeast oligosaccharyl transferase. The yeast oligosaccharyl transferase was purified according to the protocol outlined in the experimental. Samples of each purification fraction were separated by SDS-PAGE and stained with Coomassie brilliant blue R250. The fractions and the approximate amount of loaded activity are: a) yeast microsomes, 0.39 pmol/min; b) Nonidet P-40 solubilized microsomes, 0.49 pmol/min; c) concanavalin A eluate, 2.73 pmol/min; d) heparin-agarose eluate, 2.45 pmol/min; e) Q-sepharose eluate, 3.5 pmol/min; f) hydroxyapatite eluate, 8.0 pmol/min; and g) hydroxyapatite eluate digested with 0.5 unit PNGaseF, 8.0 pmol/min. In the deglycosylated sample, the oligosaccharyl transferase complex is composed of subunits with weights of 55 kDa, 43 kDa, 34, kDa, and 32 kDa; PNGaseF also has an apparent mobility of 34 kDa.

Figure 2-2. Concanavalin A binding of purification fractions. Purification fractions were separated by SDS-PAGE and then transferred to nitrocellulose. Membranes were incubated with concanavalin A-biotin and then developed with avidin-biotinylated alkaline phosphatase. Samples are: a) yeast microsomes, b) Nonidet P-40 solubilized microsomes, c) concanavalin A eluate, d) heparin-agarose eluate, e) Q-sepharose eluate, and f) hydroxyapatite eluate. The pure fraction contains two glycosylated proteins. The 55 kDa protein exists as two or three glycoforms near 64 kDa, and the 43 kDa Wbp1p protein exists as a 48 kDa glycoprotein.

Figure 2-3. A. The 48 kDa protein is Wbp1p. The amino-terminal protein amino acid sequence from the 48 kDa polypeptide is identical to the sequence derived from the *WBP1* gene (te Heesen, et al., 1991). The first twenty amino acids from the genomic sequence of Wbp1p are likely cleaved by the endoplasmic reticulum signal peptidase.

Figure 2-3. B. The 32 kDa protein is Swp1p. The amino-terminal protein amino acid sequence from the 32 kDa polypeptide is identical to the sequence derived from the *SWP1* gene (te Heesen, et al., 1991). The first nineteen amino acids from the genomic sequence of Swp1p are also likely cleaved by the signal peptidase.

Figure 2-4. Wbp1p is enriched in the purification of the oligosaccharyl transferase. Protein fractions from the purification were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was probed with antiserum raised against Wbp1p (te Heesen, et al., 1993) and then developed with an anti-rabbit alkaline phosphatase conjugate. The fractions are: a) yeast microsomes, b) Nonidet P-40 solubilized microsomes, c) concanavalin A eluate, d) heparin-agarose eluate, e) Q-sepharose eluate, and f) hydroxyapatite eluate. The second band in the first two lanes is a non-specific, cross-reacting protein stained by the antiserum (te Heesen, et al., 1992).

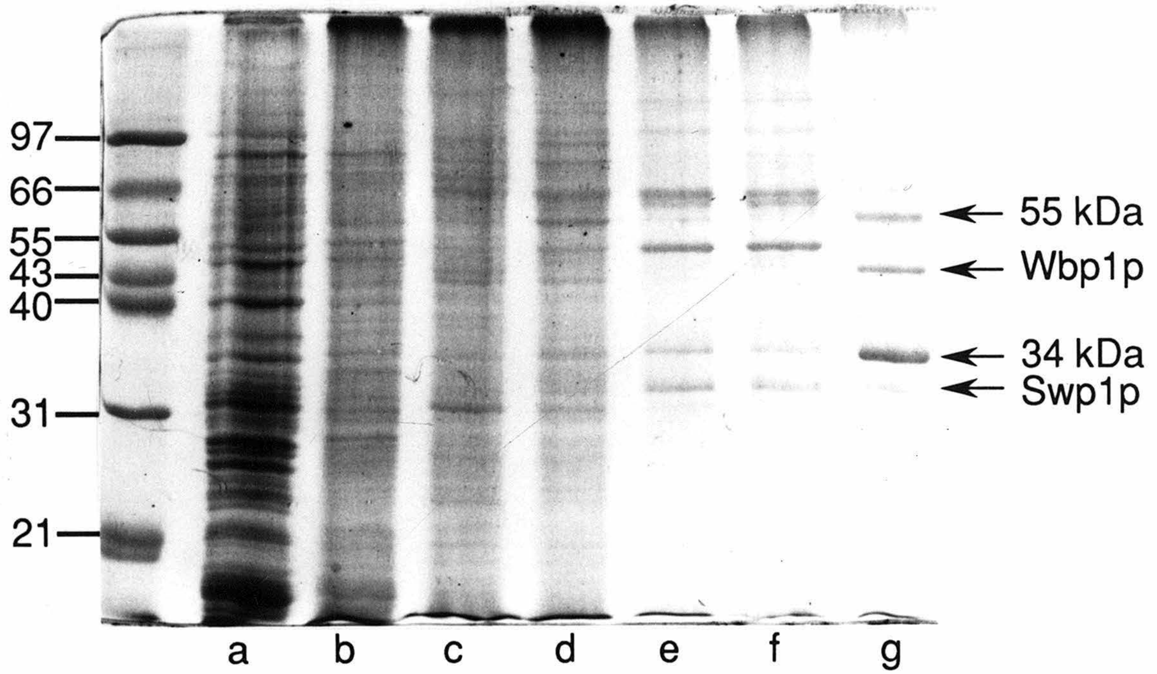

Figure 2-1. Purification of the yeast oligosaccharyl transferase.

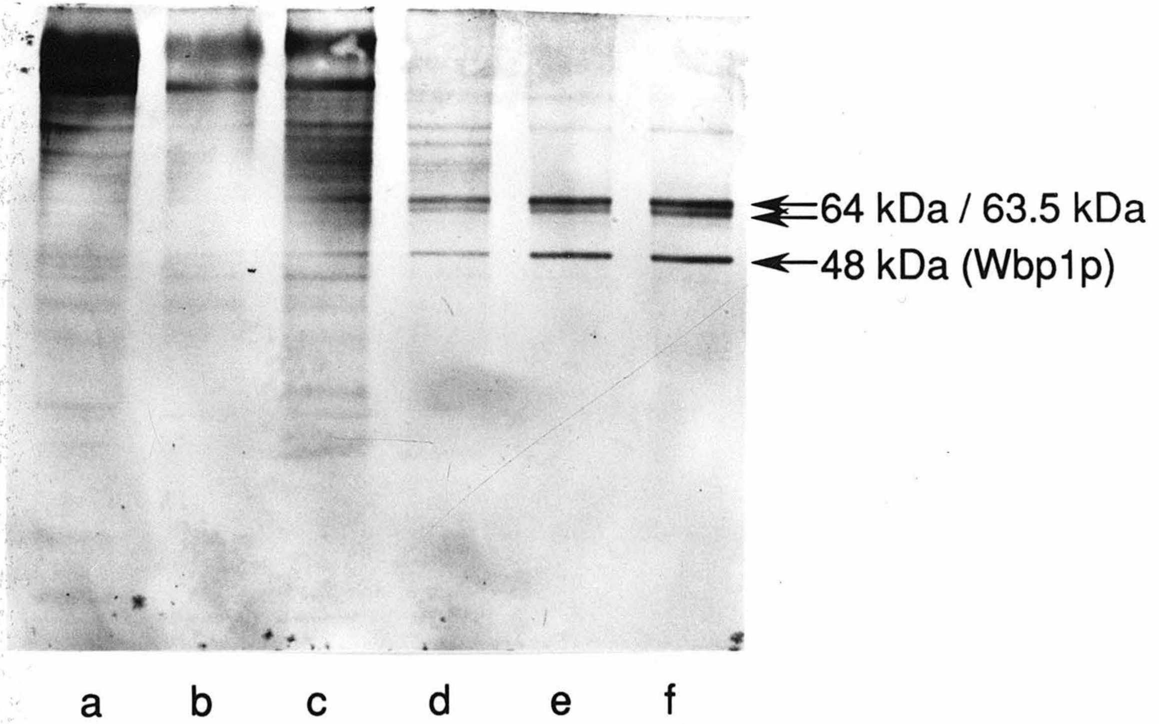

Figure 2-2. Concanavalin A binding of purification fractions.

A.	MRTDWNFFFCILLQAIFVVGTQTSRTLVLYDQSTEPLEEY	Wbp1p
	TQTSRTLVLYDQxTEPLExY	48 kDa

Figure 2-3. A. The 48 kDa protein is Wbp1p.

B.	MQFFKTLAALVSCISFVLAYVAQDVH	Swp1p
	YVAQDVH	32 kDa

Figure 2-3. B. The 32 kDa protein is Swp1p.

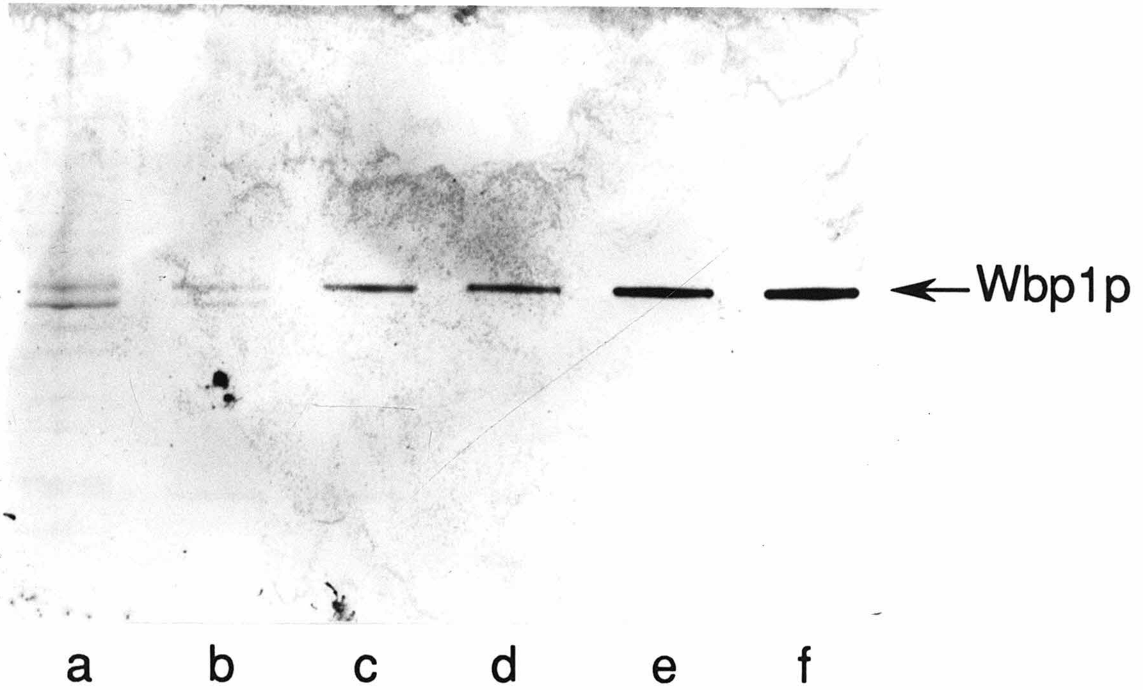

Figure 2-4. Wbp1p is enriched in the purification of the oligosaccharyl transferase.

Chapter 3

The Essential Yeast *NLT1* Gene Encodes the 64 kDa Glycoprotein Subunit of the Oligosaccharyl Transferase

The *N*-linked glycosylation of proteins is an essential processing event in eukaryotic cells; oligosaccharide chains contribute to the folding, solubility, and overall conformation of glycoproteins in addition to participating in protein function (1-3). The oligosaccharyl transferase catalyzes the transfer of a core oligosaccharide, $Glc_3Man_9(GlcNAc)_2$, from dolichol pyrophosphate to selected asparagine residues (4) of nascent polypeptides as they are translocated into the lumen of the rough endoplasmic reticulum. The enzyme has recently been purified from detergent extracts of *Saccharomcyes cerevisiae* membrane proteins (chapter 2) (5-7). The enzyme was purified either as a tetrameric complex of polypeptides with molecular weights of 64 kDa, 45 kDa, 34 kDa, and 32 kDa or as a hexameric complex with additional 16 kDa and 9 kDa subunits. The 45 kDa and 32 kDa subunits were identified as the products of the essential *WBP1* and *SWP1* genes. Previous experiments with the cloned *WBP1* and *SWP1* genes had identified Wbp1p and Swp1p as protein subunits of an oligosaccharyl transferase complex; however, simultaneous overexpression of the genes encoding both proteins failed to significantly increase oligosaccharyl transferase activity (8, 9). Presumably, overexpression of the enzyme also requires the genes encoding other subunits of purified enzyme complex. The amino acid sequences of the amino-terminal and internal peptide fragments from the 64 kDa and 34 kDa subunits indicated that they were novel yeast proteins. The 34 kDa protein, the 16 kDa protein, and the 9 kDa protein have recently been identified as the products of the *OST3, OST2,* and *OST5* genes (10-12).

The sequences of the identified components of the oligosaccharyl transferase are well conserved in eukaryotes from yeast to mammals. The mammalian enzyme was previously purified from canine pancreas microsomal membranes (13) as a complex including the proteins ribophorin

I, ribophorin II, and OST48. The amino acid sequence of the 32 kDa Swp1p is similar to the sequence of the carboxy-terminal half of the 63 kDa ribophorin II from rat and human cell lines (7), and the overall sequence of the 45 kDa Wbp1p is similar to the OST48 from canine pancreas cells (14).

Here, we describe the novel yeast protein Nlt1p, a third component of the yeast oligosaccharyl transferase. The *S. cerevisiae NLT1* gene was cloned using degenerate oligonucleotides derived from extensive amino acid sequencing of the 64 kDa subunit of the purified enzyme complex. The 1.4 kilobase open reading frame of the *NLT1* gene encodes a polypeptide with a predicted unprocessed molecular weight of 54 kDa. The amino acid sequence includes a 22 residue endoplasmic reticulum signal sequence and five potential glycosylation sites in the mature protein. Cleavage of the signal sequence and glycosylation of the acceptor sequences would yield a mature protein of 62 kDa to 67 kDa, in close agreement with the observed doublet of 64 kDa polypeptide bands in the purified complex (chapter 2) (5). The amino acid sequence predicts a type I membrane protein with an extensive soluble domain and one potential transmembrane hydrophobic domain at the carboxy-terminus of the protein. Deletion of an 800 base pair region of the *NLT1* coding region indicated that the gene is essential for vegetative growth of yeast. Consistent with the homology shown by the 45 kDa and 32 kDa yeast subunits to their mammalian counterparts, the amino acid sequence of the novel 64 kDa Nlt1p bears significant similarity to the amino acid sequences of the human and rat ribophorin I proteins (15, 16); the *NLT1* gene is the first gene encoding a ribophorin I homologue to be cloned from yeast.

The cloned *NLT1* gene, together with the *WBP1, OST3, SWP1, OST2,* and *OST5* genes, will enable a complete genetic and biochemical understanding of the essential oligosaccharyl transferase enzyme.

Materials and Methods

Yeast Strains and Media

The following yeast strains were used in this study: SEY 6210/6211 (*MAT***a**/α *leu2-3112 ura3-52 his3-200 trp1-901 lys2-801/+ ade2-101/+ suc2-9 mal*$^{-}$) (17); PRY46 (*MATα his- pep4-3 prc1-1126 prb1-1122*) (obtained from P. Robbins); YPH274 (*MAT***a**/*MAT*a *ade2-101*^{o}/*ade2-101*^{o} *lys2-801*^{a}/*lys2-801*^{a} *his3Δ200/his3 Δ200 ura3-52/ura3-52 leu2- Δ1/leu2- Δ1 trp1- Δ1/trp1- Δ1*) (18); YPH274**a** (*MAT***a** *ade2-101*^{o} *lys2-801*^{a} *his3 Δ200 ura3-52 leu2- Δ1 trp1- Δ1*) (obtained from J. Abelson); YPH274α (*MATα ade2-101*^{o} *lys2-801*^{a} *his3Δ200 ura3-52 leu2- Δ1 trp1- Δ1*) (obtained from J. Abelson); and RPY5 (*MAT***a**/*MAT*a *ade2-101*^{o}/*ade2-101*^{o} *lys2-801*^{a}/*lys2-801*^{a} *his3Δ200/his3 Δ200 ura3-52/ura3-52 leu2- Δ1/leu2- Δ1 trp1- Δ1/trp1- Δ1 Δnlt1::URA3/+*) (this study). Standard yeast media (19) were used.

Amino acid sequencing of the 64 kDa protein

The yeast oligosaccharyl transferase was purified from approximately 100 g of PRY46 cells (chapter 2) (5). For amino-terminal sequencing, approximately 10 μg of the purified protein was concentrated by chloroform / methanol precipitation (20) and separated by SDS-PAGE (21). The separated polypeptides were transferred electrophoretically (22) to a ProBlott PVDF membrane (Applied Biosystems). The 64 kDa oligosaccharyl transferase band was carefully cut out and sequenced on an Applied Biosystems automated peptide sequencer. For internal amino acid sequencing, approximately 100 μg of the purified protein was concentrated and separated by SDS-PAGE and then transferred to an Immobilon P^{SQ} membrane (Millipore). The 64 kDa band was cut out and digested *in situ* with 10 mg/mL cyanogen bromide in 70 %

formic acid for 24 hours at room temperature. The cyanogen bromide solution was removed, and the membrane fragments were washed with 70 % isopropanol and 0.2 % trifluoroacetic acid. The cyanogen bromide eluate was combined with the isopropanol wash and evaporated under a stream of nitrogen. The resultant peptide pellet was washed with water and then separated on a high resolution SDS-PAGE gel (23). The peptide fragments were transferred to a ProBlott membrane (ABI), cut out, and sequenced on an Applied Biosystems automated peptide sequencer.

Isolation of the *NLT1* gene

Standard DNA techniques were used (24). Degenerate oligonucleotides with sequences derived from the amino-terminal and internal amino acid sequences of the 64 kDa glycoprotein were synthesized (figure 3-1). Primer RP1 had the sequence 5'-G C N C A A/G T A T/C G A A/G C C N C C N G C N A C-3', and primer RP6 had the sequence 5'-T A A/G T G A/G T C N G T N G C N C C T/C T C N G G-3'. Genomic DNA prepared from yeast strain SEY 6210/6211 was used as a template for the polymerase chain reaction with primers RP1 and RP6. An 850 bp fragment of the yeast genome was amplified and cloned into pBluescript (Stratagene) to yield plasmid pBluescript-850. Partial DNA sequence of the polymerase chain reaction (PCR) product was obtained using Sequenase (U. S. Biochemical), and the amino acid translation of the sequence was consistent with the amino acid sequences from the 64 kDa protein. Probes were prepared from the amplified DNA by the polymerase chain reaction with α[^{32}P]-dCTP using pBluescript-850 as a template; the specific activity of each probe was 10^7 - 10^8 cpm/μg.

The probes were used to screen a λYES yeast genomic library (25). Lawns of *Escherichia coli* Y1090 cells were infected with the phage library at

plaque densities of 30,000 - 50,000 per 150 mm plate. The plaques were transferred to nitrocellulose filters and hybridized with 0.5 x 10^6 cpm/mL of the probe synthesized from pBluescript-850 for 16 hours at 42° C in 0.9 M NaCl, 60 mM sodium phosphate (pH 7.4), and 0.1 % SDS. The filters were then washed twice at 55° C with 0.9 M NaCl, 90 mM sodium citrate (pH 7.0), and 0.5 % SDS; positive plaques were identified by autoradiography. From a screen of approximately 300,000 plaques, 22 plaques hybridizing with the probe were identified; seven of these plaques were purified after four rounds of screening, and one (λYES-16) was selected for further study.

A plaque of λYES-16 phage DNA was suspended in 10 mM Tris-Cl (pH 7.5) and 8 mM $MgSO_4$. Recombination competent BNN132 cells (26) were infected with 1000 pfu of phage DNA and plated on LB/ampicillin media. The genomic insert of the λYES phage is within an 8 kb pYES plasmid sequence flanked by *lox* sites; infection of BNN132 cells expressing the *cre* gene allows recombination to occur at the *lox* sites yielding an intact pYES plasmid and expression of the pYES ampicillin resistance gene. Approximately 100 ampicillin-resistant colonies grew, and plasmid DNA was isolated from one colony. Restriction digestion of the plasmid DNA revealed a 3.7 kb genomic clone flanked by XhoI restriction endonuclease sites. The clone was removed from the pYES vector by digestion with XhoI and inserted into the XhoI site of pBluescript to yield plasmid pBluescript(*NLT1*). Both strands of the entire 3.7 kb genomic clone were sequenced.

Disruption of the *NLT1* gene

The 1.1 kb HindIII fragment containing the *URA3* gene from YEp24 was inserted between the StuI and MscI sites within the *NLT1* gene to yield plasmid pBluescript(Δ*nlt1*); the modified gene contained an 800 base pair

deletion within the *NLT1* open reading frame. Diploid yeast strain YPH274 was transformed with a 4.0 kb linear DNA fragment carrying the modified *NLT1* gene (27). Uracil prototrophs were selected, and successful disruption of one copy of the wild type *NLT1* gene was verified by Southern blotting. The resulting diploid yeast strain RPY5 was sporulated and subjected to tetrad analysis.

Results

Isolation of the *NLT1* gene

To clone the gene encoding the 64 kDa subunit of the oligosaccharyl transferase, amino acid sequences were obtained from the amino-terminus and from one cyanogen bromide fragment of the 64 kDa subunit (chapter 2) (5). The amino-terminus yielded 20 residues of reliable sequence, peptide 64-1 (figure 3-1). A 30 kDa cyanogen bromide fragment yielded sequence from two comigrating glycopeptides in a four to one molar ratio. The more abundant peptide gave 12 residues of reliable sequence, peptide 64-2; twelve residues of sequence were also obtained from the less abundant peptide, peptide 64-3, near the detection limits of the polypeptide sequencer.

The region of the yeast genome encoding the 64 kDa polypeptide from the amino-terminus to the start of the 30 kDa cyanogen bromide fragment was amplified by the PCR with degenerate oligonucleotide primers derived from peptides 64-1 and 64-2. An amplified 850 bp product was isolated and cloned; a partial sequence of this clone was consistent with the three sequences obtained from the 64 kDa glycoprotein. Probes synthesized from the 850 bp PCR product as a template were used to screen a λYES yeast genomic library (25). One hybridizing λ phage with a 3.7 kb genomic insert was selected for further study.

The sequence of the 3.7 kb region revealed one long 1.4 kb potential open reading frame (figure 3-2A) and one shorter potential open reading frame; the long open reading frame was assigned to the *NLT1* gene. Approximately 1 kb of the sequence upstream of the *NLT1* open reading frame is 98 % identical to the nucleotide sequence upstream of the previously sequenced *PRE3* locus (28). The *PRE3* locus contains the entire sequence of *CEN10* thereby positioning the start of the *NLT1* open reading frame 1.4 kb downstream of *CEN10* on the right arm of chromosome ten of *Saccharomyces cerevisiae*. In addition, the final 400 bp of the 3.7 kb genomic clone are 100 % identical to 400 bp from the previously sequenced *CDC35* locus (29) placing the *CDC35* locus further downstream of the *NLT1* gene also on the right arm of chromosome ten.

The *NLT1* gene encodes the 64 kDa glycoprotein

The 1.4 kb open reading frame of the novel *NLT1* gene encodes a 476 amino acid 54 kDa polypeptide (figure 3-2B). The amino acid sequence includes the exact sequences of peptides 64-1 and 64-2, and it includes all but two of the amino acids of peptide 64-3 which was sequenced at the limits of detection. The sequence matching peptide 64-3 is found immediately upstream of the sequence matching peptide 64-2; evidently, the cyanogen bromide fragments comigrated when analyzed by SDS-PAGE because of incomplete cleavage at methionine 193, the first residue of cyanogen bromide fragment 64-2. With the exception of two unclear residues out of forty-four sequenced, the predicted sequence of Nlt1p from the yeast genome exactly matches that obtained from the 64 kDa glycoprotein subunit of the oligosaccharyl transferase. Based on extensive agreement between the translation of the *NLT1* gene and the amino acid sequences obtained from the

64 kDa glycoprotein, we conclude that Nlt1p is the 64 kDa glycoprotein subunit of the oligosaccharyl transferase.

Hydropathy analysis (30) of the Nlt1p amino acid sequence reveals two potential transmembrane hydrophobic regions. At the carboxy-terminus is a potential transmembrane domain followed by a short soluble tail. At the amino-terminus is a twenty-two residue signal peptide cleaved by the endoplasmic reticulum signal peptidase. The amino-terminal amino acid sequence for the 64 kDa glycoprotein (figure 3-1) begins at residue 23 of the sequence derived from the yeast genome. In addition, a predictive weight matrix for the site of signal peptidase cleavage (31) indicates that the most likely site of signal peptidase cleavage is between residues 22 and 23 of the unprocessed protein. The predicted structure of the Nlt1p is that of a type I membrane protein with a large 435 residue soluble domain anchored in the membrane by a carboxy-terminal hydrophobic domain.

The amino acid sequence includes six potential glycosylation acceptor sites (figure 3-2B); one of these sites is in the amino-terminal signal sequence and would not be present in the mature glycoprotein. Glycosylation of the remaining five sites would yield a mature glycoprotein of 62 kDa to 67 kDa. The observed doublet of bands near 64 kDa (chapter 2) (5) would suggest that the mature Nlt1p is partially glycosylated and functions *in vivo* as two glycoforms with three or four oligosaccharides.

Nlt1p is conserved in higher eukaryotes. The protein shows 59 % amino acid similarity and 32 % amino acid identity to the mammalian ribophorin I protein from a human cell line (15); it also shows 56% similarity and 30 % identity to the rat ribophorin I (16) (figure 3-3). The amino acid similarity extends throughout the sequence of Nlt1p; it is not concentrated in any one region of the protein. However, the mammalian ribophorins are

approximately 130 amino acids larger at the carboxy-terminus than Nlt1p. Whereas the mammalian ribophorin I proteins have extensive soluble domains on either side of a potential transmembrane helix, the yeast Nlt1p includes only a one soluble domain on the amino-terminal side of a potential transmembrane anchor. The canine ribophorin I protein was purified as a subunit of the mammalian oligosaccharyl transferase complex, and the other two identified components of the yeast enzyme show homology to the other two subunits of the mammalian enzyme. Hence, the oligosaccharyl transferase is conserved throughout evolution from yeast to mammals.

The *NLT1* gene product is essential for vegetative growth

Yeast strain RPY5 was constructed with one intact and one partially deleted copy of the *NLT1* gene. To construct this strain, the 800 bp fragment between the StuI and MscI sites within the coding region of the *NLT1* gene was replaced with a 1.1 kb DNA fragment containing the selectable *URA3* gene. Southern blotting confirmed that one genomic copy of the *NLT1* gene was intact and the other contained a stable deletion and insertion (data not shown). The diploid strain was sporulated, and tetrads were dissected; segregants with the *NLT1* deletion were not viable indicating that the gene is essential for vegetative growth of yeast. Spores containing the deletion germinated and grew to colonies of only two to four cells, and spores carrying the deletion and a copy of the *NLT1* gene on an episomal plasmid were viable.

Discussion

Here we report the cloning and characterization of the yeast *NLT1* gene. This gene encodes the 64 kDa glycoprotein subunit of the oligosaccharyl

transferase complex, and it is the third oligosaccharyl transferase gene to be cloned from yeast. The *WBP1* gene encodes the 45 kDa subunit of the complex (9), and the *SWP1* gene encodes the 32 kDa subunit (8). Preliminary experiments indicate that simultaneous expression of the three genes does not yield a significant increase in transferase activity (chapter 5); evidently, characterization of the enzyme will require the cloning and expression of at least one more gene responsible for oligosaccharyl transferase activity. Previous studies have isolated the enzyme as a tetrameric (chapter 2) (5, 6) and a hexameric (7) complex of polypeptides. The genes encoding the 34 kDa Ost3p (10), the 16 kDa Ost2p (14) and perhaps the 9 kDa Ost5p (12) polypeptides which copurified with oligosaccharyl transferase activity should complete the genetic description of the enzyme.

The amino acid sequence of the 54 kDa Nlt1p is consistent with that of a type I transmembrane protein. A soluble amino-terminal domain comprises most of the protein. This domain is followed by a potential transmembrane helix and a short soluble carboxy-terminal tail. The amino-terminal domain contains four potential glycosylation sites which are consistent with the 64 kDa apparent mobility of the mature glycopeptide and the tight binding of the intact oligosaccharyl transferase complex to a concanavalin A affinity matrix (chapter 2) (5). Both Wbp1p and Swp1p have their extensive amino-terminal soluble domains in the lumen of the endoplasmic reticulum and carboxy-terminal membrane anchors; Wbp1p has one potential transmembrane domain while Swp1p has three (8, 9). If Nlt1p has a similar orientation relative to the endoplasmic reticulum membrane, then a model of the oligosaccharyl transferase emerges as that of a large, complex luminal domain anchored in the membrane by five or more transmembrane domains. These transmembrane domains could be involved

in the translocation of nascent polypeptides from the ribosome across the endoplasmic reticulum membrane or in the recognition of the dolicholpyrophosphoryl oligosaccharide substrate of the enzyme. Although the potential transmembrane domain of Nlt1p does not show homology to the putative dolichol binding consensus amino acid sequence (32), recent studies have shown that the residues of this consensus are not necessary for dolichol binding by the yeast dolichyl-phosphate-mannose synthase (33).

The oligosaccharyl transferase enzyme is conserved throughout eukaryotic evolution from yeast to mammals. The Wbp1p subunit is homologous to the 48 kDa subunit of the canine pancreas enzyme (14), and the Swp1p subunit is homologous to the carboxy-terminal half of the ribophorin II subunit of the mammalian enzyme (7). In addition, the novel Nlt1p is significantly similar to the mammalian ribophorin I protein, the final component of the purified mammalian complex. The homology of the 54 kDa Nlt1p is limited to the amino-terminal domain of the mammalian ribophorin I; the mammalian protein has an additional 130 amino acid carboxy-terminal soluble domain not found in the yeast protein (figure 3-3). The mammalian oligosaccharyl transferase might have evolved to include function on the cytoplasmic side of the endoplasmic reticulum membrane, and perhaps one of the remaining subunits of the yeast enzyme will include a more extensive carboxy-terminal domain than the three characterized polypeptides. Additionally, none of the five potential glycosylation acceptor sites in the yeast Nlt1p is conserved in the mammalian proteins, and the mammalian proteins are glycosylated at two acceptor sites which are not found in Nlt1p (figure 3-3). Yeast glycoproteins tend to be more extensively glycosylated than higher eukaryotic proteins; hence, it is not surprising that Nlt1p has more glycosylation sites than the mammalian homologues.

However, it is surprising that none of the glycosylation acceptor sites are conserved. This suggests that the oligosaccharides contribute more to the overall folding and solubility of glycoproteins and to their intracellular targeting than they do to their biochemical function.

The cloning of the yeast *NLT1* gene furthers the genetic understanding of the complex and essential oligosaccharyl transferase enzyme. This gene and the genes that encode the remaining subunits of the enzyme will enable the biochemical examination of the *N*-linked glycosylation of polypeptides in the endoplasmic reticulum of yeast cells.

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Figure 3-1. Amino acid sequences of the 64 kDa glycoprotein. The yeast oligosaccharyl transferase was purified (chapter 2) (5), and the 64 kDa glycoprotein subunit was isolated by SDS-PAGE. The sequence of peptide 64-1 was obtained from the mature amino-terminus of the protein, and the sequences of peptides 64-2 and 64-3 were obtained from two comigrating cyanogen bromide fragments of the 64 kDa glycoprotein.

Figure 3-2. A. Restriction map of the *NLT1* locus. The location of the *NLT1* open reading frame is shown with an arrow in the 3.7 kb clone of the *NLT1* locus. Selected restriction enzyme cleavage sites are shown. The XhoI sites were introduced during the preparation of the library.

Figure 3-2. B. Nucleotide sequence of the *NLT1* locus. The entire 3.7 kb of the *NLT1* locus was sequenced on both strands. The sequence in the vicinity of the *NLT1* open reading frame is shown. The numbers above the sequence indicate the nucleotides of the *NLT1* gene starting with the ATG initiation codon. The numbers to the left of the sequence indicate the amino acids of the translation of the open reading frame. Amino acid sequences corresponding to those obtained from the 64 kDa glycoprotein are underlined, and potential *N*-linked glycosylation acceptor sites are also underlined. An arrow indicates the site of signal sequence cleavage by the endoplasmic reticulum signal peptidase. A fifteen residue potential transmembrane hydrophobic domain was revealed by hydropathy analysis (30) and is indicated by a dashed underline.

Figure 3-3. Nlt1p is homologous to the mammalian ribophorin I. The sequences of the yeast Nlt1p and the human and rat ribophorin I proteins (15, 16) were compared with the program PRETTYPLOT (Computer Group, European Molecular Biology Laboratory, Heidelberg, Germany). Nlt1p shows significant amino acid similarity and identity to the amino-terminal domains of the mammalian proteins.

Peptide 64-1	A Q Y E P P A T W E N V D Y K R T I D V
Peptide 64-2	L L P E G A T D H Y F T
Peptide 64-3	R Q T H F V N V L T M L

Figure 3-1. Amino acid sequences of the 64 kDa glycoprotein.

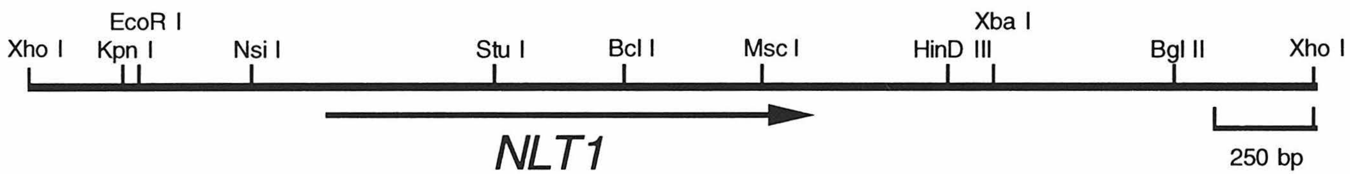

Figure 3-2. A. Restriction map of the *NLT1* locus.

```
    AAAATTCAAAAGAAATCTATCAATATAACAAAAACGCCAAATCATCAAAAATGATCGGTG
    TTGAAGAGCTTGGCACTCTTAAAGGCGGCTAGTTCATAATGCTATAGGAATTGACTCAAG
    GAAACGGACTATGTCTTGTACTGAATACTGTCTTCATTTGCCCATAGAATGCATTAGTGT
    TACTCTTCTTCGCGAGCAGGGTAAGTATGCGCGTAATGTTTTTTATTTTCTGAAAGGTTC
    AAAGTATGCAGAACAAATTAATGTTTGCTTTTTATTAAGCAAGCTACTTCTTTGACAAGT
    ACCCGATTGCTTCTTTAGCTCGGAACAAGACGCAAACTACAAAATATAGGTGCTGAAAAA
             10                   30                   50
    ATGAGGCAGGTTTGGTTCTCTTGGATTGTGGGATTGTTCCTATGTTTTTTCAACGTGTCT
  1 M  R  Q  V  W  F  S  W  I  V  G  L  F  L  C  F  F  N  V  S
             70                   90                  110
    TCTGCTGCCCAATACGAGCCACCTGCGACTTGGGAGAATGTTGATTATAAGAGGACAATA
 21 S  A  A  Q  Y  E  P  P  A  T  W  E  N  V  D  Y  K  R  T  I
          ↑ 130                  150                  170
    GACGTGTCAAACGCTTATATTTCAGAAACAATCGAAATAACTATCAAAAACATAGCAAGC
 41 D  V  S  N  A  Y  I  S  E  T  I  E  I  T  I  K  N  I  A  S
            190                  210                  230
    GAACCTGCGACTGAATACTTCACAGCCTTTGAGAGTGGCATCTTCAGTAAAGTTTCCTTT
 61 E  P  A  T  E  Y  F  T  A  F  E  S  G  I  F  S  K  V  S  F
            250                  270                  290
    TTTTCAGCCTATTTTACCAACGAGGCAACTTTTTTAAATAGCCAATTACTTGCCAATTCG
 81 F  S  A  Y  F  T  N  E  A  T  F  L  N  S  Q  L  L  A  N  S
            310                  330                  350
    ACTACAGCACCTGGTGACGATGGTGAAAGTGAAATTAGATACGGGATCATTCAATTTCCA
101 T  T  A  P  G  D  D  G  E  S  E  I  R  Y  G  I  I  Q  F  P
            370                  390                  410
    AATGCAATTTCCCCTCAGGAAGAAGTTTCTTTAGTGATTAAGAGCTTCTATAATACCGTA
121 N  A  I  S  P  Q  E  E  V  S  L  V  I  K  S  F  Y  N  T  V
                 430                  450                  470
    GGTATTCCTTATCCTGAGCACGTTGGAATGTCAGAAGAACAACACCTATTGTGGGAAACG
141 G  I  P  Y  P  E  H  V  G  M  S  E  E  Q  H  L  L  W  E  T
            490                  510                  530
    AACAGATTGCCGCTTTCTGCTTACGATACCAAGAAGGCCTCTTTTACGCTGATTGGTAGC
161 N  R  L  P  L  S  A  Y  D  T  K  K  A  S  F  T  L  I  G  S
```

Figure 3-2. B. Nucleotide sequence of the *NLT1* locus.

```
            550                 570                 590
     TCATCATTTGAGGAGTACCACCCCCCAAATGACGAGAGTTTACTGGGAAAAGCTAATGGA
181  S  S  F  E  E  Y  H  P  P  N  D  E  S  L  L  G  K  A  N  G
            610                 630                 650
     AACTCTTTTGAGTTTGGGCCTTGGGAAGATATTCCGAGATTTTCTTCAAACGAAACCTTA
201  N  S  F  E  F  G  P  W  E  D  I  P  R  F  S  S  N  E  T  L
            670                 690                 710
     GCAATTGTTTATTCCCACAATGCCCCATTGAATCAGGTAGTGAATTTGAGAAGAGATATT
221  A  I  V  Y  S  H  N  A  P  L  N  Q  V  V  N  L  R  R  D  I
            730                 750                 770
     TGGCTTTCTCATTGGGCTTCCACAATACAATTTGAGGAATATTATGAATTAACAAACAAA
241  W  L  S  H  W  A  S  T  I  Q  F  E  E  Y  Y  E  L  T  N  K
            790                 810                 830
     GCCGCAAAACTGTCTAAAGGATTTTCAAGATTAGAATTAATGAAACAGATTCAAACTCAG
261  A  A  K  L  S  K  G  F  S  R  L  E  L  M  K  Q  I  Q  T  Q
            850                 870                 890
     AATATGAGACAAACTCACTTTGTTACTGTCTTAGACATGCTCCTGCCTGAGGGAGCTACT
281  N  M  R  Q  T  H  F  V  T  V  L  D  M  L  L  P  E  G  A  T
            910                 930                 950
     GATCATTATTTCACTGATTTGGTTGGCCTTGTTTCCACGTCGCATGCAGAACGTGACCAT
301  D  H  Y  F  T  D  L  V  G  L  V  S  T  S  H  A  E  R  D  H
            970                 990                 1010
     TTCTTTATAAGACCAAGATTCCCAATCTTTGGAGGTTGGAACTACAATTTTACTGTCGGT
321  F  F  I  R  P  R  F  P  I  F  G  G  W  N  Y  N  F  T  V  G
           1030                1050                1070
     TGGACTAATAAATTGTCCGATTTCTTGCATGTATCCTCTGGCTCAGACGAGAAATTCGTT
341  W  T  N  K  L  S  D  F  L  H  V  S  S  G  S  D  E  K  F  V
           1090                1110                1130
     GCTTCTATCCCAATTCTAAACGGCCCACCGGACACTGTATATGATAATGTTGAATTATCG
361  A  S  I  P  I  L  N  G  P  P  D  T  V  Y  D  N  V  E  L  S
           1150                1170                1190
     GTATTTCTTCCGGAAGGGGCCGAAATATTCGATATTGATTCTCCAGTCCCTTTTACAAAT
381  V  F  L  P  E  G  A  E  I  F  D  I  D  S  P  V  P  F  T  N
```

Figure 3-2. B. Nucleotide sequence of the *NLT1* locus.

```
        1210                    1230                    1250
    GTTTCTATAGAAACCCAGAAATCATACTTTGACCTAAATAAAGGTCACGTTAAATTAACT
401 V  S  I  E  T  Q  K  S  Y  F  D  L  N  K  G  H  V  K  L  T
        1270                    1290                    1310
    TTCAGTTACAGAAATTTGATTAGTCAAGTTGCCAATGGCCAAGTCTTGATAAAGTACGAC
421 F  S  Y  R  N  L  I  S  Q  V  A  N  G  Q  V  L  I  K  Y  D
        1330                    1350                    1370
    TACCCGAAAAGCTCTTTTTTCAAGAAGCCTCTGTCTATTGCTTGCTATATTTTCACCGCA
441 Y  P  K  S  S  F  F  K  K  P  L  S  I  A  C  Y  I  F  T  A
        1390                    1410                    1430
    CTAATGGGAGTTTTTGTCTTAAAAACTTTGAACATGAACGTAACTAACTGAGTTCACGTT
461 L  M  G  V  F  V  L  K  T  L  N  M  N  V  T  N  *
    TGAATGATATATACCTTATGATGCAGAGCAATAAATTCAGTATTTAATTTTTTATAAGTT
    TTATAGATAAGAATTTTCCAATGAATAAAGGTATTTACAATCATTTCCTAGACATTCTCG
    TGGTCTTTTCTTTGCACAATCAATGGTCATAGTTTTTTTTGTTTAGTAGTTTAAGGACAG
    AAGAATAACAAAGATGTTCACGTTTTGAATGTTACCAGACATTCTCAGATTCATCCTTTA
    ACCTAGCGACGCGCACAGGCTCCCAATCCTCTTCAGAAAGACCTTGTAAACGATAATATT
```

Figure 3-2. B. Nucleotide sequence of the *NLT1* locus.

```
Human  MEAPAAGLFLLLLLGTWAPAPGSASSEAPPLINEDVKRTVDLSSHLAKVTAEVVLAHLGGGSTSRATSFL  70
Rat    MEAPI--VLLLLLWLALAPTPGSASSEAPPLVNEDVKRTVDLSSHLAKVTAEVVLAHPGGGSTARASSFV  68
Nlt1p  MRQVWFSWIVGLFLCFFNVSSAAQYEPPATWENVDYKRTIDVSNAYISETIEITIKNIASEP---ATEYF  67

Human  LALEPELEARLAHLGVQVKGEDE-EENNLEVRETKIKGKSG----RFFTVKLPVALDPGAKISVIVETVY  135
Rat    LALEPELESRLAHLGVQVKGEDE-EDNNLEVRETKMKGKSG----RFFTVKLPVALDPGSKISIVVETVY  133
Nlt1p  TAFESGIFSKVSFFSAYFTNEATFLNSQLLANSTTAPGDDGESEIRYGIIQFPNAISPQEEVSLVIKSFY  137

Human  THVLHPYPTQITQSEKQFVVFEGNHYFYSPYPTKTQTMRVKLASRNVESYTKLGNPT----RSEDLLDYG  201
Rat    THVLHPYPTQITQSEKQFVVFEGNHYFYSPYPTKTQTMRVRLASRNVESHTKLGNPS----RSEDILDYG  199
Nlt1p  NTVGIPYPEHVGMSEEQHLLWETNRLPLSAYDTKKASFTL-IGSSSFEEYHPPNDESLLGKANGNSFEFG  206

Human  PFRDVPAY-SQDTFKVHYENNSPFLTITSMTRVIEVSHWGN-IAVEENVDLKHTGAVLKGPFSRYDYQRQ  269
Rat    PFKDIPAY-SQDTFKVHYENNSPFLTITSMTRVIEVSHWGN-IAVEENVDLKHTGAVLKGPFSRYDYQRQ  267
Nlt1p  PWEDTPRFSSNETLAIVYSHNAPLNQVVNLRRDIWLSHWASTIQFEEYYELTNKAAKLSKGFSRLELMKQ  276

Human  PDS----GISSIRSFKTILPAAAQDVYYRDEIGNVSTSHLLILDDSVEMEIRPRFPLFGGWKTHYIVGYN  335
Rat    PDS----GISSIRSFKTILPAAAQDVYYRDEIGNVSTSHLLILDDSVEMEIRPRFGLFGGWKTHYIVGYN  333
Nlt1p  IQTQNMRQTHFVTVLDMLLPEGATDHYFTDLVGLVSTSH----AERDHFFIRPRFPIFGGWNYNFTVGWT  342

Human  LPSYEYLY---NLGDQYALKMRFVDHVFDEQVIDSLTVKIILPEGAKNIEIDSPYEISRAPDELHYTYLD  402
Rat    LPSYEYLY---NLGDQYALKMRFVDHVFDEQVIDSLTVKIILPEGAKNIQVDSPYDISRAPDELHYTYLD  400
Nlt1p  NKLSDFLHVSSGSDEKFVASIPILNGP-PDTVYDNVELSVFLPEGAEIFDIDSPVPFTNVSIETQKSYFD  411

Human  TFGRPVIVAYK-KNLVEQHIQ-DIVVHYTFNKVLMLQEPLLVVAAFYILFFTVIIYVRLDFSITKDPAAE  470
Rat    TFGRPVIVAYK-KNLVEQHIQ-DIVVHYTFNKVLMLQEPLLVVAAFYILFFTVIIYVRLDFSITKDPAAE  468
Nlt1p  LNKGHVKLTFSYRNLISQVANGQVLIKYDYPKSSFFKKPLSIACYIFTALMGVFVLKTLNMNVTN-----  476

Human  ARMKVACITEQVLTLVNKRIGLYRHFDETVNRYKQSRDISTLNSGKKSLETEHKALTSEIALLQSRLKTE  540
Rat    ARMKVACITEQVLTLVNKRLGLYRHFDETVNRYKQSRDISTLNSGKKSLETEHKAVTSEIAVLQSRLKTE  538
Nlt1p  ----------------------------------------------------------------------  476

Human  GSDLCDRVSEMQKLDAQVKELVLKSAVEAERLVAGKLKKDTYIENEKLISGKRQELVTKIDHILDAL  607
Rat    GSDLCDRVSEMQKLDAQVKELVLKSAVEAERLVAGKLKKDTYIENEKLSSGKRQELVTKIDHILDAL  605
Nlt1p  -------------------------------------------------------------------  476
```

Figure 3-3. Nlt1p is homologous to the mammalian ribophorin I.

Chapter 4

Purification of the Affinity-Tagged *Saccharomyces cerevisiae* Oligosaccharyl Transferase Complex

Introduction

Oligosaccharyl transferase is a multimeric protein complex resident in the rough endoplasmic reticulum membrane of eukaryotic cells; the molecular composition and the specific functions of the enzyme subunits have yet to be conclusively defined. The enzyme was first purified from canine pancreas cells as a complex of three polypeptides – ribophorin I, ribophorin II, and OST48 (1, 2). Transferase complexes with up to six subunits have since been isolated from a number of eukaryotic sources (3-9). Although the sizes of some of the subunits of the various transferase complexes are heterogeneous, sequence homology shows that the three subunits of the canine enzyme are conserved throughout evolution from yeast to mammals.

The enzyme has been purified from *Saccharomyces cerevisiae* as a complex of four to six polypeptides (3-5). The genes encoding five potential subunits of the enzyme have now been cloned. The essential *NLT1* or *OST1* gene encodes the 64 kDa glycoprotein subunit of the enzyme (2, 10). The 64 kDa Nlt1p is variably glycosylated in three or four positions *in vivo,* has one potential carboxy-terminal transmembrane helix, and shows significant homology to the luminal domain of the ribophorin I subunit of the mammalian enzyme (10, 11). Conditional lethal mutations in the gene cause defects in protein glycosylation *in vivo* (11). The essential *WBP1* gene encodes the 45 kDa glycoprotein subunit of the enzyme (12). Wbp1p is glycosylated in two positions *in vivo,* has one potential carboxy-terminal transmembrane helix, and shows significant overall homology to the mammalian OST48 subunit (2, 5, 13). Like mutations of the *OST1* gene, conditional lethal mutations of the *WBP1* gene also cause protein glycosylation defects; in addition, depletion of the *WBP1* gene product yields a

significant reduction in transferase activity and subsequent cell growth arrest (12). The essential *SWP1* gene was isolated as a high copy suppressor of one conditional lethal mutation in the *WBP1* gene (14). The 32 kDa Swp1p is not glycosylated, has three potential carboxy-terminal transmembrane helices, and shows significant homology to the amino-terminal half of the 64 kDa ribophorin II mammalian subunit (3, 5, 14). Depletion of the *SWP1* gene product also yields cell growth arrest and a significant reduction of transferase activity (14).

Recent research has identified further subunits of the yeast oligosaccharyl-transferase complex that have no apparent homologues in the purified mammalian enzyme complex. The hydrophobic 34 kDa subunit is encoded by the *OST3* gene (15). Unlike the other oligosaccharyl transferase genes, *OST3* is not essential for the vegetative growth of the yeast cell; however, deletion of the gene yields glycosylation defects similar to those that result from mutations in the *NLT1/OST1*, *WBP1*, and *SWP1* genes (15). Purification of the wild type enzyme indicated that the 34 kDa subunit might have been underrepresented in the multimeric complex (3, 5). An additional 16 kDa subunit was observed in one purification of the enzyme (3), but not in two subsequent purifications (4, 5). That subunit is encoded by the essential *OST2* gene. As with the other essential oligosaccharyl transferase genes, conditional mutations in the *OST2* locus yield protein glycosylation defects, and overexpression of the *OST2* gene product also suppresses a conditional mutation in the *WBP1* gene (16). Ost2p has two or three potential transmembrane domains and might have a significant cytosolic domain unlike the other three essential oligosaccharyl transferase subunits. In addition, two other genes, *OST4* and *STT3*, have been associated with transferase activity *in vivo* (17, 18), and one purification of the enzyme

yielded a 9 kDa protein subunit that has not yet been characterized genetically (3).

To expand the potential for molecular and mechanistic studies of the enzyme, we have created a yeast strain with an affinity-tagged oligosaccharyl transferase. The *NLT1* gene encoding the largest subunit of the enzyme was deleted from a *S. cerevisiae* strain and replaced with a modified version of the same gene. The coding region of the modified gene was fused in-frame to a sequence encoding a twenty-two residue carboxy-terminal affinity tag including both an eight residue FLAG epitope (19) and a six residue polyhistidine motif for purification of the enzyme on commercially available affinity media. Expression of the modified gene under the control of the *CUP1* promoter (20) from the yeast metallothionein gene under non-inducing conditions yielded an appropriate amount of the tagged subunit.

Yeast strains bearing the affinity-tagged oligosaccharyl transferase (OTtag) complex were viable and grew well on standard media; the affinity tag was stable even in high density cultures. The yields of enzyme activity and the biochemical properties of the OTtag complex were indistinguishable from those of the native enzyme. OTtag was readily purified on affinity media as a complex of five polypeptides – Nlt1p, Wbp1p, Ost3p, Swp1p, and Ost2p. All five subunits appeared to be represented in identical stoichiometric amounts including the nonessential Ost3p. The quaternary structure of the enzyme complex was stable under vigorous detergent and ionic strength conditions. The accessible purification of the OTtag complex provides an experimental system for the study of the molecular composition and ultimately the biochemical mechanism of this fascinating enzyme.

Materials and Methods

Plasmids

Standard molecular biological and polymerase chain reaction techniques were used (21). Plasmid pYES2 (Invitrogen) was modified by inserting a sequence encoding a twenty-two amino acid affinity tag including an eight residue FLAG epitope and a six residue polyhistidine tag for carboxy-terminal fusion to yeast genes. The resultant pYES2(FLAG-6His) vector included the *GAL1,10* promoter upstream and the *cyc1* terminator downstream of the polycloning region and affinity tags. The plasmid pRS414(*CUP1p*-FLAG-6His-*cyc1*) was created for expression of FLAG and 6xHis tagged proteins from the inducible *CUP1* promoter. The FLAG-6His-*cyc1* sequence from plasmid pYES2(FLAG-6His) was inserted into the polycloning region of plasmid pRS414 (22); the *CUP1* promoter was obtained from J. Abelson and inserted upstream of the FLAG-6His-*cyc1* sequence. Plasmid pRS414(*CUP1p*-*NLT1*-FLAG-6His-*cyc1*) was created by inserting the coding region from the *NLT1* gene into the vector pRS414(*CUP1p*-FLAG-6His-*cyc1*) downstream of the *CUP1* promoter and upstream of the FLAG-6His sequence such that the final codon of the *NLT1* coding region was fused to the first codon of the affinity tag sequence. The affinity tag sequence included a stop codon after the final codon of the 6His tag. A site sensitive to Factor Xa protease was placed upstream of the affinity tag to facilitate its removal in future experiments. The carboxy-terminal sequence of the encoded Nlt1p-tag fusion protein including the final four residues of the wild type polypeptide was Asn-Val-Thr-Asn-Val-Asp-Ile-Glu-Gly-Arg-Gly-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Gly-His-His-His-His-His-His-Stop.

Yeast strains and media

The yeast strains used in the experiments are listed in Table 4-1. Standard yeast genetic techniques and yeast media were used (23). Yeast strains were transformed by the lithium acetate method (24). Yeast strains JD52 and JD53 were obtained from A. Varshavsky. Diploid yeast strain JD52/53 was obtained by mating the haploid strains JD52 and JD53 and selecting zygotes by cell morphology. An NsiI-MscI fragment of the chromosomal copy of the *NLT1* gene (10) of strain JD52/53 was deleted and replaced with the *LEU2* gene to create strain RPY27; successful disruption of the locus was verified by Southern blotting. Leucine prototrophs were selected and sporulated; as expected, only two spores from each tetrad gave rise to viable colonies. Diploid strain RPY27 was transformed with plasmid pRS414(*CUP1p-NLT1*-FLAG-6His-*cyc1*) and sporulated; all four spores from several tetrads gave rise to viable colonies. All four colonies from one tetrad were transferred to media selective for leucine and tryptophan prototrophy; two strains, RPY40 and RPY41, were viable on the media indicating the presence of both the chromosomal deletion and the expression plasmid.

Isolation and solubilization of yeast membranes

Yeast cells were grown in YPD media and harvested in mid-log phase. The cells were broken with glass beads, and membranes were harvested by centrifugation at 40 krpm for 60 minutes in a Beckman Ti45 rotor (5). Membrane proteins were partially solubilized in 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 140 mM sucrose, 1.0 % Nonidet P-40, and 0.1 mM AEBSF. The solubilized fraction was clarified by centrifugation at 40 krpm for

60 minutes. The supernatant was carefully separated from a glassy pellet and an amorphous turbid pellet.

Purification of the OTtag complex

Oligosaccharyl transferase activity was monitored as described previously (5). Twenty milliliters of solubilized membrane proteins isolated from 40 g RPY40 yeast cells were applied to 6 mL of concanavalin A agarose (Vector) batchwise at 4° C for 12 hours; the media was then washed with 80 mL of the solubilization buffer with 0.5 mg/mL PC (Avanti Polar Lipids) and eluted batchwise with 750 mM α-MeMan in 80 mL of the same buffer. Eluted fractions were adjusted to 15 mM imidazole and 1 mM β-mercaptoethanol and applied to 6 mL of Ni-NTA-agarose media (Qiagen) by batch incubation. The media was applied to a 2 cm diameter column and washed with 40 mL of 20 mM HEPES pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 140 mM sucrose, 1.0 % Triton X-100, and 0.5 mg/mL PC. Bound proteins were eluted with 10 mL applications of 20 mM MES pH 5.0, 150 mM NaCl, 10 mM $MgCl_2$, 140 mM sucrose, 1.0 % Triton X-100, and 0.5 mg/mL PC; eluted fractions were immediately adjusted to 50 mM HEPES pH 7.5. Active fractions were pooled and applied to 1.0 mL M2-agarose (Eastman Kodak) batchwise. The media was washed batchwise three times with 10 mL 50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 140 mM sucrose, 1.0 % Triton X-100, and 0.5 mg/mL PC buffer and then eluted batchwise with 0.05 mg/mL FLAG peptide (Eastman Kodak) in 5.0 mL of the same buffer. Approximately ten percent of the total activity in the initial fraction of solubilized yeast membrane proteins was recovered after three steps of affinity purification.

Production of polyclonal antiserum against Nlt1p

The DNA encoding amino acids 23 through 460 of the yeast Nlt1p (10) was subcloned into vector pJC20HC (Carl Parker, personal communication) to create vector pJC-*NLT1*-HC expressing the luminal domain of Nlt1p with a carboxy-terminal six-histidine affinity tag. The fusion protein was expressed in *E. coli* BL21(DE3)pLysS cells (Novagen) from the T7 promoter. Induced cells from a 500 mL culture were lysed in 6 M guanidine-HCl, and the lysate was applied to Ni-NTA agarose beads (Qiagen). The pure recombinant protein was eluted with 30 mM EDTA and 150 mM imidazole in 6 M urea. Swiss-Webster mice were inoculated at two week intervals with 10 μg of protein each (25). After three rounds of injection, a strong response was seen against the recombinant fusion protein, and after one more injection a strong response was seen against the 64 kDa subunit of the purified oligosaccharyl transferase complex. Ascitic fluid from an induced tumor was used as a polyclonal antiserum specific for Nlt1p. Antiserum specificity enabled recognition of the glycoprotein from a crude lysate of yeast proteins.

Analytical methods

The protein composition of various biochemical fractions was visualized by SDS-PAGE (26) on 4 % - 20 % acrylamide gradient gels followed by silver staining (27). For immunoblotting, proteins were separated by SDS-PAGE and then transferred to nitrocellulose (28). The membranes were blocked with nonfat dried milk and then probed with antiserum specific for Nlt1p (above), Wbp1p (13), Swp1p (Marcus Aebi, personal communication), Ost2p (16), or the FLAG epitope (Eastman Kodak). The membranes were then

probed with alkaline phosphatase - conjugated secondary antibodies (antiNlt1p, antiWbp1p, and antiFLAG) or with horseradish peroxidase - conjugated secondary antibodies (antiSwp1p and antiOst2p). Alkaline phosphatase - conjugated secondary antibodies were visualized with 5-bromo-4-chloro-3'-indolylphosphate and nitro-blue tetrazolium (Pierce); horseradish peroxidase - conjugated secondary antibodies were visualized by enhanced chemiluminescence (Amersham).

Results

The OTtag complex is functional

To facilitate the *in vivo* and *in vitro* examination of the yeast oligosaccharyl transferase, a yeast strain bearing a tagged version of the largest enzyme subunit was created. Two affinity tags were fused to the carboxy-terminus of the *NLT1* gene. The eight residue FLAG epitope enables immunopurification of the tagged gene product with the monoclonal antibody M2 media (19), and the six-histidine motif enables binding of the subunit to immobilized nickel cations (29). The affinity tags were placed at the carboxy-terminus of the subunit to minimize interference with the function of the enzyme. The 64 kDa glycoprotein Nlt1p subunit, which assembles into the enzyme complex in the membrane of the rough endoplasmic reticulum, appears to have an extensive luminal domain followed by a potential transmembrane hydrophobic domain and a short cytoplasmic tail of a few amino acids (10). In fact, only Ost2p of the five cloned yeast oligosaccharyl transferase genes might have a significant domain on the cytoplasmic side of the endoplasmic reticulum membrane (16). The *NLT1*-FLAG-6His fusion was expressed on a low copy yeast episomal plasmid

(22) under the control of the promoter from the yeast *CUP1* gene (20). The *CUP1* promoter provided inducible expression of the tagged gene product with $CuSO_4$, and the background expression from the promoter in YPD media with no added $CuSO_4$ gave expression of the tagged Nlt1p comparable to that of the wild type *NLT1* gene.

One chromosomal copy of the *NLT1* gene was deleted from a diploid yeast strain and replaced with the selectable *LEU2* marker. The resultant strain RPY27 was heterozygous for a deletion of all but 135 bp of the coding region of the essential *NLT1* gene; sporulation of this strain yielded viable colonies from only two spores of each tetrad dissected. Strain RPY27 was transformed with the plasmid expressing the tagged *NLT1* gene product to yield strain RPY39. When strain RPY39 was sporulated and dissected, several tetrads yielded viable colonies from all four spores indicating that the *NLT1*-FLAG-6His fusion complemented the deletion of the essential *NLT1* gene. Haploid strains RPY40 and RPY41 were isolated from one tetrad and harbored both the chromosomal deletion of the *NLT1* gene and the expression plasmid.

Yeast strain RPY40 grew well on YPD media both on plates and in culture. Western blotting analysis showed that the tagged Nlt1p was stable even in high density cultures. Two 70 kDa glycoforms of Nlt1p-FLAG-6His were present when whole cell yeast extracts were probed with anti-FLAG M2 monoclonal antibody; and the two 70 kDa glycoprotein bands were revealed by probing the same fraction with anti-Nlt1p antiserum – no untagged 64 kDa Nlt1p was present. Solubilized membrane protein fractions from strain RPY40 displayed oligosaccharyl transferase activity indistinguishable from similar fractions prepared from yeast strains expressing the native Nlt1p subunit.

Affinity purification of OTtag

In order to establish a method for the accessible isolation of a stable, active oligosaccharyl transferase, the OTtag complex was purified by a series of three chromatographic steps. Two media used in the purification, Ni-NTA-agarose and M2 agarose, were specific for each segment of the affinity tag; the third, concanavalin A-agarose, was specific for the oligosaccharides of the polypeptide complex and had previously been shown to be useful for the purification of the native enzyme (3, 5). The purification was carried out under vigorous conditions including high salt and detergent concentrations to minimize the presence of any loosely interacting polypeptides that might not be essential for oligosaccharyl transferase activity. The overall activity yield of the purification of OTtag was comparable to that of the purification of the native enzyme (5).

To date, five polypeptides have been consistently enriched in the final fraction (figure 4-1). Four of the previously reported oligosaccharyl transferase subunits were conclusively identified by Western blotting. The tagged Nlt1p subunit migrated as a pair of glycoforms at roughly 70 kDa; both glycoforms were detected by antibodies specific for the recombinant Nlt1p and for the FLAG epitope (figure 4-2). The glycosylated Wbp1p was detected at 45 kDa and Swp1p was detected at 30 kDa by the appropriate antisera (figure 4-2). The recently described Ost2p (16) was conclusively detected at 16 kDa both by silver-stained SDS-PAGE (figure 4-1) and by Western blotting (figure 4-2). Ost2p was detected in one previously reported characterization (3) of the native enzyme complex but not in two other characterizations (4, 5). Based on the clear enrichment of Ost2p in the purification of the affinity-tagged

oligosaccharyl transferase complex (figure 4-2), we concluded that Ost2p is a bona fide member of the multisubunit enzyme.

A fifth polypeptide was detected at 34 kDa and has been identified as Ost3p based on molecular weight. Unlike the other four characterized subunits of the transferase, Ost3p is not essential for the vegetative growth of the yeast cell (15). However, Ost3p appeared to be present in the enzyme complex at a level comparable to the other subunits and was not depleted through the purification of the enzyme. No other polypeptides were detected at apparent stoichiometric amounts relative to the known transferase subunits. When the purified enzyme was assayed by SDS-PAGE at high protein concentrations, a few low molecular weight polypeptides were detected. One of these might be the 9 kDa oligosaccharyl transferase subunit previously described (3). The affinity-tagged purification verifies the molecular composition of the transferase and shows that the subunits form a stable quaternary structure that is maintained in the membrane and in a detergent solubilization.

Discussion

The oligosaccharyl transferase has been purified from the yeast *Saccharomyces cerevisiae* by several groups with reasonable but not complete agreement in the observed subunit composition of the enzyme. Three independent purifications of the enzyme yielded multimeric polypeptide complexes with subunits of 64 kDa, 45 kDa, 34 kDa, and 30 kDa (3-5); those subunits are encoded by the *NLT1/OST1*, *WBP1*, *OST3*, and *SWP1* genes, respectively (10-12, 14, 15). The 34 kDa subunit is not essential for the growth of the yeast cell (15), and it might be depleted during the purification of the

enzyme (3, 5). One of the three purifications also included low molecular weight subunits with weights of 16 kDa and 9 kDa (3) that were not observed in the other two (4, 5); the 16 kDa subunit is encoded by the *OST2* gene (16). In addition to those polypeptides identified as transferase subunits by direct biochemical purification of the enzyme, *STT3* and *OST4* have been identified as genes essential for proper transferase function *in vivo* (17, 18).

To address the molecular composition of the enzyme, the largest subunit was affinity tagged. The resulting OTtag complex was purified by a sequence of affinity chromatography steps. In accord with the purification of the native yeast enzyme, the four large subunits of 70 kDa , 45 kDa, 34 kDa, and 30 kDa were observed in the purified complex; the size of the 70 kDa polypeptide is consistent with that expected of the tagged Nlt1p subunit. Western blotting identified three of the subunits as the products of the *NLT1*-FLAG-6His, *WBP1*, and *SWP1* genes.

A polypeptide at 16 kDa was present in the purified OTtag complex, and this polypeptide was conclusively identified as the Ost2p by Western blotting. This observation updates the previously reported description of a four subunit oligosaccharyl transferase complex from this laboratory (5) and confirms the results of another previously reported purification of a six subunit complex (3). It is likely that the 16 kDa subunit of the enzyme was not observed on the Coomassie blue - stained linear SDS-PAGE gels used to characterize the native complex (5). Here, using silver - stained gradient SDS-PAGE gels Ost2p was consistently observed in the purified OTtag complex, and Western blotting of the native complex purified according to (5) confirms the presence of Ost2p in the enzyme (data not shown). The *OST2* gene is essential for viability and for protein glycosylation (16), and the 16 kDa subunit should be considered an integral part of the enzyme.

The final 34 kDa subunit was identified as Ost3p by molecular weight. The nonessential 34 kDa Ost3p subunit did not appear to be underrepresented in the *in vivo* complex, nor did it appear to be depleted during the independent purification of OTtag described herein. Since this subunit appears to be present in the purified OTtag complex in an amount comparable to that of the essential OTtag subunits, it is likely be present in the *in vivo* complex at the same stoichiometric amount. If it was underrepresented in the purification of the native complex (3, 5), then that underrepresentation would more likely have been due to a depletion of the polypeptide *in vitro* rather than a substoichiometric participation in the *in vivo* complex. Although this subunit is not essential for viability, its loss affects the glycosylation state of several proteins *in vivo* and reduces the measured oligosaccharyl transferase activity of membrane extracts *in vitro* (15). Therefore, loss of the Ost3p subunit during purification would reduce but not eliminate enzyme activity.

No other polypeptides were observed at apparent stoichiometric amounts relative to the known oligosaccharyl transferase subunits. However, under high protein loading conditions several additional polypeptides were observed, particularly at low molecular weights. One of these might have been the 9 kDa subunit identified in the purification of the native enzyme (3). Another might have been the product of the *OST4* gene which has a predicted weight of 3.6 kDa (17). It is possible that the products of the *OST4* and *STT3* genes are present in the complex at substoichiometric amounts, and it is also possible that they are required for the *in vivo* function of the enzyme but not the actual catalysis of the glycosylation reaction.

The OTtag system provides a setting for the comprehensive study of the oligosaccharyl transferase enzyme. OTtag has initially been used to refine

the understanding of the molecular composition of the enzyme by tagging the Nlt1p subunit of the enzyme. The short tag is equally effective when placed at the carboxy-terminus of the Wbp1p subunit (unpublished results). Further biochemical study of the yeast enzyme will be aided by the rapid and amenable purification of the tagged enzymes. Overexpressed subunits of the transferase are readily purified when tagged, and a protease sensitive site will allow removal of the tag. In addition, the stable and reversible association of the polyhistidine motif with metal cations (29) enables specific binding to immobilized media through a single site of each enzyme complex. Such binding can be used to examine the subunit interactions under mildly denaturing conditions and to assay the substrate binding properties of the individual enzyme substrates. Finally, a plasmid carrying a tagged gene encoding a transferase subunit is ideally suited for site directed mutagenesis. After mutations are introduced into the transferase gene, the plasmid can be transformed into the appropriate yeast strain where the mutant complex can be rapidly assayed for molecular composition and for catalytic efficiency.

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Table 4-1 **Yeast Strains**

Strain	Genotype	Source
JD52	*MAT***a** *leu2-3,112 his3Δ200 trp1-Δ63 ura3-52 lys2-801*	A. Varshavsky
JD53	*MATα leu2-3,112 his3Δ200 trp1-Δ63 ura3-52 lys2-801*	A. Varshavsky
JD52/53	*MAT***a***/MATα leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 lys2-801/lys2-801*	This study
RPY27	*MAT***a***/MATα leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 lys2-801/lys2-801 nlt1-Δ2::LEU2/+*	This study
RPY39	*MAT***a***/MATα leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 lys2-801/lys2-801 nlt1-Δ2::LEU2/+* pRS414(*CUP1p-NLT1*-FLAG-6His-*cyc1*)	This study
RPY40	*MATα leu2-3,112 his3Δ200 trp1-Δ63 ura3-52 lys2-801 nlt1-Δ2::LEU2* pRS414(*CUP1p-NLT1*-FLAG-6His-*cyc1*)	This study
RPY41	*MATα leu2-3,112 his3Δ200 trp1-Δ63 ura3-52 lys2-801 nlt1-Δ2::LEU2* pRS414(*CUP1p-NLT1*-FLAG-6His-*cyc1*)	This study

Figure Legends

Figure 4-1. Purification of the OTtag complex. OTtag was purified on a series of affinity media. As an initial purification step, solubilized yeast membrane proteins were separated with the lectin concanavalin A immobilized on agarose. The glycoprotein complexes were then applied to Ni-NTA agarose and eluted with a pH step gradient. After two steps of purification, the oligosaccharyl transferase complex is visible in the isolated fraction (lane 4). OTtag was then separated from the contaminating proteins by purification on an immobilized monoclonal antibody specific for the FLAG epitope of the enzyme complex. The protein content of each fraction in the purification was visualized by SDS-PAGE followed by silver staining. The fractions are: a) molecular weight standards, b) solubilized yeast membrane proteins, c) concanavalin A eluate, d) Ni-NTA eluate, and e) M2 (antiFLAG) eluate.

Figure 4-2. Identification of the subunits of OTtag. The subunits of the OTtag complex were identified by Western blotting. The tagged Nlt1p subunit migrated as a pair of glycoforms near 70 kDa; the polypeptides were recognized by antiserum specific for Nlt1p (αNlt1p) and by antiserum specific for the FLAG epitope tag (αFLAG). The 45 kDa subunit was identified as Wbp1p (αWbp1p), the 30 kDa subunit as Swp1p (αSwp1p), and the 16 kDa subunit as Ost2p (αOst2p). The fractions are: a) solubilized yeast membrane proteins, b) concanavalin A eluate, c) Ni-NTA eluate, and d) M2 (antiFLAG) eluate.

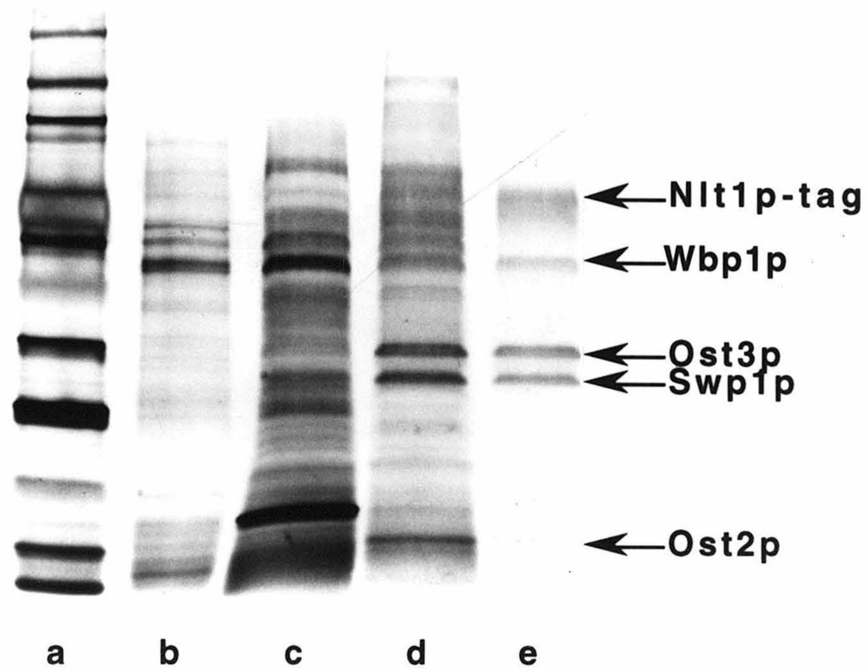

Figure 4-1. Purification of the OTtag complex.

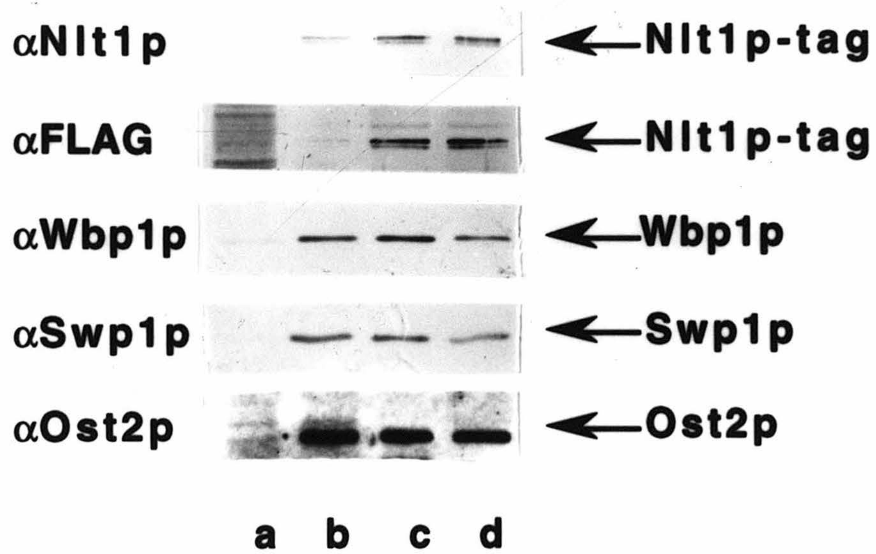

Figure 4-2. Identification of the subunits of OTtag.

Chapter 5

Molecular Characterization of the Recombinant Oligosaccharyl Transferase

The yeast oligosaccharyl transferase is a multimeric transmembrane protein complex. The enzyme has been purified from *Saccharomyces cerevisiae* as a heteromeric complex of four to six polypeptides (chapter 2) (1-3). The genes encoding all six of the putative subunits of the enzyme have now been cloned. The essential *NLT1* or *OST1* gene encodes the 64 kDa glycoprotein subunit of the enzyme (chapter 3) (4, 5). The essential *WBP1* gene encodes the 45 kDa glycoprotein subunit (6), and the essential *SWP1* gene encodes the 32 kDa protein subunit (7). The recently cloned *OST2* gene encodes the 16 kDa subunit of the enzyme; the gene is essential and more highly conserved than the other essential oligosaccharyl transferase genes (8). The *OST3* gene encodes the 34 kDa subunit; however, the *OST3* gene is not essential as deletion of the gene results in a viable yeast strain albeit with reduced oligosaccharyl transferase activity (9). *OST5* encodes the 9 kDa subunit observed in one purification of the enzyme, but the gene has yet to be fully characterized (3, 10).

The experiments described here attempt to develop a more complete understanding of the molecular and biochemical properties of the oligosaccharyl transferase. In one experiment, an affinity-purified oligosaccharyl transferase bearing a six-histidine tag on the carboxy-terminus of the Nlt1p subunit (chapter 4) (11) was exposed to series of mild denaturants in an attempt to disrupt the enzyme complex and identify those subunits that are essential for catalysis. In another, the subunits of the transferase were individually expressed in yeast and purified; an effort was made to reconstitute an active complex from purified subunits. In a third experiment, the subunits of the enzyme were overexpressed individually and multiply in yeast strains which were assayed for increases in oligosaccharyl transferase activity. Finally, an assay for the *in vitro* binding of substrates by the intact

oligosaccharyl transferase and by individual transferase subunits was developed to study the thermodynamics of substrate binding and to perhaps identify those subunits necessary for substrate binding.

Materials and Methods

Plasmids, yeast strains and media

A family of plasmids for the expression of tagged or untagged versions of yeast genes under the control of the *GAL* promoter has been created. They are based on the pRS423 - pRS426 series of vectors each containing a different selectable yeast marker and a 2 μm origin of replication (12). The vectors were modified with a *GAL* promoter, a sequence encoding in-frame fusible FLAG and six-histidine affinity tags, and a *cyc1* transcription terminator by standard PCR and molecular biological techniques (13). The various oligosaccharyl transferase genes were inserted into the vectors to create plasmids pRS423(*GAL-SWP1-cyc1*), pRS424(*GAL-WBP1-cyc1*), and pRS425(*GAL-OST2-cyc1*) encoding the gene products without the affinity tag. Plasmid pRS423(*GAL-SWP1*-FLAG-6His-*cyc1*) was created encoding a tagged version of the Swp1p. In addition, the *NLT1*, *WBP1*, *OST3*, and *OST2* genes were inserted into vector pYES2(FLAG-6His) (11) to create a series of plasmids encoding tagged versions of the oligosaccharyl transferase subunits.

The yeast strains used in the experiments are listed in Table 5-1. Standard yeast genetic techniques and yeast media were used (14). Yeast strains were transformed by the lithium acetate method (15). Yeast strain TH451 (16) – heterozygous for a deletion of the *WBP1* locus – was transformed with plasmid pYES2(*WBP1*-FLAG-6His) to yield strain RPY21;

this strain was sporulated and dissected, and the viable haploid strain RPY23 was isolated after selecting for the genomic deletion and the episomal plasmid. Yeast strain RPY27 – heterozygous for a deletion of the *NLT1* locus – was transformed with plasmid pYES2(*NLT1*-FLAG-6His) to yield strain RPY30; this strain was sporulated and dissected, and the viable haploid strain RPY32 was obtained after selecting for both the genomic deletion and the plasmid. Yeast strain ATH7 (7) – heterozygous for a deletion of the *SWP1* locus – was transformed with plasmid pRS423(*GAL-SWP1*-FLAG-6His-*cyc1*) to yield strain RPY59; this strain was sporulated and dissected, and the viable haploid strain RPY60 was obtained after selecting for both the genomic deletion and the plasmid. Yeast strain RPY47 was obtained by transforming yeast strain JD52 (A. Varshavsky) with the plasmid pYES2(*NLT1*-FLAG-6His). RPY47 was transformed with the plasmid pRS424(*GAL-WBP1-cyc1*) to yield strain RPY51. RPY51 was transformed with the plasmid pRS423(*GAL-SWP1-cyc1*) to yield strain RPY53. And RPY53 was transformed with the plasmid pRS425(*GAL-OST2-cyc1*) to yield strain RPY64.

Isolation and solubilization of yeast membranes

Yeast cells were grown in the appropriate media and harvested in mid-log phase. The cells were broken with glass beads, and membranes were harvested by centrifugation at 40 krpm for 60 minutes in a Beckman Ti45 rotor. Membrane proteins were partially solubilized in 50 mM HEPES pH7.5, 500 mM NaCl, 10 mM $MgCl_2$, 140 mM sucrose, 1.0 % Nonidet P-40, and 0.1 mM AEBSF. The solubilized fraction was clarified by centrifugation at 40 krpm for 60 minutes. The supernatant was carefully separated from a glassy pellet and an amorphous turbid pellet.

Purification of the affinity-tagged oligosaccharyl transferase complex

The affinity-tagged oligosaccharyl transferase was purified from yeast strain RPY40 as described (chapter 4) (11).

Purification of recombinant oligosaccharyl transferase subunits

Fifty milliliters of solubilized membrane proteins isolated from yeast cells expressing a tagged subunit of the oligosaccharyl transferase were adjusted to 35 mM imidazole and 1 mM β-mercaptoethanol and applied to 2 ml Ni-NTA (Qiagen) agarose batchwise. The media was applied to a 2 cm diameter column and washed with 10 ml of the solubilization buffer with 35 mM imidazole and 0.5 mg/ml PC. The media was then washed briefly with 1 M urea in the solubilization buffer with 0.5 mg/ml PC. The media was then washed with 10 ml of 20 mM HEPES pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 140 mM sucrose, 1.0 % Triton X-100, and 0.5 mg/ml PC, and then eluted with 3 x 10 ml of 20 mM glycine pH 3.5, 150 mM NaCl, 10 mM $MgCl_2$, 140 mM sucrose, 1.0 % Triton X-100, and 0.5 mg/ml PC; eluted fractions were immediately adjusted to 50 mM HEPES pH 7.5. Pooled fractions containing the recombinant subunit identified by Western blotting were applied to 0.5 ml M2 agarose (Eastman Kodak) batchwise. The media was washed with 3 x 5 ml of 50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 140 mM sucrose, 1.0 % Triton X-100, and 0.5 mg/ml PC, and then eluted with 5 ml of 50 μg/ml FLAG peptide in the same buffer.

Results

Disruption of the affinity-purified oligosaccharyl transferase complex

The yeast oligosaccharyl transferase is a complex of at least six polypeptides. It is possible that all of the subunits are not necessary for catalysis. For instance, the 34 kDa Ost3p subunit, present in several independent purifications of the enzyme, is not essential for the viability of the yeast cell (9). Also, some of the subunits might have multiple cellular functions. The essential Ost2p is more highly conserved from yeast to mammals than the other transferase subunits (8) indicating that it might be under greater selection pressure because of an additional cellular function. Hence, it might be possible to remove loosely bound, nonessential subunits without losing activity and thereby define the polypeptide composition of the minimal catalytic core of the enzyme. Oligosaccharyl transferase isolated from pig liver membranes was successfully disrupted, and activity was retained by an unstable complex of two polypeptides with molecular weights of 66 kDa and 48 kDa (17); these polypeptides are homologous to the Nlt1p and Wbp1p subunits of the yeast enzyme.

Yeast strain RPY40 expresses a tagged version of the 64 kDa Nlt1p subunit of the transferase that complements a deletion of the genomic *NLT1* locus (chapter 4) (11). The fully active, tagged enzyme complex was purified from this strain on a series of affinity chromatography media as described (chapter 4) (11). The affinity-tagged transferase allows the identification of a potential limited enzymatic core by disruption of the intact, purified complex. The enzyme is tagged on only one subunit and the interaction between the six-histidine motif and Ni-NTA agarose is stable under conditions which disrupt protein - protein interactions including 8 M urea and 6 M guanidine. A series of conditions for the disruption of the purified complex were

examined including 1 M urea, 1 M NaCl, 0 M NaCl, 0 mg/ml PC, and 5 % Triton X-100. Enzymatic activity is reduced or eliminated under most of these conditions; however, none of these conditions disrupted the enzyme complexes when visualized by silver-stained SDS-PAGE. The immobilized complex was also exposed to a gradient of up to 4 M urea in the presence of a reductant; although this treatment completely eliminated enzymatic activity, none of the subunits were removed from the complex in a significant amount.

Reconstitution of the enzyme from purified recombinant subunits

Another approach to defining the catalytic core of the enzyme is to build a minimal complex from the expressed recombinant subunits of the enzyme. Three of the subunits of the enzyme have been expressed in separate yeast strains from the *GAL* promoter and purified. The subunits were purified via the same dual affinity tags used for the purification of the enzyme complex (chapter 4) (11). Each subunit was expressed in a yeast strain that contains a background of the native oligosaccharyl transferase complex; an effort was made to separate the independent, tagged polypeptides from the complexed, tagged polypeptides. The background imidazole concentration in the application of the proteins to Ni-NTA agarose was increased to prevent the binding of the complexed subunit to the media. In each purification, oligosaccharyl transferase activity did not bind to the media and could be detected in unbound fractions; no activity was present in the purified fractions. The purified fractions also did not contain any additional transferase subunits when probed by Western blotting and by silver-stained SDS-PAGE. Approximately 50 μg of the recombinant Nlt1p was purified from 100 g RPY32, 200 μg of Wbp1p was purified from 100 g RPY23, and perhaps 1

µg of Swp1p was purified from 100 g RPY60; attempts to purify the expressed Ost2p were not successful.

The limited amounts of Swp1p and Ost2p hindered the reconstitution experiment. No activity was observed when the three purified recombinant subunits were incubated together in roughly equimolar amounts. An aliquot of the purified oligosaccharyl transferase complex with a similar amount of total protein yielded an easily measurable amount of activity. The reconstituted enzyme was analyzed by native PAGE. Only the Wbp1p migrated into the native gel; all or almost all of the recombinant Wbp1p did not appear to be complexed with any other protein. In addition, the reconstituted samples were exposed to the cross-linking reagent ethylene glycobis(succinimidylsuccinate) (EGS; Pierce); SDS-PAGE of the cross-linked sample indicated a fraction of each subunit was shifted into a higher molecular weight species, but most of each subunit remained uncomplexed.

Coexpression of the oligosaccharyl transferase genes

In addition to building a catalytically competent enzyme complex *in vitro*, the enzyme can also be examined by building such a complex *in vivo* through overexpression of the enzyme subunits. Yeast strains were created in which the essential oligosaccharyl transferase genes were expressed under the control of the *GAL* promoter including one strain in which all four essential genes were coexpressed. Yeast strain RPY64 harbored separate plasmids each expressing one of the four genes. In this strain, the Nlt1p was expressed by 5 - 10 fold under the control of the *GAL* promoter. The Wbp1p expressed well under *GAL* control yielding a 10 - 50 fold enrichment of the enzyme subunit. Swp1p expression was also increased several fold. Ost2p expressed well; the protein was not detectable in Western blots of extracts from wild type cells,

but it was detectable in Western blots from the expression strains. Coexpression of Ost2p improved the expression of other transferase subunits. In strains without the overexpressed Ost2p, Wbp1p was expressed as a mixture of two glycoforms; when Ost2p was coexpressed with Wbp1p, the fully glycosylated form of Wbp1p predominated. Strains bearing the *GAL-SWP1* fusion alone did not have detectable Swp1p in a crude extract, but when *GAL-SWP1* was coexpressed with *GAL-OST2* the protein was readily detectable by Western blotting. However, strain RPY64 coexpressing all four genes yielded only a 16 % increase in transferase specific activity relative to the parent yeast strain even though each of the four polypeptide subunits were overexpressed by at least five fold; strains overexpressing fewer than four genes yielded similar results. The coexpression strain included the dual affinity tags on only the Nlt1p subunit. All four subunits copurified with Nlt1p-tag on Ni-NTA agarose indicating that the expressed subunits did form a complex *in vivo*. It is possible that these four subunits are not sufficient for oligosaccharyl transferase activity.

Substrate binding assay

An assay for the binding of the dolicholpyrophosphoryl oligosaccharide substrate was established. An aliquot of the purified, six-histidine tagged active complex (chapter 4) (11) or a purified subunit was incubated with the radiolabeled substrate (chapter 2) (1) and Ni-NTA beads. The beads were washed with enzyme buffer and eluted with imidazole and EDTA. Fractions collected from the media were examined by scintillation counting to monitor copurification of the substrate with the protein sample. A similar assay could be established for the binding of a labeled peptide substrate.

In one such experiment, the substrate was present at a concentration of 2.4 nM and the enzyme was present at a concentration of roughly 10 nM. No binding of substrate to the enzyme was observed even though the oligosaccharide is readily transferred to a peptide substrate at that concentration. When the sugar substrate concentration was increased to 1 μM, binding was still not measurable; the expected 1 % substrate binding should have been detected under the experimental conditions. When the immobilized enzyme was chased with unlabeled substrate after the incubation with the labeled substrate, binding was still not improved. The substrate binding assay might be improved by co-incubation with an inhibiting analog of the transferase peptide substrate (18).

Discussion

To date, only one experiment has shown that some of the oligosaccharyl transferase subunits are dispensable for enzymatic activity. The pig liver oligosaccharyl transferase was purified as a heterotetrameric complex and then disrupted with a high ionic strength buffer while bound to an immobilized lectin (19). Catalytic activity was transiently retained by a heterodimeric complex of two subunits, the homologues of the yeast Nlt1p and Wbp1p subunits (19). It is conceivable that the minimal requirements for catalysis are contained within one or two of the six potential yeast subunits.

The six subunits of the enzyme form a tight complex *in vitro*. During the three step affinity purification (chapter 4) (11) using the detergents Nonidet P-40 and Triton X-100, none of the subunits, including the nonessential Ost3p, of the complex appeared to be depleted relative to the other subunits. Attempts to disrupt the complex using denaturants such as urea and reductants were not successful. The enzyme complex might be

disrupted in more severe denaturants, but concentrations of urea which do not disrupt the complex *do* interfere with enzyme activity. SDS certainly disrupts the complex, but it is not likely that activity can be recovered from an SDS disrupted enzyme. Perhaps a detergent more severe than Nonidet P-40 but more gentle than SDS will be useful for the disruption of the enzyme.

Efforts to establish enzymatic activity with fewer than the six subunits of the complex have not been successful. Reconstitution of a limited complex from the Nlt1p, Wbp1p, and Swp1p complexes failed to show any enzymatic activity. In addition, coexpression of Nlt1p, Wbp1p, Swp1p, and Ost2p did not yield a significant increase in transferase activity even though all the proteins were measurably overexpressed and formed a complex. It is likely that other components such as the 9 kDa polypeptide and perhaps a polypeptide which copurifies in substoichiometric amounts are necessary for the full overexpression of the enzyme. Experiments such as reconstitution and coexpression are hampered by the poor expression of some of the enzyme subunits, in particular the more hydrophobic subunits. Expression of the yeast subunits in a heterologous organism or expression of the mammalian subunits in yeast might yield better expression levels by less interference with essential cellular processes.

Efforts to monitor the binding of substrates by the immobilized enzyme were not successful. At substrate amounts above the Michaelis - Menten K_M concentration little or no binding was detected. This result is consistent with other attempts to observe substrate binding by the transferase such as affinity chromatography with immobilized substrates and substrate analogs. The binding of the enzyme to its individual substrates seems to require significantly higher concentrations of enzyme and substrate than those required for effective catalysis. The dolicholpyrophosphoryl chitobiose

binding experiment can now be repeated in the presence of a recently synthesized and characterized tight-binding inhibitor based on the transferase peptide substrate (18). Similarly the peptide binding might be measured in the presence of an inhibiting analog of the sugar substrate when one is available. If the substrate binding of the intact complex can be measured, then perhaps a parallel experiment with individual transferase subunits will identify those subunits capable of binding each substrate.

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Table 5-1 **Yeast Strains**

Strain	Genotype	Source
TH451	*MAT***a***/MAT*α *ade2-101/ade2-101 his3*Δ*200/his3*Δ*200 ura3-52/ura3-52 lys2-801/+ +/tyr1 wbp1::HIS3/+*	(16)
ATH7	*MAT***a***/MAT*α *ade2-101/ade2-101 his3*Δ*200/his3*Δ*200 ura3-52/ura3-52 lys2/+ +/tyr1* Δ*swp1::URA3/+*	(7)
JD52	*MAT***a** *leu2-3,112 his3*Δ*200 trp1-*Δ*63 ura3-52 lys2-801*	A. Varshavsky
RPY21	*MAT***a***/MAT*α *ade2-101/ade2-101 his3*Δ*200/his3*Δ*200 ura3-52/ura3-52 lys2-801/+ +/tyr1 wbp1::HIS3/+* pYES2(*WBP1*-FLAG-6His)	This study
RPY23	*MAT*α *ade2-101 his3*Δ*200 ura3-52 lys2? tyr1? wbp1::HIS3* pYES2(*WBP1*-FLAG-6His)	This study
RPY27	*MAT***a***/MAT*α *leu2-3,112/leu2-3,112 his3*Δ*200/his3*Δ*200 trp1-*Δ*63/trp1-*Δ*63 ura3-52/ura3-52 lys2-801/lys2-801 nlt1-*Δ*2::LEU2/+*	(11)
RPY30	*MAT***a***/MAT*α *leu2-3,112/leu2-3,112 his3*Δ*200/his3*Δ*200 trp1-*Δ*63/trp1-*Δ*63 ura3-52/ura3-52 lys2-801/lys2-801 nlt1-*Δ*2::LEU2/+* pYES2(*NLT1*-FLAG-6His)	This study
RPY32	*MAT? leu2-3,112 his3*Δ*200 trp1-*Δ*63 ura3-52 lys2-801 nlt1-*Δ*2::LEU2* pYES2(*NLT1*-FLAG-6His)	This study
RPY40	*MAT*α *leu2-3,112 his3*Δ*200 trp1-*Δ*63 ura3-52 lys2-801 nlt1-*Δ*2::LEU2* pRS416(*CUP1p-NLT1*-FLAG-6His-*cyc1*)	(11)

Table 5-1 **Yeast Strains**

Strain	Genotype	Source
RPY47	*MAT***a** *leu2-3,112 his3Δ200 trp1-Δ63 ura3-52 lys2-801* pYES2(*NLT1*-FLAG-6His)	This study
RPY51	*MAT***a** *leu2-3,112 his3Δ200 trp1-Δ63 ura3-52 lys2-801* pYES2(*NLT1*-FLAG-6His) pRS424(*GAL-WBP1-cyc1*)	This study
RPY53	*MAT***a** *leu2-3,112 his3Δ200 trp1-Δ63 ura3-52 lys2-801* pYES2(*NLT1*-FLAG-6His) pRS424(*GAL-WBP1-cyc1*) pRS423(*GAL-SWP1-cyc1*)	This study
RPY59	*MAT***a**/*MAT*α *ade2-101/ade2-101 his3Δ200/ his3Δ200 ura3-52/ura3-52 lys2/+ +/tyr1 Δswp1::URA3/+* pRS423(*GAL-SWP1*-FLAG-6His-*cyc1*)	This study
RPY60	*MAT*α *ade2-101 his3Δ200 ura3-52 Δswp1::URA3* pRS423(*GAL-SWP1*-FLAG-6His-*cyc1*)	This study
RPY64	*MAT***a** *leu2-3,112 his3Δ200 trp1-Δ63 ura3-52 lys2-801* pYES2(*NLT1*-FLAG-6His) pRS424(*GAL-WBP1-cyc1*) pRS423(*GAL-SWP1-cyc1*) pRS425(*GAL-OST2-cyc1*)	This study

Chapter 6

Photoaffinity Labeling of the Yeast Oligosaccharyl Transferase

The yeast oligosaccharyl transferase is a complex enzyme with at least five subunits (1-3). Four of these five subunits are essential for the growth of the yeast cell (4-8), and deletion of the nonessential subunit yields a yeast strain with glycosylation defects (9). In addition, point mutations in three of the four essential subunits yield cell strains with glycosylation defects and temperature sensitive phenotypes (4, 8, 10). Each of the subunits of the purified oligosaccharyl transferase most likely makes some contribution to the overall function of the enzyme. For instance, the 45 kDa Wbp1p and 30 kDa Swp1p subunits might contribute to the structure and stability of the enzyme (2, 7) while the 64 kDa Ost1p / Nlt1p subunit might be less important for the stability of the complex (4). Other important facets of the overall function of the transferase include cellular localization, interaction with the translocation proteins, substrate binding, and catalysis.

One of the most interesting problems in the study of the oligosaccharyl transferase is the development of a complete description of its catalytic mechanism. The enzyme manages to transfer an elaborate oligosaccharide from a pyrophosphate carrier to the not-so-reactive side chain amide of an asparagine residue within the consensus sequence Asn-Xaa-Ser/Thr. Study of transferase acceptor substrates has led to a model for the structure of the peptide recognition sequence accounting for the increased reactivity of the asparagine side chain (11), but to date little insight has been gathered on the participation of the enzyme in the reaction. Identification of the residues at the sites of catalysis and substrate binding would provide targets for further experiments to elucidate the biochemistry of the transferase reaction. One experiment indicated that one or more of the three cysteine residues of the 45 kDa Wbp1p subunit might be involved in binding the lipid-linked oligosaccharide substrate (12), and here I describe a series of experiments to

photolabel the site of peptide substrate binding and perhaps transferase catalysis.

Three peptides were designed and synthesized to bind and label the oligosaccharyl transferase. Two of the peptides were substrates, and the third was a peptide substrate analog that inhibited the enzyme. Biotin was coupled to each sequence to allow visualization of any polypeptide subunit cross-linked by the peptides. The photoactivatable benzophenone group was used as the cross-linker in the two peptide substrates, and the photoactivatable *p*-azidobenzoic acid group was used in the inhibitor peptide. Unfortunately, all three peptides failed to specifically identify any oligosaccharyl transferase subunit as the site of peptide binding.

Materials and Methods

Peptide Synthesis

All peptides were synthesized on a 0.119 mmole scale by solid phase methods using N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) amino acids with diisopropylcarbodiimide / hydroxybenzotriazole (DIPCDI / HOBt) activation on a Milligen 9050 automated peptide synthesizer. Asparagine was incorporated as the pre-activated pentafluorophenyl ester. Fmoc-PAL-PEG-PS resin (0.17 - 0.37 mmol/g) was used to afford carboxy-terminal primary amides. Deprotection of Fmoc-protected amine groups was performed using a seven minute 20% piperidine / dimethylformamide (DMF) wash. Peptides were either *N*-benzoylated on the resin using seven equivalents of benzoic anhydride and seven equivalents of triethylamine (TEA) in 3 mL DMF for 2

hours or coupled to a photoactivatable group at the terminal amine as described below. The resin was then washed with DMF and dichloromethane overnight.

The peptides were then deprotected and cleaved from the resin either by treatment with reagent R (90:5:3:2 trifluoroacetic acid / thioanisole / ethanedithiol / anisole) (13) or by treatment with reagent B (88:5:5:2 trifluoroacetic acid / phenol / water / triisopropylsilane) (14) for two hours. The resin was filtered, washed with trifluoroacetic acid, and the combined filtrates were concentrated to 2 mL and precipitated with 1:1 ether / hexanes. The supernatant was decanted and the peptides were triturated with 1:1 ether / hexanes (5 x 20 mL). The peptides were lyophilized overnight and purified as described below.

Commercially available starting materials and reagents were purchased from MilligenBiosearch, EM Science, NovaBiosearch, Aldrich Chemical Co., or Bachem.

BPA30 Synthesis

The protected heptameric peptide Asn-Leu-Thr(tBu)-Val-Thr(tBu)-Lys(Boc)-Glu was synthesized on resin. The free amino terminus of the peptide was coupled to 4-benzoylbenzoic acid by reaction with three equivalents of 4-benzoylbenzoic acid *N*-hydroxysuccinimide ester, three equivalents of HOBt, and three equivalents of TEA in DMF; ninhydrin staining (15) verified that the reaction was complete within eight hours. The peptide was cleaved from the resin with reagent R, dissolved in water at pH 7.0, and dried under vacuum. The ε-amine of the lysine side chain was coupled to biotin by reaction of the peptide with 1.5 equivalents of biotin, 1.5

equivalents of benzotriazole-1-yloxytris(dimethylamino)-phosphoniumhexafluorophosphate (BOP), and 1.5 equivalents of TEA in DMF for twelve hours. The resulting peptide was extracted twice with MeOH and then dissolved in water at pH 7.0. The desired product was purified away from a significant contaminant by reversed phase HPLC. MALDI-MS: [MH$^+$] calculated for $C_{58}H_{84}N_{12}O_{16}S$ as the Na$^+$ adduct, 1261.42; observed 1262.

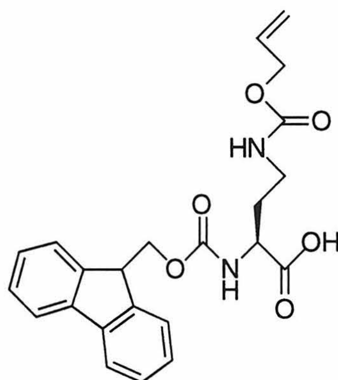
BPA40 Synthesis

The protected heptameric peptide Bz-Asn-Leu-Thr(tBu)-Phe(pBz)-Thr(tBu)-Lys(Boc)-Glu was synthesized on resin. The unnatural amino acid *p*-benzoylphenylalanine was incorporated in the fourth position by coupling with *N*-Fmoc-*p*-benzoyl-phenylalanine. The peptide was cleaved from the resin with reagent B. The cleaved peptide was dissolved in water at pH 7.0 and dried under vacuum. The ε-amine of the lysine side chain was coupled to biotin by reaction of the peptide with 1.5 equivalents of biotin, 1.5 equivalents of BOP, and 1.5 equivalents of triethylamine for twelve hours. The resulting peptide was extracted twice with methanol and then dissolved in water at pH 7.0. The desired product was purified by reversed phase HPLC. ESI-MS: [M$^-$] calculated for $C_{62}H_{83}N_{12}O_{16}S$, 1283.6; observed, 1283. MALDI-MS: [MH$^+$] calculated for $C_{62}H_{83}N_{12}O_{16}S$ as the Na$^+$ adduct, 1308.6; observed, 1308.6.

***N*-α-Fmoc-*N*-γ-aloc-L-α,γ-diaminobutyric Acid Synthesis**

N-α-Fmoc-L-α,γ-diaminobutyric acid (500 mg, 1.5 mmol; Bachem) was dissolved in 20 mL 1:1 tetrahydrofuran / water. Allyl chloroformate (160 μL,

1.6 mmol) and diisopropylethylamine (260 μL, 1.6 mmol) were dissolved in an additional 6 mL 1:1 tetrahydrofuran / water at 0° C. To this solution were added the diaminobutyric acid solution and sodium carbonate (2.0 g) slowly over 0.5 hour. The reaction slurry was stirred on ice for 1 hour, it was then warmed to room temperature and stirred for 1 hour. Allyl chloroformate (16 μL, 0.16 mmol) and diisopropylethylamine (26 μL, 0.16 mmol) were added, and the reaction was stirred at room temperature for another hour. The organic solvents were removed under reduced pressure, and hydrochloric acid was added to the aqueous solution to pH 3.2. The product was extracted with dichloromethane (3 x 50 mL). The combined extracts were dried over magnesium sulfate and evaporated under reduced pressure. The desired product was purified by silica gel chromatography eluting with 94:5:1 chloroform / methanol / acetic acid. The pure compound was dried from toluene under vacuum. FAB-MS: [MH+] calculated for $C_{23}H_{25}N_2O_6$, 425.1707; observed, 425.1712.



N-$^{\alpha}$-Fmoc-N-$^{\gamma}$-aloc-L-$^{\alpha,\gamma}$-diaminobutyric Acid

AZB10 Synthesis

The protected heptameric peptide Amb(Aloc)-Leu-Thr(tBu)-Val-Thr(tBu)-Lys(Boc)-Glu was synthesized on resin using the protected amino acid *N*-α-Fmoc-*N*-γ-aloc-L-α,γ-diaminobutyric acid in the first position. The amino terminus of the peptide was coupled to 4-azidobenzoic acid by reaction with three equivalents of 4-azidobenzoic acid *N*-hydroxysuccinimide ester, three equivalents of HOBt, and three equivalents of TEA in DMF for 8 hours. The peptide was cleaved from the resin with reagent B and subsequently dried under vacuum. ESI-MS: [M^-] calculated for $C_{45}H_{70}N_{13}O_{14}$, 1016.5; observed 1017. MALDI-MS: [MH^+] calculated for $C_{45}H_{72}N_{13}O_{14}$, 1018.5; observed 1018. The cleaved peptide was reacted with 1.5 equivalents of biotin, 1.5 equivalents of BOP, and 1.5 equivalents of TEA in DMF for 12 h. The product was washed with methanol two times, and then dried from water under vacuum. ESI-MS: [M^-] calculated for $C_{55}H_{84}N_{15}O_{16}S$, 1242.6; observed 1243. MALDI-MS: [MH^+] calculated for $C_{55}H_{86}N_{15}O_{16}S$, 1244.6; observed 1242.4. The aloc protecting group was removed by reaction of the peptide with 0.5 equivalents of dichlorobis(triphenylphosphine)palladium and 3 equivalents of tributyltin hydride (16) in 500 μL of 9:9:2 dimethylsulfate / terahydrofuran / acetic acid for 0.5 hour under nitrogen at room temperature. The desired product was identified by ultraviolet spectroscopy and purified by reversed phase HPLC.

Photoaffinity labeling of the oligosaccharyl transferase

The affinity-tagged oligosaccharyl transferase was purified from *Saccharomyces cerevisiae* strain RPY40 as described (12). For a typical labeling experiment, 100 μL of the purified protein was added to a borosilicate test tube in a buffer of 50 mM HEPES pH 7.5, 150 mM NaCl, 140 mM sucrose, 10 mM

$MgCl_2$, 10 mM $MnCl_2$, 1.0 % Triton X-100, and 0.5 mg/mL phosphatidylcholine. To this was added the peptide affinity label in 10 μL DMSO. The sample was irradiated on ice 3 cm away from a 400 W ultraviolet lamp for 20 to 30 min. For the benzophenone peptides, the lamp was shielded with a pyrex sleeve to limit irradiation below 300 nm. The protein samples were precipitated with chloroform and methanol (17) and then separated on a 12.5 % SDS-PAGE gel (18). The separated proteins were transferred to a nitrocellulose membrane (19) which was developed with an ABC - alkaline phosphatase reagent (Pierce) to identify biotinylated proteins.

Results

Peptide design and synthesis

A series of peptides were synthesized to characterize the peptide binding site of the multisubunit yeast oligosaccharyl transferase (chapter 2 and chapter 4). Peptide BPA30 (figure 6-1A) is a substrate for the transferase containing a consensus glycosylation Asn-Leu-Thr sequence in the first three positions. The benzophenone group at the amino terminus is a photoactivatable cross-linking moiety that does not interfere with oligosaccharide transfer as aromatic groups are tolerated and even favored at this position in many transferase peptide substrates (11). Upon irradiation of the peptide at 350 nm, the benzophenone is excited to a triplet state which can form a covalent bond with adjacent atoms of the transferase active site (20). The similar peptide BPA40 (figure 6-1B) contains a glycosylation consensus in its first three positions and the photoactivatable benzophenone group on the side chain of the fourth residue. Both peptides have biotin coupled to the

side chain of a lysine residue for the visualization of cross-linked polypeptides with an avidin conjugate. Glutamate was included at the seventh position to allow the peptide to bear a negative charge at neutral pH and to improve solubility.

The bulk of the two peptides were synthesized by standard solid phase peptide synthetic techniques. The benzophenone group was incorporated into BPA30 by coupling the *N*-hydroxysuccinimide ester of 4-benzoylbenzoic acid to the terminal amine of the peptide; the benzophenone group was incorporated into BPA40 by coupling a commercially available protected version of the amino acid *p*-benzoylphenylalanine at the fourth position of the sequence. Cleavage of the peptides from the resin was achieved with trifluoroacetic acid / phenol / water / triisopropylsilane; cleavage with ethanedithiol resulted in the formation of an undesired dithiane adduct of the benzophenone. Biotin was coupled to the lysine side chain as the BOP-activated ester. Both peptides were successfully glycosylated *in vitro*, and both showed Michaelis-Menten constants of approximately 10 μM.

Peptide AZB10 (figure 6-1C) was designed as an inhibitor of the oligosaccharyl transferase with a photoactivatable cross-linking group. The sequence of aminobutyrate followed by leucine and then threonine at the amino terminus of a short peptide has been shown to form a potent inhibitor of the oligosaccharyl transferase with a Ki of 37 nM (21). The photoactivatable cross-linking *p*-azidobenzoic acid group was incorporated at the amino terminus of the peptide. The azide moiety can be activated by irradiation at 250 nm to form a nitrene that will readily react with any nearby atoms including those in an enzyme active site. The *p*-azidobenzoic acid group at the amino terminus of the peptide is similar to the benzoic acid cap of many transferase peptide substrates and is thus appropriate for enzyme binding.

Like BPA30 and BPA40, AZB10 was biotinylated for visualization of cross-linked polypeptides.

AZB10 was synthesized by solid phase techniques employing the orthogonal aloc protecting group (22) on the side chain amine of the aminobutyrate residue. The backbone of the peptide was synthesized on a resin and coupled at the amino terminus with an *N*-hydroxysuccinimide activated ester of *p*-azidobenzoic acid. Cleavage and deprotection of the peptide afforded a free lysine side chain and a protected aminobutyrate side chain. The lysine side chain was coupled to the BOP-activated ester of biotin. The aminobutyrate side chain was then deprotected with a dichlorobis(triphenylphosphine)palladium catalyst (16); this Pd(II) catalyst removed the aloc protecting group more effectively than a similar Pd(0) catalyst. Peptide AZB10 was a strong inhibitor of the transferase with an apparent IC_{50} of 30 nM.

Photoaffinity labeling of the oligosaccharyl transferase

In an attempt to label residues of the oligosaccharyl transferase adjacent to the peptide substrate binding site, the enzyme was incubated with the photoaffinity labels described above. Peptides BPA30 and BPA40 were incubated with the enzyme at concentrations of up to 100 μM in a buffer appropriate for the transferase reaction lacking only the lipid pyrophosphoryl oligosaccharide substrate. A concentration of 100 μM is at least ten fold higher than the Michaelis-Menten K_M for each peptide. The enzyme-substrate mixture was exposed to ultraviolet irradiation from a 400 W lamp for up to 30 minutes. Unfortunately, only faint and inefficient labeling of the four high molecular weight transferase subunits was observed. When the

labeling sensitivity was increased, none of the four oligosaccharyl transferase subunits with molecular weights greater than 30 kDa (chapter 4) were preferentially labeled.

The enzyme was also incubated with the photoactivatable transferase inhibitor AZB10. The inhibitor was added to the enzyme buffer at concentrations of up to 250 nM, significantly higher than the measured IC_{50} of 30 nM. The lipid pyrophosphoryl oligosaccharide substrate was included in some of the experiments to allow synergistic binding of this substrate with the peptide substrate analog. The enzyme-substrate mixtures were exposed to ultraviolet irradiation from a 400 W lamp for up to 30 minutes. With a highly reactive nitrene group, the photoactivated AZB10 peptide efficiently labeled the oligosaccharyl transferase. Unfortunately, none of the subunits were preferentially labeled as all five polypeptides were detected at similar intensities when probed for biotin (figure 6-2). Experiments were also conducted at reduced concentrations of the photolabel; at a concentration of 25 nM, the labeled polypeptides were barely detectable, but none of the subunits showed specific binding of the peptide. Excess competing peptide and competing biotin did not improve specificity. Competition with bovine serum albumin in the cross-linking reaction led to intense labeling of the competing protein.

Discussion

Three peptides were designed and synthesized to probe the peptide binding site of the multisubunit oligosaccharyl transferase. Two peptides were substrates of the oligosaccharyl transferase and included the photoactivatable benzophenone moiety. Benzophenone is chemically stable

until it is activated by irradiation at 350 nm. Absorption of a photon excites the ketone to a diradicaloid triplet state that can either react preferentially with adjacent C-H bonds or relax to the ground state (20). Biotin was coupled to the peptides to allow visualization of cross-linked polypeptides. A similar design was successfully used to cross-link the insulin receptor. Native insulin was substituted with benzoylphenylalanine, an amino acid analog bearing the benzophenone group on its side chain, at one position and coupled to biotin at another. The insulin peptide bound the receptor and specifically cross-linked the protein upon irradiation (23). However, oligosaccharyl transferase peptide substrates bearing the benzophenone cross-linking group and the biotin label failed to efficiently and specifically label any of the transferase subunits. The benzophenone peptides might have shown weak labeling due to the limited reactivity of the benzophenone photophore. The benzophenone activation is inefficient, and the activated triplet species has limited distance and orientation constraints for the formation of a covalent cross-link with an adjacent C-H bond (20); such limitations might reduce the usefulness of the benzophenone group for the cross-linking of the oligosaccharyl transferase.

Two changes were made in the design of the photolabels to yield peptide AZB10. The photoactivatable *p*-azidobenzoic acid group was incorporated at the amino terminus of the peptide instead of the benzophenone group of the previous peptides. Irradiation of this group forms a highly reactive nitrene with little discrimination for the distance and orientation of nearby bonds for the formation of a covalent cross-link. In one example, the insulin receptor from whole cells was successfully photolabeled on a fifteen amino acid region of one subunit by an insulin peptide modified with *p*-azidophenylalanine and a biotin label (24). The second change in the

design was the replacement of the asparagine residue of the glycosylation consensus sequence with aminobutyrate. Recent studies have shown the substitution of aminobutyrate for asparagine at this position affords a tight-binding inhibitor of the transferase (21). The use of a photoactivatable inhibitor enabled incubation at lower photolabel concentrations to promote specificity and allowed co-incubation with the lipid-linked oligosaccharide substrate. The use of the *p*-azidobenzoic acid group increased the efficiency of labeling significantly, but it also possibly reduced the specificity of labeling. In all labeling experiments with AZB10, the five oligosaccharyl transferase subunits were equally labeled (figure 6-2).

These experiments suffer from two major problems. The efficiency of labeling is a problem for the benzophenone group, but not for the *p*-azidobenzoic acid group. Specificity of binding is a problem for the *p*-azidobenzoic peptide and might also be a problem for the benzophenone peptides. These peptides are hydrophobic molecules being used to label a detergent solubilization of a hydrophobic membrane protein complex. It is conceivable that the peptides decorate the hydrophobic surfaces of the complex and thereby label each subunit non-specifically. The use of a radiolabel instead of biotin would reduce the hydrophobicity of the peptides dramatically and perhaps reduce the non-specific binding. Hopefully, the peptides do bind to the transferase active site more strongly that they do to the non-specific sites. If so, then the experiment can be altered to favor the specific binding to the desired site. Incubation of the enzyme complex with the photolabel at a one to one ratio should favor labeling at the specific site. Reducing the concentration of the photolabels provided limited improvement. For instance, reducing the concentration of AZB10 in the experiment ten-fold to 25 nM, a concentration at the limits of inhibition and

detection, does not improve specificity. What is needed is a greater concentration of the oligosaccharyl transferase. If concentrations of up to 10 nM become accessible after overexpression of the multisubunit enzyme, then specific labeling and a description of the peptide binding site will become amenable.

Acknowledgment

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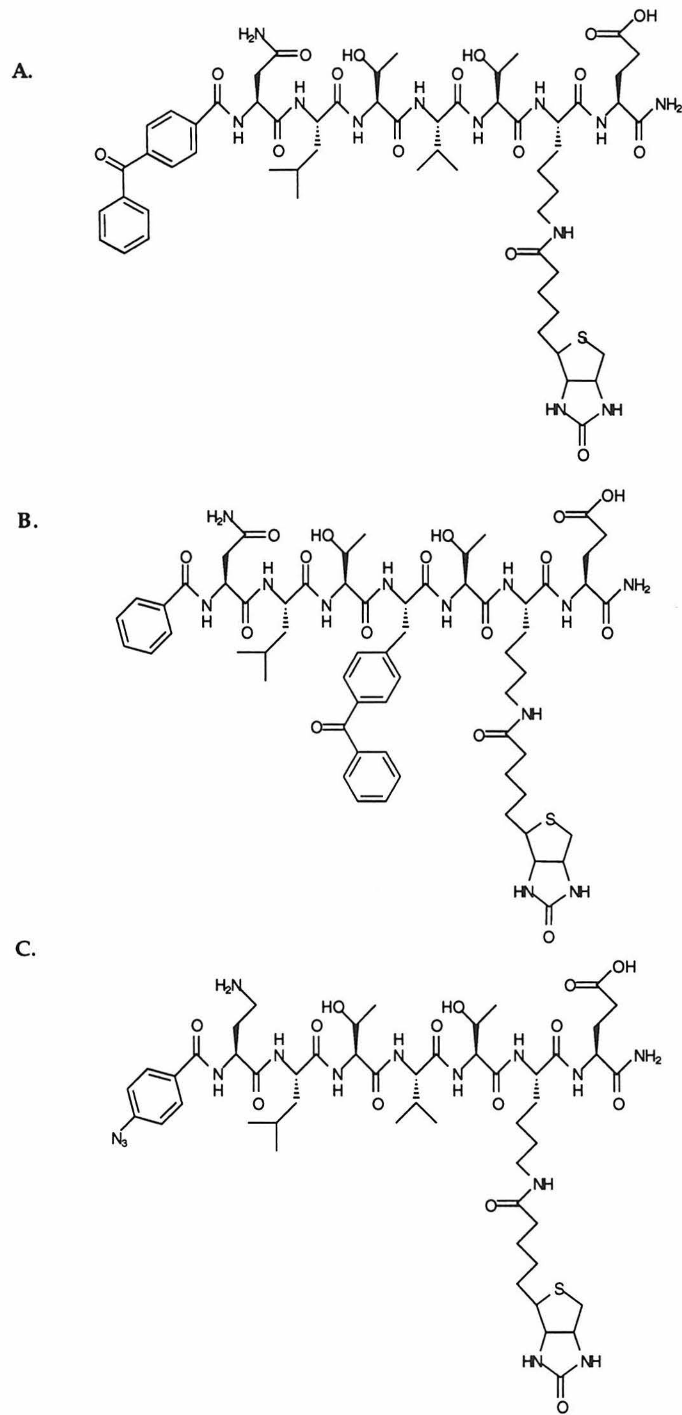
Figure Legends

Figure 6-1. Oligosaccharyl transferase peptide photoaffinity labels. Three peptides were designed and synthesized to identify the polypeptide subunit of the oligosaccharyl transferase associated with peptide substrate binding. **A.** BPA30 is a competent substrate for the enzyme with a benzophenone cross-linking group at the amino terminus and a biotin group near the carboxy-terminus. **B.** BPA40 is also a competent substrate for the enzyme. It has a benzophenone cross-linking group at the fourth residue and a biotin group on the lysine side chain at the sixth position. **C.** AZB10 is a potent inhibitor of the enzyme; the sequence of aminobutyric acid followed by leucine and threonine at the amino terminus of the peptide form a competitive inhibitor of the transferase peptide substrate. The peptide also contains the photoactivatable azidobenzoic acid cross-linking group at the amino-terminus and biotin near the carboxy-terminus.

Figure 6-2. AZB10 photoaffinity labeling of the oligosaccharyl transferase. The purified affinity-tagged oligosaccharyl transferase was labeled by the biotinylated photoactivatable inhibitor AZB10. The inhibitor was incubated with the enzyme at a concentration of 250 nM and exposed to ultraviolet irradiation from a 400 W lamp for 30 minutes. Labeled polypeptides were separated on a 12.5 % polyacrylamide gel and transferred to nitrocellulose; the nitrocellulose membrane was developed with an avidin conjugate to visualize biotinylated polypeptides. Development showed that all five oligosaccharyl transferase subunits were labeled with equal intensities.



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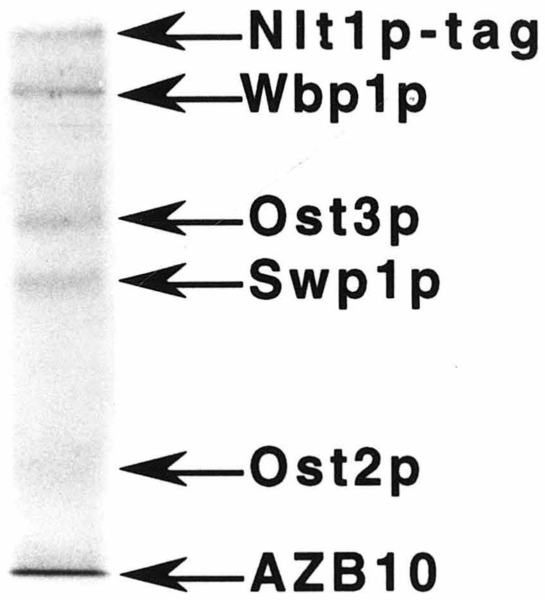

Figure 6-2. AZB10 photoaffinity labeling of the oligosaccharyl transferase.