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The oligosaccharyltransferase complex of
Saccharomyces cerevisiae

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Abbreviations

Asn	asparagine
CTP	cytidine 5'-triphosphate
CDP	cytidine 5'-diphosphate
CPY	carboxypeptidase Y
Dol	dolichol
Dol-PP	dolichylpyrophosphate
ER	endoplasmic reticulum
GalNAc	N-acetyl-D-galactosamine
GDP	guanosine diphosphate
Glc	glucose
GlcNAc	N-acetyl-D-glucosamine
GPI	glycosylphosphatidylinositol
HIV	human immunodeficiency virus
kb	kilobase(s)
kDa	kilodalton
LLO	lipid-linked oligosaccharide
Man	mannose
NLO	N-linked oligosaccharide
OD	optical density
ORF	open reading frame
OTase	oligosaccharyltransferase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
Pro	proline
rpm	revolutions per minute

SDS	sodium dodecyl sulfate
Ser	serine
Thr	threonine
Tyr	tyrosine
UDP	uridine diphosphate
wt	wild-type

Summary

The work described in this thesis pertains to the process of N-linked glycosylation in eukaryotes focusing on the oligosaccharyltransferase (OTase), the key enzyme of this process. The budding yeast *Saccharomyces cerevisiae* was used as a model organism.

N-linked glycosylation is an essential and highly conserved process in eukaryotic cells. The oligosaccharide $Glc_3Man_9GlcNAc_2$ is assembled in a stepwise process on a dolichylpyrophosphate carrier at the membrane of the endoplasmic reticulum and transferred *en bloc* onto selected asparagine residues of polypeptide chains. This transfer reaction is catalyzed by the multimeric enzyme complex oligosaccharyltransferase.

An active oligosaccharyltransferase complex was purified and shown to consist of six proteins: Ost1p (64/62 kDa), Wbp1p (45 kDa), Ost3p (34 kDa), Swp1p (30 kDa), Ost2p (16 kDa), and Ost5p (9 kDa). The genes encoding these subunits have been cloned. *OST1, WBP1, SWP1,* and *OST2* are essential, whereas *OST3* and *OST5* are dispensable for growth but required for optimal glycosylation *in vivo*. Genetic screens have identified two other loci, *STT3* and *OST4*, which are required for full oligosaccharyltransferase activity *in vivo*. Most recently, a protein termed Ost6p, showing siginificant similarity to the *OST3* protein, was revealed in a homology search in the database. However, it was not clear whether or not this protein represented a further subunit of the yeast OTase.

The *STT3* gene product has a molecular weight of 78 kDa and is essential for vegetative growth of yeast. It is highly conserved in eukaryotic cells and proteins with significant similarity have been found in both archaeal and eubacterial genomes. Although Stt3p is required for OTase activity *in vivo*, it was not present in highly purified OTase preparations. In the first part of this his thesis project, using affinity purification with a tagged *STT3* protein, other known components of the complex specifically copurified

with the *STT3* protein. Additionally, different conditional *stt3* mutations could be suppressed by overexpression of either *OST3* or *OST4* in an allele specific manner. These genetic and biochemical data demonstrated that Stt3p is a structural component of the yeast OTase. With regard to suppression of mutations in different OTase subunits by overexpression of other components of the OTase, the subunits of the OTase were grouped into three subcomplexes.

In a further part of this work, the *S. cerevisiae* oligosaccharyltransferase complex was analyzed using the recently described technique of blue native gel electrophoresis that allows the separation of proteins and protein complexes under native conditions according to their molecular weight. Using this analytical method in combination with a set of yeast mutant strains, defined subcomplexes of the OTase were visualized. In addition, it was demonstrated that the *OST6* protein indeed represented an additional subunit of the OTase complex.

OST4 codes for a very small, 3.4 kDa hydrophobic protein. Its deletion leads to a temperature-sensitive phenotype and a marked hypoglycosylation of N-glycoproteins. Using blue native gel electrophoresis in combination with high copy number suppression studies with *Δost3* and *Δost4* mutant strains, it was demonstrated that Ost4p was required for incorporation of either Ost3p or Ost6p into the complex suggesting that this small protein functions as an assembly factor. Furthermore, it was demonstrated that two distinct fully assembled oligosaccharyltransferase complexes existed in yeast which contained, in addition to a set of shared proteins, either Ost3p or Ost6p. Finally, a model was proposed which describes an ordered stepwise *in vivo* assembly pathway of the yeast OTase with defined subcomplexes.

In the final part of this thesis, suppression of oligosaccharyltransferase assembly mutations by different mechanisms was investigated. Multicopy suppression screens

using either *stt3* or *Δost4* strains revealed that both overexpression of other OTase subunits and of integral ER membrane proteins not part of the OTase, rescued the temperature sensitive phenotype. The high copy number suppression by OTase subunits was explained by a shift of the assembly equilibrium towards fully assembled complex. High copy number suppression by the *ALG7* protein that catalyzes the first step in the biosynthesis of the lipid linked oligosaccharide, was explained by an elevation of the substrate level overcoming the defective OTase. Overexpression of the *UBC6* protein that is a part of the ubiquitine-dependent proteasomal protein degradation pathway as well as a *UBC6* deletion both resulted in suppression of the *stt3* mutation. It was shown that non-assembled OTase subunits were rapidly degraded in *stt3* mutant strains and a block in the ubiquitin-dependent proteasomal protein degradation pathway prevented this degradation. Moreover, a deletion of the *MNS1* locus that is involved in the trimming of N-linked oligosaccharides in the ER was able to suppress the *stt3* mutation, suggesting that degradation of ER-located membrane glycoproteins was controlled by N-linked oligosaccharide trimming, as it is known for soluble glycoproteins. In conclusion, it was demonstrated that oligosaccharyltransferase assembly mutations could be suppressed by an inhibition of protein degradation.

Zusammenfassung

Das Thema dieser Doktorarbeit ist die N-Glykosylierung von Proteinen in Eukaryonten mit Schwerpunkt auf der Oligosaccharyltransferase (OTase), dem Schlüsselenzyms dieses Prozesses. Die Sprosshefe *Saccharomyces cerevisiae* diente dabei als Modell-Organismus.

N-Glykosylierung ist ein essentieller und hochkonservierter Prozess, der in allen eukaryotischen Zellen vorkommt. Das Oligosaccharid $Glc_3Man_9GlcNAc_2$ wird dabei in einem schrittweisen Prozess an einem Dolicholpyrophosphat-Träger an der Membran des Endoplasmatischen Retikulums assembliert und anschliessend *en bloc* auf spezifische Asparagin-Reste von Polypeptid-Ketten übertragen. Diese Transfer-Reaktion wird vom multimeren Enzym-Komplex Oligosaccharyltransferase katalysiert.

Ein aktiver gereinigter Oligosaccharyltransferase Komplex besteht aus sechs Proteinen: Ost1p (64/62 kDa), Wbp1p (45 kDa), Ost3p (34 kDa), Swp1p (30 kDa), Ost2p (16 kDa), und Ost5p (9 kDa). Die für diese Untereinheiten codierenden Gene wurde kloniert. *OST1, WBP1, SWP1* und *OST2* sind essentiell, während *OST3* und *OST5* für das Wachstum nicht benötigt, jedoch für eine optimale Glykosylierung *in vivo* gebraucht werden. Auf genetischem Weg wurden zwei weitere Gene identifiziert, *STT3* und *OST4*, die für eine vollständige *in vivo* Oligosaccharyltransferase-Aktivität benötigt werden. Vor kurzem wurde zudem in einer Suche nach Ost3p ähnlichen Proteinen in der Datenbank ein weiteres Protein, Ost6p, identifiziert, das signifikante Aehnlichkeit zum *OST3* Protein aufweist. Es war jedoch nicht klar, ob dieses Protein eine weitere Untereinheit des OTase Komplexes ist oder nicht.

Das *STT3* Gen-Produkt hat eine molekulare Masse von 78 kDa und ist essentiell ist für das vegetative Wachstum von Hefe. Es ist hochkonserviert in eukaryotischen Zellen, und

Proteine mit signifikanter Aehnlichkeit wurden in den Genomen von Archaea und Eukarya gefunden. Obwohl es für die *in vivo* Aktivität der OTase benötigt wird, war es dennoch in gereinigten OTase Präparationen nicht nachweisbar. Unter Anwendung von Affinitätsreinigung eines markierten *STT3* Proteins wurden in einem ersten Teil dieser Doktorarbeit andere bekannte Komponenten des Komplexes mit dem *STT3* Protein spezifisch mitgereinigt. Zudem konnten verschiedene konditionelle *stt3* Mutationen auf eine allel-spezifische Art durch *OST3* oder *OST4* supprimiert werden. Diese genetischen und biochemischen Daten zeigten, dass Stt3p eine strukturelle Komponente der Hefe-OTase darstellt. In Anbetracht der Suppressionsdaten von Mutationen in verschiedenen OTase Untereinheiten durch Ueberexpression von anderen OTase Komponenten wurden die Untereinheiten der OTase in drei Subkomplexe gruppiert.

In einem weiteren Teil dieser Arbeit wurde der Oligosaccharyltransferase-Komplex von *S.cerevisiae* mit Hilfe der neulich beschriebenen Technik "Blue Native Gel Elektrophoresis" analysiert, die eine Auftrennung von Proteinen und Protein-Komplexen unter nativen Bedingungen nach ihrem Molekulargewicht erlaubt. Unter Verwendung dieser analytischen Technik in Kombination mit einer Reihe von Hefe-Mutanten wurden definierte Subkomplexe der OTase visualisiert. Zudem konnte gezeigt werden, dass das *OST6* Protein tatsächlich eine weitere Komponente des OTase-Komplexes darstellt.

OST4 kodiert für ein sehr kleines hydrophobes Protein mit einem Molekulargewicht von 3.4 kDa. Die Deletion dieses Lokus führt zu einem temperatur-sensitiven Phänotyp und einer starken Unterglykosylieung von N-Glykoproteinen. Unter Verwendung der "Blue Native Gel Elekrophoresis" in Kombination mit Suppressions-Studien mit Δ*ost3* and Δ*ost4* Mutanten-Stämmen wurde gezeigt, dass Ost4p für die Inkorporierung von Ost3p oder Ost6p in den Komplex benötigt wird . Es wurde deshalb vorgeschlagen, dass Ost4p als Assemblierungs-Faktor funktioniert. Weiter wurde gezeigt, dass in Hefe zwei verschiedene vollständig assemblierte Oligosaccharyl-transferase-Komplexe existeren, die

zusätzlich zu einer gemeinsamen Anzahl Proteine entweder Ost3p oder Ost6p enthalten. Schliesslich wurde ein Modell vorgeschlagen, das eine geordnete und schrittweise *in vivo* Assemblierung der Hefe-OTase mit definierten Subkomplexen beschreibt.

Im letzten Teil dieser Doktorarbeit wurde durch verschiedene Mechanismen verursachte Suppression von Oligosaccharyltransferase-Assemblierungsmutationen untersucht. "High Copy Number Suppression Screens", bei denen *stt3* und *Δost4* Stämme verwendet wurden, zeigten, dass sowohl eine Ueberexpression anderer OTase-Untereinheiten als auch die Ueberexpression von integralen ER-Membranproteinen, die nicht Teil der OTase sind, den temperatur-sensitiven Phänotyp supprimieren konnten. Die Suppression durch OTase-Untereinheiten wurde durch eine Verschiebung des Assemblierungs-Gleichgewichts auf die Seite des voll-assemblierten Komplexes erklärt. Die Suppression durch das *ALG7* Protein, das den ersten Schritt in der Biosynthese des Lipid-gebundenen Oligosaccharides katalysiert, wurde durch eine Erhöhung des Substrat-Spiegels erklärt, was die Aktivität der schadhaften OTase positiv beeinflusste. Ueberexpression des *UBC6* Proteins, das am Ubiquitin-abhängigen proteosomalen Protein-Abbau-Weg beteiligt ist, sowie eine *UBC6* Deletion resultierten ebenfalls in einer Suppression der *stt3* Mutation. Es wurde gezeigt, dass nicht-assemblierte OTase Untereinheiten in *stt3* Stämmen rasch abgebaut werden. Eine Blockierung des Ubiquitin-abhängigen proteosomalen Protein-Abbau-Weg konnte diesen Abbau verhindern. Ueberdies konnte auch eine Deletion des *MNS1* Lokus, der am Trimming der N-gebundenen Oligosaccharide im ER beteiligt ist, die *stt3* Mutation supprimieren. Dies wies darauf hin, dass der Abbau von ER Membran-Glykoproteinen - wie schon für lösliche Proteine bekannt - durch das Trimming der N-gebundenen Oligosaccharide kontolliert wird. Es wurde also gezeigt, dass Oligosaccharyltransferase-Assemblierungsmutationen durch eine Verhinderung von Protein-Abbau supprimiert werden konnten.

CHAPTER 1

Introduction

INTRODUCTION

1. Glycosylation of proteins in eukaryotes

The glycosylation of proteins is the most complex type of protein modification known. This covalent modification modulates the structure and the function of secretory and membrane proteins. Hundreds of different enzymes are involved in the biosynthesis of oligosaccharides. Almost all proteins associated with elements of the secretory pathway, the cell surface, and secreted proteins are glycoproteins. Ten to twenty percent of glycoproteins normally consist of saccharides, sometimes this may even exede 80%. The obviously high cost for a cell to synthesize these carbohydrates implies an important function of these sugars. Carbohydrates have been shown to play a role in diverse biological processes such as protein folding, stability, solubility, and targeting, cell-matrix and cell-cell interactions, and cell cycle progression as well as polarisation of growth.

Three types of glycosylation occur in eukaryotic cells. Glycosylphosphatidyl inositol (GPI) anchors are attached to the C-terminus of proteins providing a membrane anchor. In O-linked glycosylation, glycans are attached to the hydroxyl group of serine or threonine residues, and in the process of N-glycosylation, branched oligosaccharides are attached to secretory proteins via the amide group of an asparagine residue.

The yeast *Saccharomyces cerevisiae* has proved to be an excellent model organism for studying different cellular processes. Its advantages are ease and low costs of handling and a variety of strategies for the characterization and manipulation of genes. Consequently, this organism is characterised very well. The complete genome of *Saccharomyces cerevisiae* was sequenced by 1996 and is now available in the database.

Yeast glycosylation mutants have been isolated by several different strategies and provide a valuable tool for studying this complex process.

1.1 Protein modification by GPI anchors

Many membrane glycoproteins are linked to the membrane by a glycosylphosphatidylinositol (GPI)-anchor (Ferguson, 1992). GPI achored proteins are predominantly found on the cell surface. GPI anchors are supposed to play a role in intracellular targeting of proteins to the cell surface. GPIs from all organisms seem to have a common tri-mannosyl core attached to a glucosamine. This residue forms the link to phosphatidylinositol. GPIs are pre-assembled in the rough endoplasmic reticulum, and are rapidly attached to the nascent polypeptide chain by a putative GPI:protein transamidase. The mechanism and the sequence requirement for GPI attachement are conserved (Nuoffer et al., 1991; Gerber et al., 1992). GPI anchored proteins show a hydrophobic N-terminal signal sequence and a hydrophobic C-terminal peptide which is concomitantly cleaved and replaced by the GPI anchor. GPI anchoring of mannoproteins is an essential process (Orlean, 1997). Recently, Gaa1p and Gpi8p, two essential ER membrane proteins, have been suggested to be part of the putative GPI:protein transamidase complex (Hamburger et al., 1995; Benghezal et al., 1996). Modification of the C-terminal domain of the *GAS1*/gp115 protein eliminating GPI anchoring results in secretion of the protein into the medium (Nuoffer et al., 1991). In certain mouse and human cell lines, however, GPI anchoring is not required for growth and cell division (Hyman, 1988; Hirose et al., 1992). Despite this, a human acquired hematopoietic disease that is characterized by abnormal blood cell populations, paroxysmal nocturnal hemoglobinuria, has been shown to be due to a defect in GPI anchoring (Takeda et al., 1993; Bessler et al., 1994).

1.2 O-linked glycosylation

Modification of Ser and Thr residues by attachement of O-linked glycans is abundant in all eukaryotes. It is found in nuclear and cytosolic as well as in secretory proteins. There is no obvious consensus sequence for the attachement of O-linked sugars (Tanner and Lehle, 1987). Often both O-linked and N-linked glycans are present in the same protein.

Nuclear and cytosolic proteins are glycosylated by the addition of one or more N-acetylglucosamine residue(s) (GlcNAc) onto selected serine or threonine residues (Hart et al., 1989; Hart, 1992). This type of protein modification is an ubiquitous and abundant process in eukaryotic cells and has been shown to be highly dynamic, with rapid cycling response to cellular signals or cellular stages, analogous to phosphorylation. There is evidence that O-GlcNAcylation is an important regulatory modification that may modulates many biological processes in eukaryotes (Roquemore et al., 1996; Hart, 1997). Examples of O-GlcNAc-bearing glycoproteins are nuclear pore proteins, chromatin proteins, RNA polymerase II and its transcription factors, viral proteins, heat-shock, tumor-suppressor, nuclear-oncogene proteins, and cytoskeletal proteins (Hart, 1992; Hart, 1997).

In the case of mucine-type O-glycosylation, N-acetylgalactosamine (GalNAc) derived from UDP-GalNAc is transferred to the side chains of serine and threonine residues in polypeptides. The initiation of this type of glycosylation is controled by a family of UDP-GalNAc:polypeptide N-acetylgalactosaminyl-transferases located in the Golgy apparatus (Clausen and Bennett, 1996; Gabius and Gabius, 1997). Mucin-type O-glycosylation is one of the most abundant forms of glycosylation in animal cells. It is most often found on mucin-like glycoproteins that are the major glycoproteins of the mucus gel, but is not restricted to them (Clausen and Bennett, 1996).

In the pathway of O-linked glycosylation of <u>secretory proteins</u> in yeast, one to five mannose residues are added to selected Ser or Thr residues. For mammalian cells, O-linked mannose has only been reported in brain proteoglycan (Krusius et al., 1987). The two first mannose residues are linked by α-1,2 glycosidic bonds, whereas the terminal residues are linked by α-1,3 glycosidic bonds (Figure 1) (Lehle and Tanner, 1995).

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linkage:        α-1,3    α-1,3    α-1,2    α-1,2

       Man  -  Man  -  Man  -  Man  -  Man  -  Ser/Thr

gene:                  MNN1    KRE2            PMT1-7
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FIGURE 1. Attachement of mannose (Man) residues onto Ser/Thr sites of polypeptides in O-linked glycosylation of *Saccharomyces cerevisiae*. The first and probably also the second Man are added in the ER. Additional Man residues are added in the Golgi apparatus. The linkages and the genes encoding the corresponding mannosyltransferases are indicated.

The first mannosyl residue is donated by Dol-P-Man in the lumen of the endoplasmic reticulum and the reaction is catalyzed by a family of seven Dol-P-Man protein O-mannosyl transferases (Pmp1p - Pmt7p) (Tanner and Lehle, 1987; Orlean, 1990; Strahl-Bolsinger et al., 1993; Gentzsch and Tanner, 1997) . The second and subsequent mannose residues are donated directly by GDP-Man (Tanner and Lehle, 1987). The cellular compartment where the second mannose is added is still unclear. Extension of the O-linked chain occurs in the Golgi apparatus and is catalyzed by the Kre2p α-1,2-mannosyl transferase (Häusler et al., 1992; Häusler and Robbins, 1992) and the Mnn1p α-1,3-mannosyl transferase (Yip et al., 1994). Examples of yeast O-mannosylated

proteins are the a agglutinin (Cappellaro et al., 1991; Lipke and Kurjan, 1992), chitinase (Kuranda and Robbins, 1991), as well as the *GAS1*/gp115 protein (17, 27).

1.3 N-linked glycosylation

N-linked glycosalation is highly conserved in eukaryotic cells. The process starts in the rough endoplasmic reticulum as a co- or post-translational (co- or post-translocational) process and proceeds as the glycosylated proteins are delivered through the Golgi machinery to their final destination. N-linked oligosaccharides are found on nearly all secreted and surface proteins. The only known eukaryotic organism that seems not to synthesize N-linked glycoproteins is the asexual intraerythrocytic stage of the malarial parasite *Plasmodium falciparum* (Dieckmann-Schuppert et al., 1991).

Three main classes of asparagine-linked oligosaccharides exist: <u>High mannose-type N-linked oligosaccharides</u> contain mannose as predominant constituent and usually contain only mannose or glucose residues at the nonreducing termini. <u>Hybrid-type oligosaccharides</u> are similar to high mannose-type chains but contain monosaccharides other than mannose or glucose at the nonreducing termini. <u>Complex-type oligosaccharide chains</u> contain only three mannose residues and usually many other monosaccharides in addition. None of the mannose residues are present at the nonreducing termini.

Yeast glycoproteins containing N-linked glycans can also be categorized according to their destination of delivery: Cell wall proteins, secreted proteins, and vacuolar proteins. Cell wall mannoproteins (100-200 mannose residues) are structural components and carry antigenic determinants. Secreted proteins (containing intermediate-length chains of more than 50 mannose residues) such as invertase and acid phosphatase, are involved in nutrient acquisition. Finally, vacuolar proteins such as carboxypeptidase Y (CPY) have digestive functions and harbour only short carbohydrate moieties.
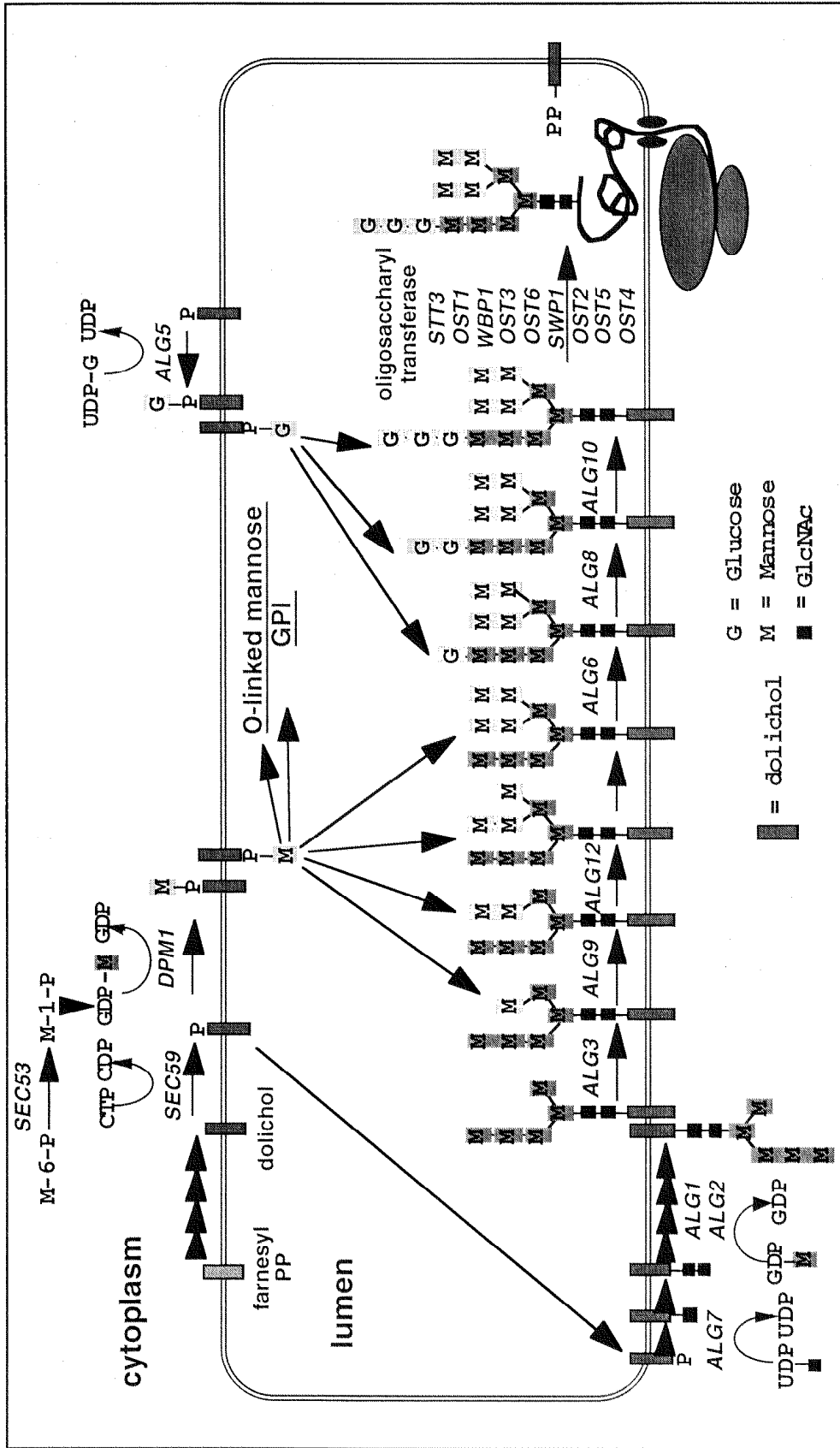
FIGURE 2. Stepwise assembly of the lipid-linked oligosaccharide Glc$_3$Man$_9$GlcNAc$_2$ at the membrane of the endoplasmic reticulum in *Saccharomyces cerevisiae* and transfer of the oligosaccharide onto polypeptides by the oligosaccharyltransferase complex. The loci required for the individual enzymatic reactions are indicated.

The pathway of N-linked glycosylation in eukaryotes can be devided into four processes: (1) the synthesis of dolichylphosphate, (2) the assembly of the dolichylphosphate-linked oligosaccharide at the membrane of the endoplasmic reticulum, (3) its transfer from the lipid carrier dolichylpyrophosphate to selected asparagine residues of polypeptides, and (4) the trimming and modification reactions of the oligosaccharide, occuring in the ER as well as in the Golgi apparatus.

1.3.1 Synthesis of dolichylphosphate

Dolichylphosphate (Dol-P) is a polyisoprenoid consisting of 18-20 isopren units in vertrebrates and 15-16 units in *S. cerevisiae* (Parodi, 1977). Its synthesis can be devided into the assembly of the polyisoprenol chain, a reduction step leading to dolichol, and finally dolichol phosphorylation.

The first steps in dolichol biosynthesis are common to the synthesis of sterol and ubiquinone (Choinacki and Dallner, 1988; Hemming, 1995). The two pathways diverge after the synthesis of farnesylpyrophosphate. Elongation of farnesylpyrophosphate is catalyzed by cis-prenyl transferase by the sequential addition of isopentenylpyrophosphate units. The length of the dolichol molecule varies from organism to organism; the mechanism determing the length is not known. Polyprenylpyrophosphate is converted to dolichol by the reduction of the α-isoprenol unit by an α-reductase (Sagami et al., 1993; Sagami et al., 1996). Finally, dolichol is phosphorylated on the cytosolic face of the ER membrane by the CTP-dependent dolichol kinase. In yeast, this activity is encoded by the *SEC59* locus (Heller et al., 1992; Szkopinska et al., 1996). Dolichylphosphate is the substrate of several enzymes which are involved in the glycosylation pathway, namely Dol-P-Man synthase encoded by *DPM1* (Orlean et al., 1988), Dol-P-Glc synthase encoded by *ALG5* (te Heesen et al., 1994), and GlcNAc-PP-Dol synthase encoded by the *ALG7* locus (Rine et al., 1983),

and its availability was shown to be a rate-limiting factor in lipid-linked oligosaccharide synthesis in higher eukaryotes (Hubbard and Robbins, 1980; Crick et al., 1991; Crick and Waechter, 1994; Carlberg et al., 1996).

1.3.2 Assembly of lipid-linked oligosaccharides

The assembly of the oligosaccharide $Glc_3Man_9GlcNAc_2$ at the membrane of the endoplasmic reticulum was shown to occur by an ordered and stepwise addition of monosaccharide residues (Kornfeld and Kornfeld, 1985; Kukuruzinska et al., 1987; Tanner and Lehle, 1987; Herscovics and Orlean, 1993; Burda et al., 1996) (Figure 2). The oligosaccharide is assembled on the lipid carrier dolichylphosphate (Dol-P) that is embeded in the ER membrane. The first seven monosaccharides (two N-acetylglucosamine (GlcNAc) and five mannose (Man) residues) derive from UDP-GlcNAc and GDP-Man, respectively, whereas the following seven residues (four mannose and three glucose residues) are provided by the lipid intermediates Dol-P-Man and Dol-P-Glc, respectively. For each transfer reaction, an individual glycosyltransferase encoded by different ALG (asparagine linked glycosylation) loci has been postulated (Kornfeld and Kornfeld, 1985; Kukuruzinska et al., 1987; Tanner and Lehle, 1987; Herscovics and Orlean, 1993; Burda et al., 1996). The glycosyltransferases for the early steps catalyzing the addition of the first seven sugars are essential in yeast, whereas the elongation steps leading to the fully assembled $Glc_3Man_9GlcNAc_2$ structure are not essential.

The pathway has a remarkable topology. The first seven sugars are added on the cytosolic side of the ER membrane, resulting in the $Man_5GlcNAc_2$ structure, whereas the following seven glycosyltransfer reactions take place in the ER lumen and lead to the fully assembled $Glc_3Man_9GlcNAc_2$ (Hirschberg and Snider, 1987). This topology has been

determined in rat liver microsomes (Snider et al., 1980) but it has never been experimentally determined in yeast. The topology implies that the dolichylpyrophosphate-linked oligosaccharide $Man_5GlcNAc_2$ as well as the sugar donors for the late assembly reactions, Dol-P-Man and Dol-P-Glc, which are both synthesized at the cytoplasmic side of the ER membrane as well (Abeijon and Hirschberg, 1992), have to be translocated across the membrane to the ER lumen. It has long be speculated that the synthesis of Dol-P-Man is followed by a transmembrane "flipping" of the product (Haselbeck and Tanner, 1982; Rush and Wächter, 1995) and it has also been demonstrated that the translocation of the Dol-P-linked sugars does not occur spontaneously (McCloskey and Troy, 1980).The requirement of a "flippase" for the translocation of the $Man_5GlcNAc_2$-PP-Dol structure is postulated but only few experimental evidence supports this proposal (Kukuruzinska et al., 1987; Tanner and Lehle, 1987; Abeijon and Hirschberg, 1992; Herscovics and Orlean, 1993). If the "flipping" reactions are indeed protein-mediated, the enzyme catalyzing the translocation of $Man_5GlcNAc_2$-PP-Dol as well as Dol-P-Man to the ER lumen should be essential, whereas a Dol-P-Glc "flippase" is not expected to be essential but might result in a phenotype similar to the phenotype of Δalg5 or Δalg6 strains. The observation that alg1 and alg2 mutant cells transfer truncated oligosaccharides onto proteins indicates that the postulated "flippase" does not show a stringent specifity towards $Man_5GlcNAc_2$-PP-Dol (Jackson et al., 1989).

1.3.3 Transfer of the oligosaccharide to the polypeptide by the enzyme complex oligosaccharyltransferase

1.3.3.1 Catalytic activity of the oligosaccharyltransferase

After the $Glc_3Man_9GlcNAc_2$ structure is fully assembled, this oligosaccharide is transferred from the dolichylpyrophosphate carrier onto selected asparagine residues of

polypeptides in the lumen of the endoplasmic reticulum. This enzymatic reaction which requires handling of two large substrates, the lipid-linked oligosaccharide and the polypeptide chain, is catalyzed by the multimeric enzyme complex oligosaccharyltransferase (OTase) (for review see (Imperiali and Hendrickson, 1995; Silberstein and Gilmore, 1996)).

The asparagine residue lies in the consensus Asn-Xaa-Ser/Thr where Xaa can be any natural amino acid exept proline (Kornfeld and Kornfeld, 1985; Tanner and Lehle, 1987; Cummings, 1992; Herscovics and Orlean, 1993). Consensus sites located less than 12-14 residues away from a transmembrane domain are not glycosylated, and sites located near the C-terminus are modified less frequently. An early study indicated that only 30 to 40% of all such sequons were glycosylated (Struck and Lennarz, 1980). When more carefully evaluated by eliminating non-glycosylated sequons in soluble cytoplasmic proteins or in cytoplasmic tails and transmembrane regions of membrane proteins, and by eliminating the sequons from homologous proteins, the level of non-glycosylated Asn-X-Ser/Thr sequences was about 10% (Gavel and von Heijne, 1990; Trimble and Verostek, 1995). Threonine is found twice as frequent as Serine at the +2 position of glycosylated sites.

The prefered sugar donor for the enzyme complex of all organisms other than trypanosomatid protozoa is the oligosaccharide $Glc_3Man_9GlcNAc_2$ linked to dolichylpyrophosphate. Incompletely assembled dolichol-linked oligosaccharides, however, can be transferred by the OTase *in vitro* and *in vivo* (Trimble et al., 1980; Sharma et al., 1981; Huffaker and Robbins, 1983). Dolichylpyrophosphate activated chitobiose ($Dol-PP-GlcNAc_2$) can serve as a glycosyl donor for the porcine (Breuer and Bause, 1995) and the yeast enzyme (Sharma et al., 1981) *in vitro* as well. However, for the yeast and mammalian OTase, the *in vitro* transfer rate for $Dol-PP-GlcNAc_2Man_9Glc_3$ is about 25-fold faster than for $Dol-PP-GlcNAc_2Man_9$ (Trimble et al., 1980).

OTase activity can be measured by an *in vitro* assay. A short oligopeptide containing the N-glycosylation consensus sequence is glycosylated upon incubation with solubilized microsomes in the presence of an exogenous glycosyl donor (Sharma et al., 1981; Kelleher et al., 1992).

1.3.3.2 Subunit composition of the oligosaccharyltransferase

In *Saccharomyces cerevisiae* at least nine proteins are required for optimal transfer of the lipid-linked oligosaccharide onto protein. The OTase from yeast has been purified as a complex composed of six subunits (Kelleher and Gilmore, 1994): Ost1p (64/62 kDa), Wbp1p (45 kDa), Ost3p (34 kDa), Swp1p (30 kDa), Ost2p (16 kDa), and Ost5p (9 kDa). The genes encoding all these six subunits have been cloned (te Heesen et al., 1992; te Heesen et al., 1993; Karaoglu et al., 1995; Silberstein et al., 1995a; Silberstein et al., 1995b; Reiss et al., 1997). Among these, four subunits (Ost1p, Wbp1p, Swp1p, Ost2p) are essential, wherease Ost3p and Ost5p are dispensable for growth. Genetic screens have identified two other loci, *STT3* and *OST4*, which are required for full oligosaccharyltransferase activity *in vivo* (Zufferey et al., 1995; Chi et al., 1996). A homology search in the database recently revealed a homologue for the 34 kDa *OST3* protein (Knauer, 1997). This protein, termed Ost6p, has now been demontrated to be part of the mature OTase, representing the ninth subunit of the complex (Spirig et al., submitted for publication).

The OTase has been purified from three mammalian sources (canine pancreas) (Kelleher et al., 1992), porcine liver microsomes (Breuer and Bause, 1995), and human liver microsomes (Kumar et al., 1995) and one avian source (hen oviduct microsomes (Kumar et al., 1994)). These OTase purifications consist of the subunits: ribophorin I (66 kDa), ribophorin II (64 kDa), and OST48 (48 kDa). Comparison of the subunits from each of

these purifications evidenced a high degree of conservation. Ribophorin I shows sequence homology to the yeast Ost1p, ribophorin II to the yeast Swp1p, and OST48 to the *WBP1* protein. Additionally, a subunit of the OTase from *Drosophila melanogaster* (*DmOST50* homologuous to Wbp1p and *OST48*) has recently been identified and sequenced using PCR-mediated cloning with the help of the consereved regions of the canine and yeast enzymes (Stagljar et al., 1995). Furthermore, DAD1, the defender against apoptotic cell death, initially identified as a negative regulator of programmed cell death (Nakashima et al., 1993; Sugimoto et al., 1995) and showing 40% sequence identity to the yeast Ost2p, has recently been demonstrated to be part of the mammalian oligosaccharyltransferase (Kelleher and Gilmore, 1997).

1.3.4 Processing of the asparagine-linked oligosaccharide within the ER, and its role in protein folding, quality control, and degradation of proteins

After transfer to protein, the N-linked oligosaccharide is subjected to trimming reactions while still in the ER lumen. Three glycosidases are involved in these trimming reactions. Glucosidase I removes the terminal $\alpha 1,2$-linked glucose residue. Glucosidase II sequentially cleaves the two remaining $\alpha 1,3$-linked glucose residues. Finally, in *Saccharomyces cerevisiae*, mannosidase I selectively removes one $\alpha 1,2$-linked mannose residue. After these trimming steps, the glycoprotein can exit the ER (Herscovics and Orlean, 1993; Moremen et al., 1994; Roth, 1995). Yeast mutants defective in glucosidase I or glucosidase II are viable and lack obvious phenotypes such as slow growth (Esmon et al., 1984; Trombetta et al., 1996). It is interesting to note that mutants with defects in the *ALG6*, *ALG8*, and *ALG10* loci encoding the glucosyltransferases which sequentially add the three glucose residues also lack such a phenotype. Yeast mutants harbouring a deletion in the *MNS1* locus lack such a phenotype as well (Puccia et al., 1993).

CHAPTER 1

In various eukaryotes, a UDP-Glc:glycoprotein glucosyltransferase reglucosylates the glucose-free N-linked oligosaccharides (Trombetta and Parodi, 1992; Fernandez et al., 1994; Parker et al., 1995). The opposing activities of glucosidase II and UDP-Glc:glycoprotein glucosyltransferase generate a cycle of deglucosylation and reglucosylation of the protein-bound $GlcNAc_2Man_9$. UDP-Glc:glycosylprotein glucosyltransferase, however, acts only on unfolded/misfolded proteins. Once the glycoprotein attains its native conformation, it ceases to be a substrate for this enzyme. UDP-Glc:glycosylprotein glucosytransferase has not been detected in *S. cerevisiae*, but the enzyme termed $Gpt1^+p$ has been purified from *Schizosaccharomyces pombe* (Fernandez et al., 1994). Intriguingly, the $Gpt1^+p$ shows sequence similarity to Krc5p, an ER-located protein required for the initiation of β-1-6 glucan synthesis in *S.cerevisiae* (Meaden et al., 1990). However, no glycoprotein glucosyltransferase activity has been determined in *S. cerevisiae*.

In higher eukaryotes, a quality control system first postulated by Hammond (Hammond and Helenius, 1994) for mammalian cells monitors the folding status of a glycoprotein in the endoplasmic reticulum (Figure 3A). This system ensures that misfolded glycoproteins are retained in the ER and are either refolded or degraded. Only correctly folded proteins may exit the ER. In this system, misfolded glycoproteins containing a $Glc_1Man_9GlcNAc_2$ structure are recognized and bound by the ER resident chaperones, calnexin and calreticulin, and thereby are retainded in the folding environment of the ER. Release of the protein from calnexin/calreticulin then occurs through glucosidase II mediated removal of the single glucose residue (Hebert et al., 1995; Helenius et al., 1997). If a protein remains unfolded, incompletely folded, or misfolded, UDP-Glc:glycoprotein glucosytransferase again targets the protein to bind to calnexin/calreticulin through addition of a glucose residue to the $Man_9GlcNAc_2$ structure (Sousa et al., 1992).

Recently, a similar quality control mechanism has also been described for *Saccharomyces cerevisiae* (Jakob et al., 1998) (Figure 3B). The study proposes that the action of the ER mannosidase I establishes the time frame for correct folding of glycoproteins. It was shown that the $Man_8GlcNAc_2$ structure, as the final product of the trimming in the ER, is mandatory for efficient degradation suggesting that the ER $\alpha1,2$-mannosidase represents the key enzyme for timing the onset of degradation. Transport and maturation of correctly folded carboxypeptidase Y was not dependent on oligosaccharide structure. However, degradation of misfolded carboxypeptidase Y was dependent on specific trimming steps. Degradation of misfolded carboxypeptidase Y with N-linked oligosaccharides containing glucose residues was less efficient when compared to misfolded CPY bearing the correctly trimmed $Man_8GlcNAc_2$ oligosaccharide. In particular, $Man_8GlcNAc_2$ oligosaccharides supported degradation, suggesting the presence of a $Man_8GlcNAc_2$-binding lectin which would be involved in targeting misfolded glycoproteins to degradation. However, the existence of an alternative, glycosylation independent degradation pathway for misfolded glycoproteins in the ER in yeast was shown as well (Jakob et al., 1998).

FIGURE 3. (see following page) **A model for the ER-located protein quality control in higher eukaryotes (A) and in yeast (B).**

(A) A glycoprotein folds in the lumen of the endoplasmic reticulum with the help of molecular chaperones. The N-linked oligosaccharide of the glycoprotein is trimmed by glucosidase I and II to $Glc_1Man_9GlcNAc_2$. This structure is recognized by the lectins calnexin and calreticulin, the glycoprotein is bound and thereby retained in the ER. The N-linked oligosaccharide structure is subsequently trimmed to $Man_9GlcNAc_2$ by glucosidase II. The correctly folded glycoprotein is subjected to mannosidase I trimming and exported to the Golgi. An incorrectly folded glycoprotein harbouring $Man_9GlcNAc_2$ is reglucosylated by the UDP-Glc:glycoprotein glucosyltransferase (GlcTase) leading to repeated binding to calreticulin/calnexin, generating a cylce of deglucosylation and reglucosylation of $Man_9GlcNAc_2$. If the protein finally fails to fold correctly within the time frame of glucosidase II, GlcTase, and mannosidase I action, it is exported to the cytosol for degradation. Adapted from (Hebert et al., 1995).

(B) In *S. cerevisiae*, no UDP-Glc:glycoprotein glucosyltransferase exists. The action of mannosidase I sets the time frame for correct folding of the glycoprotein. A correctly folded protein is exported and targeted to the Golgi apparatus. If folding of the protein is not completed within the time frame of complete oligosaccharide trimming, it is targeted for export to the cytosol, where degradation by the proteasome takes place. A suggested lectin recognizing $Man_8GlcNAc_2$ mediates the targeting of the malfolded protein to the degradation pathway. Adapted from (Jakob et al., 1998).
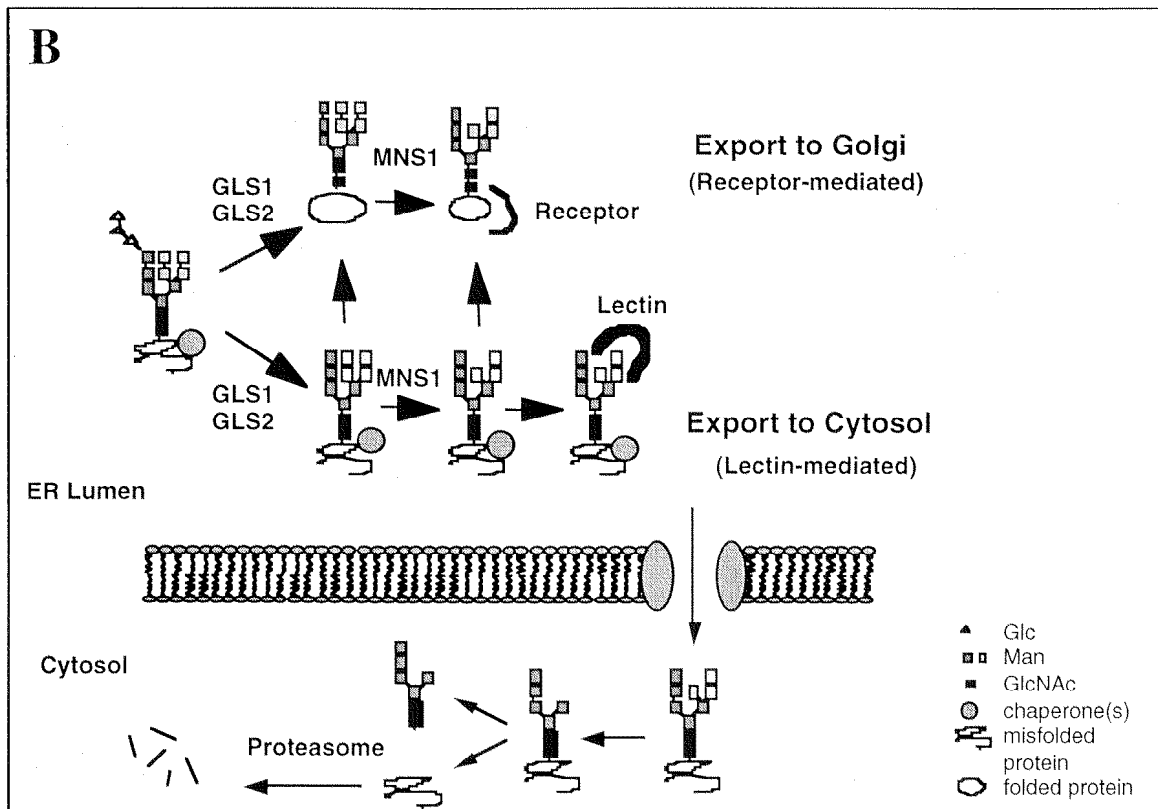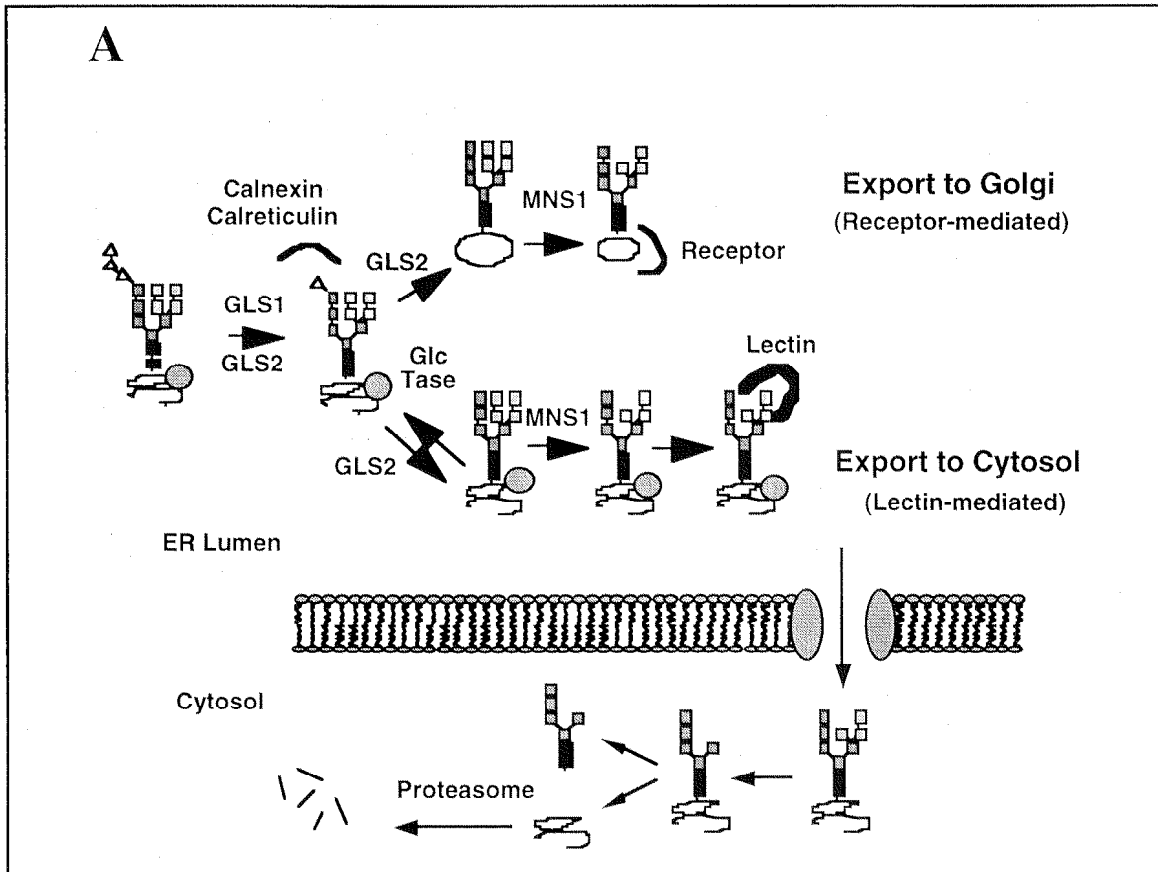
Proteins that finally fail to fold correctly or to assemble into oligomeric complexes are in most cases retained in the endoplasmic reticulum (Rose and Doms, 1988; Hurtley and Helenius, 1989). In order to maintain homeostasis, proteins retained in the ER must somehow be removed. All these proteins are eventually degraded.

In many cases, protein degradation takes place by the ubiquitin-proteasome pathway which tags the proteins for degradation by ligating ubiquitin to them (for review see (Hochstrasser, 1996; Varshavsky, 1997)). Attachment of ubiquitin to proteins involves a series of enzymatic steps. Ubiquitin-activating enzymes (E1 or Uba) form a high-energy thiol ester bond with the C-terminus of ubiquitin. Ubiquitin is then transferred to the thiol of a ubiquitin-conjugating enzyme (E2 or Ubc) and is subsequently transferred from the Ubcs to the target protein. In some cases, the last step is assisted by a ubiquitin protein ligase (E3). Usually, several cycles of ubiquitination attach a multiubiquitin chain to a protein. The ubiquitin-tagged molecules are then degraded by the 26S proteasome, an ATP-dependent multisubunit protease located in the cytoplasm (Jentsch and Schlenker, 1995; Hilt and Wolf, 1996). The 26S proteasome consists of a 20S "core" proteasome and a complex containing multiple ATPases at both ends of the 20S proteasome. 20S proteasomes with very similar basic structural features can be purified from all eukaryotes, archaebacteria, and even some eubacteria (Tamura et al., 1995).

For degradation of proteins by the cytoplasmatic proteasome, a retrograde translocation system is required. It was demonstrated that retrograde transport of proteins is mediated by a functional translocon. The *SEC61* protein, a component of the translocation machinery, is required for retro-translocation in yeast (Pilon et al., 1997; Plemper et al., 1997) and mammalian cells (Wiertz et al., 1996).

1.3.5 Maturation of N-linked oligosaccharides in the Golgi apparatus

Synthesis of the outer chain of the N-linked oligosaccharide in yeast takes place in different Golgi apparatus compartments (Preuss et al., 1992) by a stepwise and controlled addition of mannose residues catalyzed by several distinct compartmentalized mannosyltransferases. Evidence for the localization of the mannose addition was provided by the observation that mannose addition does not occur in *sec18* mutants (Esmon et al., 1981). GDP-Man has long been known as the monosaccharide donor for the Golgi mannosyltransferases (Parodi, 1979). While the $Man_{9-14}GlcNAc$ oligosaccharides have structurally been defined (Trimble and Atkinson, 1986; Alvarado et al., 1990), very little is known about the pathway of outer chain synthesis and subcompartmentation of enzymes involved beyond the ER. However, structural studies on oligosaccharides of *sec* and *mnn* mutants (for review see (Ballou, 1990)) have provided a working model for outer chain maturation. It proposes that an α-1,6 transferase (Reason et al., 1991) initiates the outer chain elongation by the addition of the first mannose residue. The next steps appear to involve the coordinated action of an α-1,6 elongating transferase and an α-1,2-transferase which introduce the side chains on the backbone. If the terminal α-1,6-mannose in the growing chain obtains an α-1,2-mannose before the next α-1,6-elongation then outer chain synthesis is terminated. A "capping" α-1,3-transferase completes the side chains (Lehle and Tanner, 1995). Recently, it has been shown that two multi-protein complexes with α-1,6-mannosyltransferase activity exist in the cis-Golgi of *S.cerevisiae* suggesting that these complexes are responsible for the synthesis of the long α-1,6-linked backbone of hypermannose structures of N-linked glycans (Jungmann and Munro, 1998).

2. Glycosylation of proteins in prokaryotes

Glycosylation of a protein in prokaryotes was first described in 1976 (Mescher and Strominger, 1976). The glycoprotein and cell surface component CSG (cell surface glycoprotein) was described for the archaeon *Halobacterium salinarium* . Prokaryotes were long considered to be unable to glycosylate proteins. However, it is now clear that glycoproteins are widespread in both archaea and eubacteria. N- as well as O-glycosyl linkage units analogous to those found in eukaryotes have been described for archaea as well as eubacteria (Lechner and Wieland, 1989; Bugg and Brandish, 1994; Sumper and Wieland, 1995; Messner, 1997) .

As in eukaryotic organisms, the oligosaccharides in prokaryotes are always attached to a protein via the amide nitrogen of an Asn residue (N-glycosylation) or via the hydroxyl groups of mainly Ser or Thr (sometimes Tyr) residues (O-glycosylation) (Moens and Vanderleyden, 1997). Many of the glycan chains of archaebacteria are linked to the protein by a N-glycosidic linkage, however, O-glycosidic linkages via Thr are also common (Mescher and Strominger, 1976; Lechner and Wieland, 1989; Messner, 1997) . In eubacterial glycoproteins, however, O-glycosidic linkages are much more frequently observed than N-glycosidic likages (Messner et al., 1995; Messner, 1997). The N-glycosylation consensus sequence Asn-Xaa-Ser/Thr found in all eukaryotes has also been reported for *H. halobium* (Lechner and Wieland, 1989) and *Methanothermus fervidus* (Bröckl et al., 1991). As in eukaryotes, O-glycosylation has been described to take place mainly on Ser/Thr in proline-rich regions for *Mycobacterium tuberculosis, Cellulomonas fimi, Clostridium thermocellum,* and *Bacteroides cellulosolvens.* In other prokaryotes, however, several distinct consensus sequences have been proposed.

A main feature of prokaryotic glycoconjugates is the extreme variety of the glycan structures. For example, there is no common structure such as the chitobiose core of

eukaryotic N-glycoproteins. The fact that prokaryotes lack the organelles which are involved in eukaryotic glycosylation raises the question how prokaryotic glycosylation and delivery of glycoproteins takes place. In Halobacteria, oligosaccharides are completed and sulfated while still attached to dolichylphosphate on the cytosolic side of the cell membrane. Thereafter the oligosaccharides are translocated to the cell surface. The transfer of oligosaccharide to the protein occurs at the cell surface (Lechner and Wieland, 1989). With this generation of N-glycosyl linkages, the halobacterial cell surface is functionally equivalent to the luminal side of the eukaryotic endoplasmic reticulum membrane.

ALG proteins, which catalyze the assembly of the lipid-linked oligosaccharide, are highly conserved in eukaryotic cells. Interstingly, sequence similarities to prokaryotic proteins have also been found. UDP-GlcNAc:dolichyl-phosphate GlcNAc-1-phosphate transferase (GPT), that performs the first step in the LLO biosynthesis, has significant sequence similarity to bacterial UDP-N-acetylmuramoyl-pentapeptide transferases that are involved in murein biosynthesis and to UDP-GlcNAc:undecaprenol-P GlcNAc-1-P transferases involved in lipopolysaccharide biosynthesis of enterobacteria. Similarity was also detected between the *S. cerevisiae ALG1* and *ALG2* proteins and bacterial glycosyltransferases (Burda and Aebi, 1998). These bacterial enzymes are involved in the biosynthesis of lipopolysaccharides and exopolysaccharides. Furthermore, archaebacterial ORF with high degree of sequence identity to the *ALG5* protein, the Dol-P-Glc synthase of yeast, have been identified (Burda and Aebi, 1998). Dol-P-Man synthase has been purified from archaea (Zhu and Laine, 1996). However, no bacterial proteins with significant sequence similarity to yeast *ALG* proteins acting on the lumenal side of the ER membrane (Alg3p, Alg9p, Alg6p, Alg8p, and Alg10p) have been found, suggesting that only the part of LLO biosyntheses occuring on the cytoplasmic side of the ER membrane is conserved between eukaryotes and prokaryotes.

Proteins with significant similarity both in the predicted overall stucture and primary sequence to the eukaryotic oligosaccharyltransferase subunit Stt3p were also detected in archaeal (Spirig et al., 1997; Burda and Aebi, 1998) and eubacterial genomes (Fry et al., 1998). Interestingly, two gene clusters which contain all the elements needed for N-glycosylation were observed in the genome of the hyperthermophilic archaeon *Archaeoglobus fulgidus* (Burda and Aebi, 1998). The arrangement of the ORF of one of these gene clusters as well as the putative functions of the corresponding proteins in protein glycosylation is depicted in Figure 4. It has been speculated that N-linked protein glycosylation in archaea follows a similar pathway as it is shown for eukaryotes (Lechner and Wieland, 1989; Sumper and Wieland, 1995). *STT3*-like proteins may represent central components of the oligosaccharyltransferase in both archaeal and eukaryotic organisms. However, since no proteins were found in the archaeal genomes showing similarity to other eukaryotic OTase subunits, the *STT3* homologue found in archaea might represent the archaeal OTase *per se*.
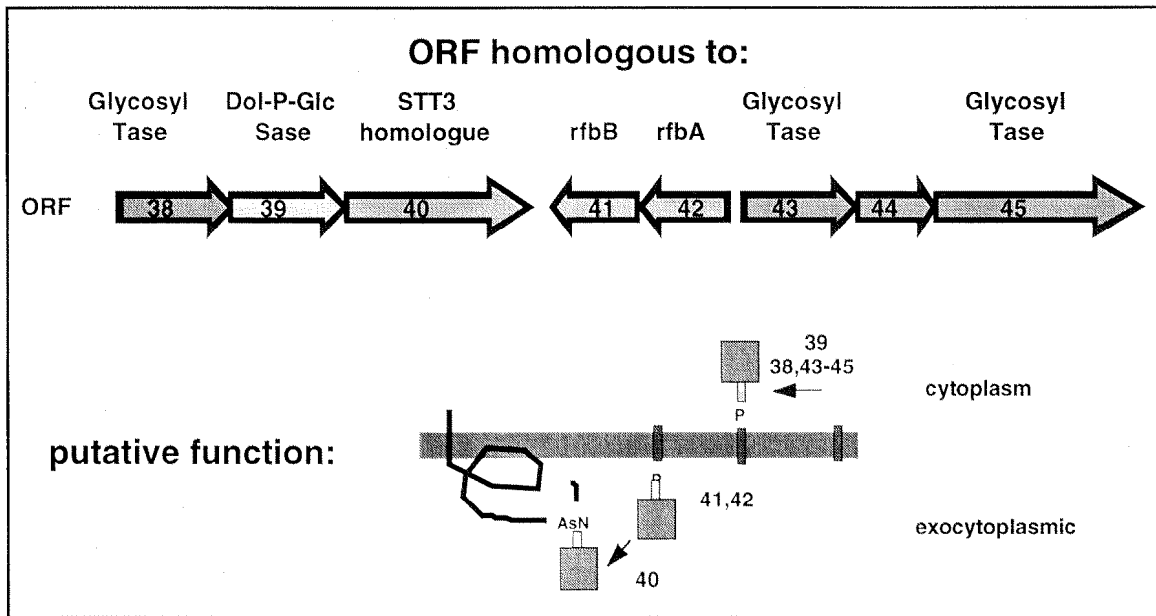
FIGURE 4. *Archaeoglobus fulgidus* **gene cluster containing all the elements needed for N-glycosylation.**

The arrangement of the different ORF in the genome of *A. fulgidus* is shown by arrows. ORF 38, 43, and 45 code for proteins with high similarity to glycosyltransferases. The ORF 39 product shows similarity to a Dol-P-Glc synthase from yeast, ORF 40 is similar to eukaryotic *STT3* proteins, and ORF41 and 42 to the O-antigen transporter *rfbA* and *rfbB* from *K. pneumoniae*. Below the gene map the putative function of each enzyme in protein glycosylation is shown. Within this model, ORF 38 and 39 and ORF 43-45 are involved in assembly of the oligosaccharide structure, ORF 41 and 42 in translocation of the lipid linked oligosaccharide across the membrane ("flipping"), and ORF40, as the *STT3* homologue, in transfer of the oligosaccharide to Asn residues of the polypeptide chain. Adapted from (Burda and Aebi, 1998).

3. Glycosylation and human diseases

In the past decade, an increasing interst in the associations of oligosaccharides with diseases could be observed, and substantial advances have been made in the design of carbohydrate based therapies and diagnostic techniques (for reviews see (Axford, 1997; Brockhausen and Kuhns, 1997; McDowell and Gahl, 1997)). The identification and investigation of these glycosylation diseases are not only important for the patients but they also offer important insight into fundamental biological processes. However, only a few glycan synthesis disorders have been described until now. Far more disorders of glycan catabolism have been described. Possibly, many glycan synthesis disorders are prenatally lethal due to the critical role of carbohydrates in development.

The oligosaccharides of mammalian glycoproteins show significant changes in their structure and occurrence during growth, development, differentiation and upon oncogenic transformation. Since glycoproteins are involved in many parts of the immune system, in fertilization, hormon action and receptor functions, cell adhesion as well as in bacterial and viral binding and infectivity, it is not surprising that altered carbohydrate structures can lead to abnormalities in many cell functions. Alterations of the sugar moieties of glycoproteins have been reported in conditions such as cancer and metastasis (Muramatsu, 1993), rheumatoid arthritis (Furukawa et al., 1990), leukemia, infection, inflammatory and other diseases, as well as alcoholism (Stibler et al., 1986). However, in most cases only associations and correlations and not causative relationships between glycoprotein glycan structures, their biosynthesis and the phenotype of a diseased cell can be established. Thus glycoprotein changes may be secondary to the true disease-triggering factors. However, the carbohydrate changes may contribute to the disease process.

An example of an inherited disease showing abnormalities in oligosaccharide synthesis is the I-cell disease first described in 1967 (Leroy and Demars, 1967). It is characterized by a lack of mannose-6-phosphate recognition marker in lysosomal enzymes which bind to specific receptors located in the Golgi, resulting from a defect in the phosphotransferase. Another example is the carbohydrate-deficient glycoprotein syndrome (CDGS) (Jaeken et al., 1984). Four different disease types have been described for this syndrome (CDGS 1 to IV). The vast majority of CDGS patients identified have type I which is characterized by a deficiency in N-linked glycosylation due mainly to a phophomannomutase (PMM) deficiency. In hereditary erythroblastic multinuclearity with positive acidified-serum (HEMPAS) (Verwilghen et al., 1973), a major erythrocyte membrane protein, the band 3 glycoprotein, was found to be abnormally glycosylated. Evidences suggest that a Golgi α-mannosidase II (α-MII) deficiency is the primary cause of HEMPAS.

The knowledge of the structures and biosynthesis of glycoproteins in disease may contribute to designing new diagnosis and therapy. Compounds inhibiting normal glycan synthesis are being considered as possible treatments for both acquired and inherited disorders. One example is N-butyldeoxynojirimycin (NBDNJ), an inhibitor of the Golgi α-glucosidase I, which has been proposed as an antiviral therapy against hepatitis B (Block et al., 1994) and HIV (Karlsson et al., 1993).

4. Outline of the work described in this thesis

The goal for the first part of this thesis was to determine whether or not the highly conserved, essential *STT3* protein required for oligosaccharyltransferase activity *in vivo* but not present in highly purified OTase preparations, indeed is a structural component of the complex.

In a second part of this thesis, the composition, structure, and function of the yeast oligosaccharyltransferase complex was investigated, applying the novel technique of blue native gel electrophoresis that allows the separation of proteins and protein complexes under native conditions. Using this technique, defined subcomplexes were revealed and a newly identified protein (Ost6p) was shown to be part of the complex, representing a ninth subunit. Furthermore, we demonstrated the existence of two distinct wild-type oligosaccharyltransferase complexes in yeast and suggested a function of the smallest 3.4 kDa Ost4p OTase subunit in complex assembly. Finally, a model was proposed which describes a stepwise and ordered *in vivo* assembly pathway of the yeast OTase.

In the last part of this thesis, different suppression mechanisms of the mutant phenotype of yeast strains carrying a mutation in oligosaccharyltransferase complex assembly were investigated. We showed that elevation of the lipid-linked oligosaccharide pool, overexpression of defined OTase subunits or other membrane proteins, influencing the ER-located N-linked oligosaccharide trimming, or a block in the ubiquitin-dependent proteasomal protein degradation pathway led to a suppression of this mutant phenotype.

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CHAPTER 2

The *STT3* protein is a component of the yeast oligosaccharyltransferase complex

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ORIGINAL PAPER

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The *STT3* protein is a component of the yeast oligosaccharyltransferase complex

Abstract N-linked protein glycosylation is an essential process in eukaryotic cells. In the central reaction, the oligosaccharyltransferase (OTase) catalyzes the transfer of the oligosaccharide $Glc_3Man_9GlcNAc_2$ from dolicholpyrophosphate onto asparagine residues of nascent polypeptide chains in the lumen of the endoplasmic reticulum. The product of the essential gene *STT3* is required for OTase activity in vivo, but is not present in highly purified OTase preparations. Using affinity purification of a tagged Stt3 protein, we now demonstrate that other components of the OTase complex, namely Ost1p, Wbp1p and Swp1p, specifically co-purify with the Stt3 protein. In addition, different conditional *stt3* alleles can be suppressed by overexpression of either *OST3* and *OST4*, which encode small components of the OTase complex. These genetic and biochemical data show that the highly conserved Stt3p is a component of the oligosaccharyltransferase complex.

Key words N-linked glycosylation · Endoplasmic reticulum · Oligosaccharyltransferase · *STT3* · *Saccharomyces cerevisiae*

Introduction

The transfer of the oligosaccharide $Glc_3Man_9GlcNAc_2$ to asparagine residues of nascent polypeptide chains is a highly conserved process in the secretory pathway of eukaryotic cells. The oligosaccharide is assembled on the lipid carrier dolicholpyrophosphate and transferred to asparagine residues specified by the Asn-X-Ser/Thr sequence, where X can be any amino acid except proline

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(Kornfeld and Kornfeld 1985; Tanner and Lehle 1987; Cummings 1992; Herscovics and Orlean 1993). The transfer is catalyzed by the enzyme oligosaccharyltransferase (OTase) and recently, components of this enzyme complex have been identified both in higher and lower eukaryotes (for a review, see Silberstein and Gilmore 1996). Purification of active OTase from ER membranes of higher eukaryotes showed that this activity is associated with a heterotrimeric complex consisting of 66-kDa (Ribophorin I), 64-kDa (Ribophorin II) and 48-kDa subunits (OST48), whereas purified yeast oligosaccharyltransferase consists of six different subunits (Kelleher and Gilmore 1994). However, active complexes lacking the two smallest subunits, a 16-kDa and a 9-kDa protein, were also reported (Knauer and Lehle 1994; Pathak et al. 1995). The yeast oligosaccharyltransferase complex is composed of Ost1p, which is homologous to ribophorin I of higher eukaryotes (Silberstein et al. 1995b), Wbp1p (OST48) (te Heesen et al. 1992; Silberstein et al. 1992), Swp1p (ribophorin II) (te Heesen et al. 1993; Kelleher and Gilmore 1994), the 34-kDa protein Ost3p (Karaoglu et al. 1995), the 16-kDa Ost2p (DAD1) (Silberstein et al. 1995a; Kelleher and Gilmore 1997) and the 9-kDa Ost5p (Reiss et al. 1997). In addition to these six proteins, genetic screens have identified two other loci, *OST4* and *STT3*, required for optimal oligosaccharyltransferase activity in vivo (Zufferey et al. 1995; Chi et al. 1996). *OST4* encodes a very small, 3.4-kDa hydrophobic protein. Depletion of this protein causes a temperature-sensitive phenotype at 37°C and results in hypoglycosylation of N-glycoproteins (Chi et al. 1996). The *STT3* product is highly conserved in eukaryotes and essential for vegetative growth of yeast cells. Stt3p is a transmembrane protein with a hydrophobic N-terminal domain spanning the membrane several times and a hydrophilic, lumenal domain at the C-terminus. The phenotype of a Stt3p depletion and of *stt3* mutations is a reduced OTase activity in vivo (Zufferey et al. 1995). Among all proteins suggested to be necessary for oligosaccharyltransferase activity, Stt3p is the most conserved one, because both

human and murine proteins as well as a putative protein in *Caenorhabditis elegans* are more than 50% identical in amino acid sequence (Zufferey et al. 1995). The apparant lack of Stt3p in purified OTase preparations (Kelleher et al. 1992; Knauer and Lehle 1994; Pathak et al. 1995) and the specific phenotypes of *stt3* mutations have given rise to the hypothesis that Stt3p is required for stability and/or assembly of the OTase complex (Zufferey et al. 1995). To clarify the role of Stt3p in the essential process of N-linked glycosylation, we addressed the question whether or not Stt3p is a component of the OTase complex in vivo. Here we report genetic and biochemical data which show that this protein makes contacts with other components of the OTase complex in vivo and therefore is indeed a component of the OTase complex.

Materials and methods

Yeast strains and media

Yeast strains used are listed in Table 1. Standard yeast media and genetic techniques were used (Guthrie and Fink 1991). G418-resistant strains were grown on yeast extract/peptone/dextrose (YPD) plates containing 200 mg/l G418 (Geneticin. GIBCO-BRL).

Sequencing of *stt3* mutations

The mutant *stt3* loci were amplified by PCR using genomic DNA (prepared with Kristal Yeast KY. MWG-Biotech) as templates and the two primers: 5′-CATCCGCTGCAGCATTCA CAGCCGTA-AATCCTAAC-3′ (primer 1) and 5′-TATTTTCTCGAGGTCATT-TCCTTT AATGTGTGGCCAATT-3′ (primer 2). Primer 1 is complementary to position −61 to −27 (ATG = +1). A PstI restriction site (*bold*), not present in the genomic sequence was introduced. Primer 2 is complementary to the region directly downstream of the stop codon but also has a XhoI restriction site (*bold*) not present in the genomic sequence. In the PCR, performed in the buffer recommended for Vent DNA polymerase by the supplier. a mixture of Taq DNA polymerase (Fermentas) and Vent DNA polymerase (New England Biolabs) in a ratio of 5:1 (units/units) was used. The 2255-bp PCR product was cloned into the vector pBS KS(−) (Stratagene) and the sequence was determined (Microsynth. Balgach. Switzerland). Besides the allele-specific mutations (Fig. 6), one nucleotide change (T to G) as compared to the published sequence (Yoshida et al. 1995) (position 1816. ATG = 1)) was found in all alleles. This changes the proposed tyrosine residue to an aspartic acid. This alteration was also found

in the wild-type strain SS328. To exclude PCR-induced mutations. the wild-type and *stt3* loci were amplified again and the mutant regions were sequenced directly from the PCR fragments using specific primers.

Disruption of the *OST4* gene

The *OST4* gene was disrupted according to the PCR-based gene disruption method using the KanMX4 module (Wach et al. 1994). A linear DNA fragment was amplified from pFA6a-KanMX4 as template with the two primers 5′-CTTGCAAGTCAGTAGA-GAAACCCAGTACAGAGCAAGCAAAAG **TCGATGAATTC-GAGCTC**-3′ and 5′ATAATAAAAACTGA AGTTCGCTATCC AAATGTAACCACTTTACGTACGCTGCAGGTCGAC-3′. These primers contain 17- and 18-bp sequences, respectively, derived from pFA6a-KanMX4 at their 3′ end (*bold*) and a 42-nucleotide sequence at the 5′ end derived from *OST4* (Chi et al. 1996). The linear fragment was used to transform the wild-type strain SS328, selecting for geneticin resistance (200 µg/ml). Correct replacement of the *OST4* locus by the KanMX4 cassette was confirmed by whole-cell PCR (Sathe et al. 1991) using *OST4*- and KanMX4-specific primers. and the resulting strains were tested for temperature sensitivity and a hypoglycosylation phenotype induced by the loss of *OST4* function.

Construction of a strain expressing Protein A-tagged Stt3p

A 410-bp DNA fragment encoding two IgG-binding domains of *Staphylococcus aureus* Protein A (Moks et al. 1987; Fleischmann et al. 1996) was amplified and used to exchange the *XhoI-XbaI SUC2/HIS4* fragment in the plasmid pSTT3-HIS4 (Zufferey et al. 1995). This generated pSTT3-ProtA, encoding an Stt3-ProtA fusion protein. The sequence at the fusion junction is: Stt3p-GLRV*LELAQHDEA*VDN-ProtA (residues in *italics* represent linker sequences). To construct the integrative plasmid pRS303(Δstt3-ProtA). pSTT3-ProtA was cut with *PvuII* and *SpeI*, generating a DNA fragment containing the *STT3* locus with a 276-bp deletion at the 5′ end. This fragment was cloned into the vector pRS303. The resulting plasmid, pRS303(Δstt3-ProtA). was linearized within the *stt3* coding region with *NcoI* and used to transform the diploid yeast strain SS328xSS330, selecting for histidine prototrophs. Sporulation and tetrad dissection resulted in strain YG469. Correct integration of the fragment into the genome and expression of the STT3-ProtA fusion protein was verified by Western analysis as described (Fleischmann et al. 1996).

Purification of the *STT3* protein using IgG-Sepharose column

The basic method has been described (Grandi et al. 1993). Strain YG469 was grown in 6 l of YPD medium at 30° C for 16 h. The cells were harvested at OD_{546} = 1–2 by centrifugation and washed once with 500 ml of ice-cold 50 mM TRIS-HCl. pH 7.4, 0.2 M mannitol. 0.1 M NaCl. 1 mM MgCl₂, 1 mM CaCl₂, and 1 mM

Table 1 Yeast strains used in this study

Strain	Genotype	Reference
SS328	*MATα ade2-101 ura3-52 his3Δ200 lys2-801*	Vijayraghavan et al. (1989)
SS330	*MATa ade2-101 ura3-52 his3Δ200 tyr1*	Vijayraghavan et al. (1989)
YG170	*MATα ade2-101 ade3 ura3-52 his3Δ200 alg3-1 stt3-3*	Zufferey et al. (1995)
YG176	*MATa ade2-101 ade3 ura3-52 his3Δ200 tyr1 leu2 stt3-3*	Zufferey et al. (1995)
YG390	*MATa ade2-101 ura3-52 his3Δ200 lys2-801 stt3-5*	This study
YG393	*MATα ade2-101 ura3-52 his3Δ200 tyr1 stt3-6*	This study
YG397	*MATa ade2-101 ura3-52 his3Δ200 lys2-801 stt3-4*	This study
YG437	*MATα ade2-101 ura3-52 his3Δ200 lys2-801 ProtA-ALG3*	O. Meier and S. te Heesen
YG469	*MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δstt3::STT3-ProtA*	This study
YG493	*MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δost4::kanMX4*	This study
YG535	*MATa ade2-101 ura3-52 his3Δ200 leu2 lys2-801 stt3-7*	This study

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MnCl₂. Lysis was achieved in the same buffer containing 1 mM PMSF, 0.1 mM DTT and protease inhibitors (aprotinin, antipain, chymostatin, leupeptin and pepstatin, 2 μg/ml each) by disrupting with glass beads under constant cooling with ice in a Bead-Beater (BioSpec) using six 1-min pulses at 1-min intervals. The lysate was centrifuged for 8 min in a Sorvall GS3 rotor at 2000 rpm and the supernatant centrifuged for 30 min at 22 000 rpm in a Beckman Ti45 rotor. The pellet was resuspended in 50 ml of ice-cold 50 mM TRIS-HCl, pH 7.4, 0.2 M mannitol, 0.1 M NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM MnCl₂ and 10% glycerol, adjusted to 1% Triton-X-100, incubated on ice for 30 min and centrifuged for 30 min in a Beckman Ti45 rotor at 36 000 rpm. This 100 000 × g supernatant was loaded onto a 10 mm × 200 mm column (Sigma) packed with 5 ml of IgG-Sepharose (Pharmacia), equilibrated in the above buffer. The column was run by gravity flow at 4°C. The flowthrough fraction was applied a second time. The column was then washed with 50 ml of 50 mM Tris-HCl, pH 7.4, 0.2 M mannitol, 0.1 M NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM MnCl₂, 10% glycerol, 1% Triton-X-100, followed by an additional wash with 10 ml of 5 mM ammonium acetate (pH 5), 1% Triton-X-100, 10% glycerol. Bound Protein A fusion protein was eluted with 10 ml of 0.5 M acetic acid (pH 3.5), 1% Triton X-100 and 10% glycerol, and immediately neutralized with 3 M TRIS-HCl (pH 7.5). Aliquots of the homogenate, the 100 000 × g supernatant, the flowthrough, the wash, and the eluted fractions were analysed by Western analysis using anti-Ost1p, anti-Wbp1p, anti-Swp1p, and anti-Alg3p antibodies or PAP, a soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase (Dakopatts, Denmark). As a control, the same procedure was carried out with a yeast strain carrying a Protein A-tagged *ALG3* protein (YG437, O. Meier and S. te Heesen, unpublished).

Immunological methods

Analysis of carboxypeptidase Y (CPY) by in vivo labeling and immunoprecipitation (te Heesen et al. 1992) and Western blot analysis (Burda et al. 1996) have been described.

Determination of OTase activity

OTase activity of membranes of wild-type and *stt3* mutant cells was measured as described (Reiss et al. 1997), except that the reaction was performed for 30 min at 22°C.

Results

stt3 alleles with a temperature-sensitive phenotype

In order to study the basis of the requirement for Stt3p for OTase activity, we took advantage of a large collection of mutant alleles of the *STT3* locus. These alleles were detected in screens designed to identify mutants with alterations in N-linked glycosylation processes in the endoplasmic reticulum (Zufferey et al. 1995; Reiss et al. 1997). Among approximately 50 independent *stt3* alleles, we recovered four which lead to a temperature-sensitive phenotype. *stt3-4*, *stt3-5* and *stt3-6* were identified as being lethal in combination with the *Δalg5* mutation (Reiss et al. 1997), whereas *stt3-7* was found in a synthetic lethal screen using the *wbp1-2* mutation (Zufferey et al. 1995) (P. Burda, unpublished). The original isolates were backcrossed up to four times to the wild-type strain SS328. Segregation analysis in these crosses revealed that the individual *stt3* mutations did indeed cause the temperature-sensitive phenotype. We

sequenced the different conditional *stt3* alleles and found that all encoded mutations in the C-terminal, hydrophilic domain of the *STT3* protein. Surprisingly, the *stt3-4* and *stt3-6* alleles had the same mutation, resulting in a change from glycine at position 520 (Yoshida et al. 1995) to a glutamic acid, while the *stt3-5* allele affected the same glycine residue, changing it to a serine. The *stt3-7* mutation is located in close proximity to this glycine residue and affects serine at position 552. This serine is changed to a proline. In contrast to the ts alleles, the non-conditional *stt3-3* allele (Zufferey et al. 1995) is characterized by a mutation in the N-terminal domain of the protein and results in a change from threonine to isoleucine at position 85 (see Fig. 6). It is interesting to note that in all mutant alleles sequenced, an amino acid residue that is conserved in yeast and higher eukaryotes (Zufferey et al. 1995) is affected. The mutations are located in hydrophilic, lumenal domains of the protein.

We tested the effect of the conditional alleles on OTase activity in vivo and in vitro. For this purpose, we examined the expression of the glycoprotein carboxypeptidase Y (CPY). This highly expressed vacuolar protease contains four N-linked oligosaccharides (Hasilik and Tanner 1978) and changes in N-linked glycosylation are visualized by the alterations in the mobility of CPY in SDS-PAGE (te Heesen et al. 1992; Stagljar et al. 1994). Cells harboring the different *stt3* alleles were grown at permissive temperature (23°C) and shifted to the non-permissive temperature (37°C). Cells were metabolically labeled for 1 h at the shift, and for 3, 6 and 9 h after the shift, and CPY molecules were analyzed after specific immunoprecipitation and SDS-PAGE (Fig. 1). Already at permissive temperature, we observed a hypoglycosylation of CPY molecules in the different *stt3* strains, indicated by the appearance of faster moving bands, representing molecules lacking N-linked sugars (verified by treatment with endo-
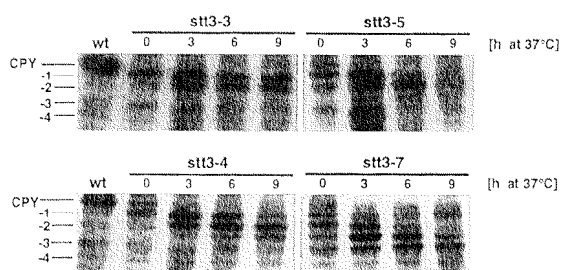

Fig. 1 Underglycosylation of carboxypeptidase Y (CPY) in *stt3* mutant cells. Strains were grown at 23°C and shifted at time 0 to 37°C. Samples were taken at the times indicated, cells were labeled for 1 h with [³⁵S]methionine and cysteine, extracts were prepared and used for CPY-specific immunoprecipitations. The wild-type strain SS328 (wt) served as control. The position of mature CPY (CPY) and the glycoforms lacking one (−1), two (−2), three (−3), or four (−4) oligosaccharide units are indicated. The *stt3* genotype of the strains used is indicated. Strains: SS328 (wt), YG176 (*stt3-3*), YG397 (*stt3-4*), YG390 (*stt3-5*), YG535 (*stt3-7*).

glycosidase H, data not shown). However, there was a further reduction in glycosylation when *stt3-4*, *stt3-5* and *stt3-7* cells were grown under non-permissive conditions: CPY molecules lacking up to all four oligosaccharides were produced. The non-conditional *stt3-3* allele did not induce a temperature-dependent hypoglycosylation of CPY. We conclude that, in vivo, oligosaccharyltransferase activity is reduced in the conditional *stt3* strains at permissive temperature but is further diminished at 37° C. The most severe underglycosylation is observed in *stt3-7* cells. We also tested the in vitro activity of the OTase in extracts derived from *stt3* cells grown under permissive conditions. A mixture of incomplete lipid-linked oligosaccharides derived from bovine pancreas was used as oligosaccharide substrates (Badet and Jeanloz 1988). As reported previously for the *stt3-3* allele, we observed a strong reduction in OTase activity in vitro in *stt3* extracts (Table 2). The use of incomplete oligosaccharide donors resulted in a drastic decrease in OTase activity in the novel *stt3* mutant strains. However, we did not assay the activity using the same conditions with complete oligosaccharide, which partially restored activity in the *stt3-3* strain (Zufferey et al. 1995).

Allele-specific suppression of *stt3* alleles by overexpression of OST3p and OST4p

It is a characteristic of mutations in components of the OTase complex that they can be suppressed by overexpression of other OTase subunits: the *wbp1-2* mutation is rescued by overexpression of either Swp1p (te Heesen et al. 1993) or Ost2p (Silberstein et al. 1995a), whereas a mutation in the 64-kDa subunit Ost1p is specifically suppressed by overexpression of Ost5p (Reiss et al. 1997). We therefore wanted to know whether or not such a suppression can also be observed for the *stt3* mutations and tested all known OTase components for their suppressing activity when expressed from the high-copy-number vector YEp352 (Hill et al. 1986). Overexpression of Ost1p, Wbp1p, Swp1p, Ost2p and Ost5p did not alter the temperature-sensitive phenotype of the different *stt3* strains (data not shown), but additional

Table 2 In vitro activity of oligosaccharyltransferase in wild-type and *stt3* mutant cell extracts

Yeast strain	Relative glycosyltransfer to peptide (%)[a]
SS328 (wild type)	100
YG176 (*stt3-3*)	3
YG397 (*stt3-4*)	3
YG390 (*stt3-5*)	5
YG535 (*stt3-7*)	2

[a] Strains were grown at 23° C to mid-log phase in YPD complete medium and oligosaccharyltransferase activity was determined using lipid-linked oligosaccharides from bovine pancreas and a synthetic radiolabeled acceptor peptide as a substrate. The value obtained from wild-type extracts was taken as 100%. The relevant genotype of each strain is given

copies of *OST3* and *OST4* were able to restore growth at 37° C (Fig. 2A). This suppression affected the N-linked glycosylation process directly, because we observed improved glycosylation of CPY in the *OST3* and *OST4*-transformed cells (Fig. 2B). It is interesting to note that in the most severely affected *stt3* mutant, *stt3-7*, overexpression of both *OST3* and *OST4* improved glycosylation efficiency very significantly, whereas in both *stt3-4* and *stt3-5* cells less suppression is observed. We conclude that *stt3* mutations can be suppressed by overexpression of either *OST3* or *OST4* in an allele-specific manner. Importantly, the suppression efficiency with respect to growth correlated very well with the improved glycosylation efficiency: *OST3* overexpression only slightly improved growth of *stt3-4* or *stt3-5* cells at 37° C and resulted in a weak improvement of glycosylation as well.

In contrast to the conditional *stt3* alleles, the *stt3-3* allele is characterized by a mutation in the N-terminal domain of the *STT3* protein. However, no growth defect is associated with this particular allele. To determine whether overexpression of *OST3* and *OST4* has a suppressive effect on the *stt3-3* allele, we took advantage of the synthetic phenotype of the *stt3-3* mutation in combination with *alg3-1* (Zufferey et al. 1995). This synthetic phenotype is characterized by temperature-sensitive growth. Suppression of the *stt3-3* mutation can be monitored by growth at 37° C because the *alg3-1* mutation alone has no growth defect. However, we did not observe any suppression of the growth-defect of the double mutant *stt3-3 alg3-1* at 37° C (Fig. 2C). In addition, no suppression of hypoglycosylation in *stt3-3* mutant strains by overexpression of Ost3p and Ost4p was observed (data not shown).

Overexpression of *OST3* rescues a deletion of the *OST4* locus

The similarity in the behaviour of *OST3* and *OST4* with respect to the suppression of *stt3* alleles led to the question whether these two proteins perform redundant functions. Neither of these loci encodes an essential component of the OTase complex, but the deletion of *OST4* results in hypoglycosylation of secretory proteins and a temperature-sensitive growth phenotype (Chi et al. 1996), whereas *Δost3* cells do not have any growth phenotype and show a mild hypoglycosylation defect in the ER (Karaoglu et al. 1995). Due to this lack of a selectable phenotype we only tested for the suppression of *Δost4* by overexpression of *OST3*. Indeed, this overexpression suppressed the ts defect in a *Δost4* strain (Fig. 3).

Stt3p interacts with components of the OTase complex

Allele-specific suppression indicates a direct physical interaction between the corresponding proteins (Huffa-
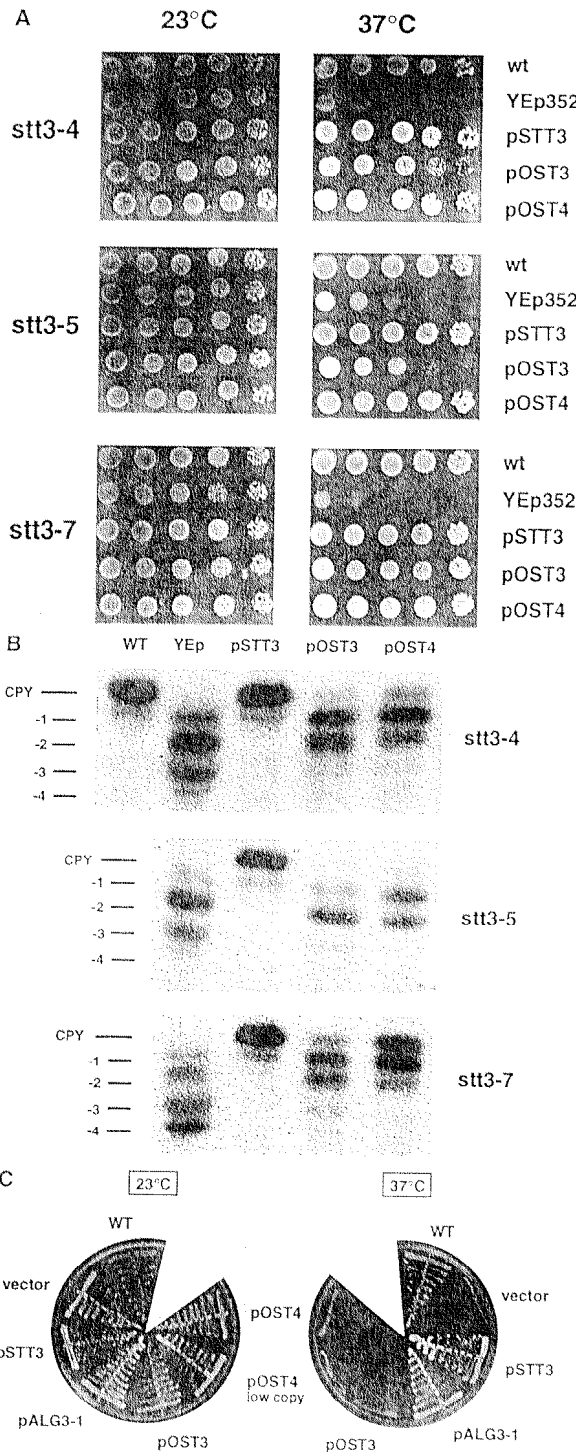
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A

Fig. 2A–C OST3 and OST4 are allele-specific, high-copy-number suppressors of stt3 mutants. A Growth of stt3 mutant cells harboring YEp352, pSTT3, pOST3, or pOST4. stt3-4, stt3-5, and stt3-7 cells were transformed with the 2μ plasmids YEp352 (vector), pSTT3, pOST3, or pOST4, and resulting uracil prototrophs were cultured to mid-log phase in liquid minimal medium lacking uracil. Serial 1:10 dilutions starting at 5×10^5 cells were spotted onto plates containing minimal medium lacking uracil. Plates were incubated for 4 days at 23°C or 37°C and photographed. SS328 + YEp352 (wt) is included in each set for comparison. B OST3 and OST4 can suppress CPY underglycosylation in stt3 mutant cells at 37°C. The transformants described in A were grown at 23°C in minimal medium lacking uracil to mid-log phase, shifted to 37°C, diluted after 3 h to an OD_{546} of 0.5 and reincubated for another 3 h at 37°C. Proteins were then labeled for 1 h with ^{35}S-methionine and cysteine, cell extracts prepared and used for CPY-specific immunoprecipitation. SS328 + YEp352 (wt) is shown for comparison. The position of mature CPY (CPY) and the different glycoforms lacking one to all four N-linked oligosaccharides (−1 to −4) are indicated. (C) OST3 and OST4 are not able to suppress the synthetic temperature-sensitive growth phenotype of stt3-3 alg5-1 cells. The double mutant stt3-3 alg5-1 (YG170) was transformed with the plasmid YEp352 (vector), YEp352/STT3 (pSTT3), YEp352/ALG3-1 (pALG3-1), YEp352/OST3 (pOST3), YEp352/OST4 (pOST4) or pRS316/OST4 (pOST4 low copy). Transformants were streaked on plates lacking uracil and incubated for 4 days at 23°C or 37°C. SS328 + YEp352 (wt) served as control
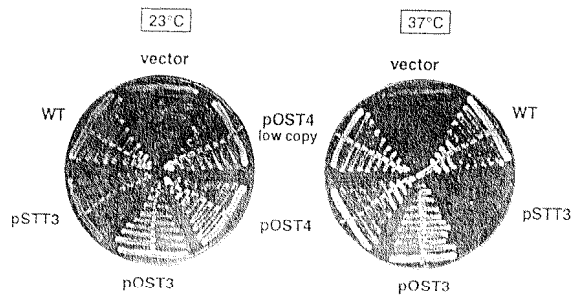
B

C

Fig. 3 OST3 is a high-copy-number suppressor of an ost4 deletion. The Δost4 strain YG493 was transformed with the plasmid YEp352 (vector), pSTT3, pOST3, or pOST4 or pRS316/OST4 (pOST4 of low copy number). Transformants were streaked on plates containing minimal medium lacking uracil and incubated for 4 days at 23°C or 37°C. SS328 + YEp352 (wt) is shown for comparison

ker et al. 1987) and our in vivo experiments suggest that Stt3p is required for OTase activity (Zufferey et al. 1995). Our data therefore favor a model in which Stt3p is a component of the OTase complex. However, Stt3p has not been detected in highly purified OTase preparations from yeast or from other sources (Silberstein and Gilmore 1996). To resolve this discrepancy, we asked whether or not Stt3p physically interacts with other OTase components. We prepared a yeast strain which carries a tagged version of the STT3 protein. A 410-bp DNA encoding two IgG-binding domains of Staphylococcus aureus Protein A (Moks et al. 1987; Grandi et al. 1993) was fused in frame to the 3′-end of the STT3 ORF. The hybrid gene was used to functionally replace the endogenous STT3 locus in the wild-type yeast strain SS328. We did not observe any alteration of either growth rate or N-linked protein glycosylation in the

modified strain (data not shown), suggesting that the tagged version of the *STT3* protein is fully functional. Membrane fractions from strain YG469 carrying the tagged *STT3* locus were solubilized and the *STT3* protein was bound to IgG-Sepharose columns. Western blot analysis revealed that the tagged protein was bound

quantitatively to the column (Fig. 4A). This bound protein was eluted by lowering the pH (Fig. 4A). Immunoblot analysis of the different column fractions using different antibodies revealed that not only the tagged *STT3* protein was retained on the column but also all other tested components of the OTase complex, namely Ost1p, Wbp1p and Swp1p (Fig. 4B–D). This retention was not quantitative, because major fractions of these proteins were not bound to the column. However, the retention was specific for OTase components because other membrane proteins of the ER, such as Alg3p (encoding a mannosyltransferase located in the ER) (Aebi et al. 1994) (Fig. 4E) and Alg5p (encoding Dol-P-Glc synthase) (te Heesen et al. 1994) (data not shown), were not retained. Importantly, the retention of the different OTase components was mediated by the tagged *STT3* protein, because binding of a tagged *ALG3* protein did not result in retention of either Ost1p, Wbp1p and Swp1p (Fig. 4B–D). We therefore conclude that Stt3p interacts specifically with other components of the OTase complex in vivo.

Fig. 4A–E Stt3p specifically interacts with other components of the oligosaccharyltransferase complex. Membranes of a yeast strain (YG469) carrying an *STT3* protein tagged with two IgG-binding domains from *S. aureus* Protein A (STT3-ProtA) were prepared, solubilized under non-denaturating conditions and used for affinity purification of the *STT3* protein by IgG-Sepharose chromatography. Solubilized membrane fractions (M) were loaded onto the IgG-Sepharose column. The flowthrough (F) was reapplied to the column. The column was then washed once in the same buffer (W) and with a buffer at pH 5 (1). Bound proteins were eluted at pH 3.5 (2). Aliquots of the individual fractions were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. As a control, the same procedure was performed with strain YG437 carrying a ProtA-ALG3 fusion (ProtA-ALG3). The proteins bound to nitrocellulose were detected with: **A** PAP, a soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase, which detects the IgG-binding domains of Protein A; **B** anti-Ost1p antibodies; **C** anti-Wbp1p antibodies; **D** affinity-purified anti-Swp1p antibodies; and (**E**) anti-Alg3p antibodies. The positions of two to four proteins from a broad-range prestained marker set (Biolabs) are indicated. The positions of the proteins detected using the different antibodies are indicated by *arrows*. STT3-ProtA protein and ProtA-ALG3 protein are detectable with every serum, due to the presence of IgG-binding domains in the fusion protein. ProtA-ALG3 blots are overexposed to demonstrate the absence of OTase components in the bound fractions

Discussion

The *STT3* protein is the most highly conserved protein implicated in the function of the oligosaccharyltransferase reaction. The hypothesis of a direct involvement of the *STT3* protein in the OTase reaction was based on
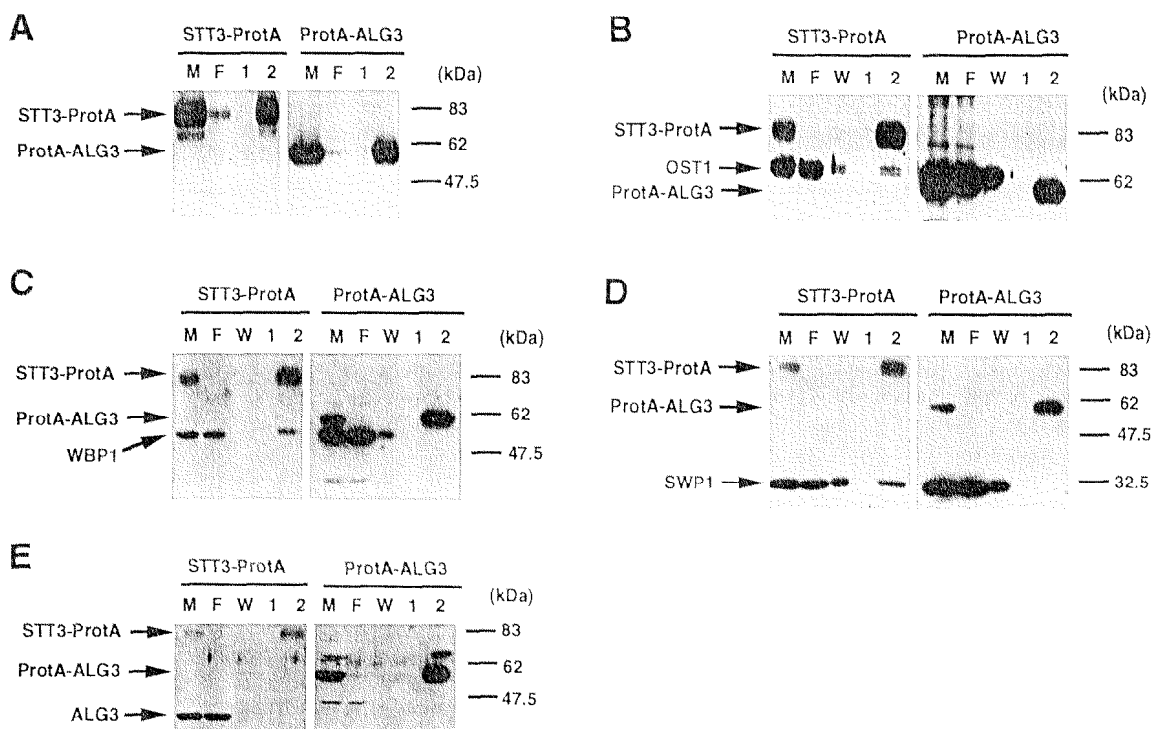
the phenotype of *stt3* mutations and the loss of OTase activity upon Stt3p depletion (Zufferey et al. 1995). Our present results now strongly suggest that this protein is indeed a functional component of the OTase complex. The allele-specific suppression of temperature-sensitive *stt3* alleles by overexpression of two other OTase components, Ost3p and Ost4p, provides a strong genetic argument for a direct interaction of Ost3p and Ost4p with the *STT3* protein. In addition, the specific physical interaction of this protein with other components of the OTase complex, namely Ost1p, Wbp1p and Swpp, points to a direct role for Stt3p in the OTase complex. The partial retention of OTase components on the Stt3p column can be explained by the harsh solubilization conditions used or the presence of substoichiometric amounts of Stt3p in the OTase complex. It is interesting to note that the three proteins which were shown to physically interact (either directly or indirectly) with Stt3p do not suppress *stt3* mutations on overexpression. It is most likely that affinity purification of the tagged *STT3* protein results in retention of the whole OTase complex; however, we did not assay for OTase activity in these preparations. Due to the lack of Ost3p and Ost4p-specific antibodies, we were not able to assay for a physical interaction of these two proteins with Stt3p, but our genetic data provide strong although indirect evidence for such an interaction. Why has Stt3p remained undetectable in purified preparations of the OTase complex, if it is an essential component of the activity? Tagging of the *STT3* protein revealed an aberrant mobility of this protein in SDS-PAGE (Fig. 4). Instead of 96 kDa, the tagged protein has a mobility indicative of a 83-kDa protein. Therefore, unmodified Stt3p, with a calculated molecular mass of 78 kDa, may possibly co-migrate with another component of the OTase complex. Ost1p (64 kDa) and might therefore escape detection. In addition, we note that the tagged *STT3* protein appears as a relatively broad band in SDS-PAGE (see Fig. 4) and stains poorly with Commassie Blue (data not shown). All these characteristics of the *STT3* protein may contribute to the apparent lack of this protein in purified OTase preparations. Indeed, recent results from R. Gilmore's group also suggest that Stt3p is a component of the purified complex (D. Karaoglu and R. Gilmore, personal communication). Genetic and biochemical experiments show that the OTase complex consists of eight different subunits, and a direct interaction of these proteins in the OTase complex has been demonstrated for seven of these subunits (Stt3p, Ost1p, Wbp1p, Ost3p, Swp1p, Ost2p and Ost5p). For the small Ost4p subunit, biochemical evidence is still lacking (Chi et al. 1996), but the role of *OST4* as an allele-specific high-copy-number suppressor of *stt3* mutations, and the suppression of a *Δost4* mutation by overexpression of the OTase component Ost3p further support the hypothesis that Ost4p is also a component of the OTase complex.

The different temperature-sensitive alleles which are suppressed by overexpression of either Ost3p or Ost4p are characterized by mutations in a hydrophilic domain which is possibly exposed on the lumenal side of the ER membrane (Zufferey et al. 1995). This contrasts with the purely hydrophobic properties of the high-copy-number suppressor Ost4p. At present, we have no direct experimental evidence that might explain the suppressing activity of Ost4p and Ost3p overexpression. It is possible that these small transmembrane proteins are important for the integration of Stt3p into the OTase complex, and overexpression of these auxiliary proteins might allow the assembly of mutant *STT3* protein into this complex. Ost3p and Ost4p perform partially redundant functions, because a loss of Ost4p can be rescued by overexpression of *OST3*. The phenotype of the specific *stt3* alleles differs with respect to the degree of hypoglycosylation, temperature sensitivity and suppressibility. Multiple functions of Stt3p in the glycosylation process might account for this observation.

With regard to suppression of mutations in different subunits of the OTase complex by overexpression of other OTase components, a clear picture is emerging: a *wbp1* mutation is suppressed by overexpression of either *SWP1* or *OST2* (te Heesen et al. 1993; Silberstein et al. 1995a), whereas suppression of an *ost1* mutation was observed solely for *OST5* (Reiss et al. 1997). We now demonstrate that specific *stt3* alleles are suppressed by either *OST3* or *OST4*, and overexpression of *OST3* suppresses the ts phenotype of an *ost4* deletion. This type of analysis allows the sorting of the eight OTase subunits into three distinct groups (Wbp1p, Swp1p and Ost2p; Ost1p and Ost5p; Stt3p, Ost3p and Ost4p, Fig. 5). It remains to be seen whether these groups represent subcomplexes of the OTase with different functions in vivo.

In genetic screens directed towards the identification of components required for the process of N-linked glycosylation in the ER, we have identified more than 50 independent isolates of *stt3* alleles, four of which lead to a temperature-sensitive phenotype. It is interesting to note that all four of these mutations cluster around a highly conserved region of the *STT3* protein. Three of the mutations affect the same amino acid residue, two of these lead to the same amino acid exchange. This extreme bias in the amino acids affected in the different alleles might be explained by a bias in the selection procedure. However, the *stt3-4*, *stt3-5* and *stt3-6* alleles were identified based on their synthetic lethality in combination with *Δalg5*. (Reiss et al. 1997) whereas *stt3-7* was isolated in a screen based on synthetic lethality in combination with *wbp1-2* (Zufferey et al. 1995). The common characteristic of all these *stt3* alleles is their temperature-sensitive phenotype. It is possible that only mutations in this area of the protein can lead to such a conditional phenotype.

The area of the protein in which the temperature-sensitive mutations are located is highly conserved between all eukaryotic *STT3* homologs (Zufferey et al. 1995). A sequence homology search using the BLAST algorithm reveals that in the archaebacterium *Methanococcus*
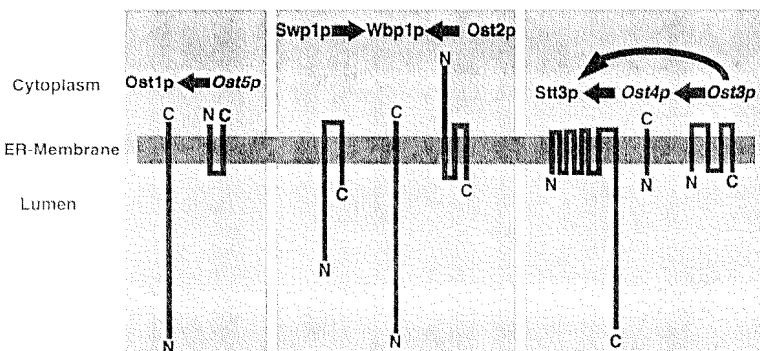
jannaschii, an ORF encoding a putative 933-amino acid protein contains sequences highly similar to the *STT3* protein family. The hydrophobicity analysis of the *M. jannaschii* protein shows a very similar overall structure to that of the eukaryotic *STT3* proteins: the protein can be divided into a hydrophobic N-terminal region and a hydrophilic C-terminal domain covering one third of the protein (Fig. 6). Most interestingly, the region of highest sequence similarity between the eukaryotic *STT3* proteins and the archaeal protein is in the area in which all the conditional *stt3* mutations are located: the glycine residue affected in the products of the *stt3-4*, *stt3-5* and *stt3-6* alleles is also found in the archaebacterial sequence, and the serine residue affected in the *stt3-7* allele is a threonine residue in the *M. jannaschii* sequence. In this respect it is noteworthy that in *Halobacterium*, another member of the Archaea, N-linked protein glycosylation of a surface glycoprotein has been reported (for a review, see Lechner and Wieland 1989). Two types of glycosylation occur in this bacterium: in both types sulfated oligosaccharides are

Fig. 5 The eight oligosaccharyltransferase subunits can be grouped into three subcomplexes. The pattern of suppression of mutant alleles by overexpression of other OTase subunits (the *arrows* point *towards* the subunit in which a defect can be suppressed) allows the grouping of the OTase subunits into three distinct groups (gray blocks). The postulated topology of the different subunits is also shown, but only for Ost1p and Wbp1p does direct experimental evidence support the topology proposed. For all other subunits, the proposed topology is based on suggestive experimental evidence and/or theoretical calculations. With the exception of Ost3p, Ost4p and Ost5p (indicated in *italics*), all components are essential proteins. The drawing is not to scale

Fig. 6 Hydropathy analysis of *S. cerevisiae* Stt3p and the *Methanococcus jannaschii* MJ1525 gene product (Accession No. 1511526) according to Kyte and Doolittle (1982) using a window of 19 residues. The region of highest similarity between the two primary sequences is given in detail. Sequence identities are listed, conservative changes are indicated by *crosses*. All sequenced *stt3* mutations are indicated in the *S. cerevisiae* hydropathy plot by *dashed lines*, the conditional mutations are also marked in the primary sequence

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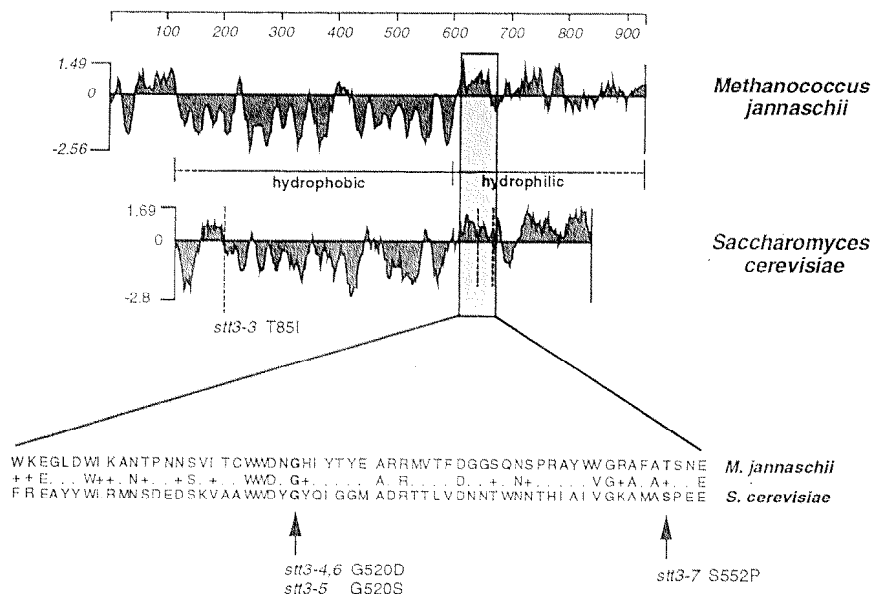
attached to the Asn residues of the typical AsN-X-Ser/Thr sequence, either via the linkage unit asparagine-N-acetylglucosamine or via asparagine-glucose. In the first case, the transferred oligosaccharide is assembled on the lipid carrier dolicholpyrophosphate, whereas for the second type of oligosaccharide, dolicholphosphate serves as a carrier in the biosynthesis of the oligosaccharide. The similarity between halobacterial glycoprotein biosynthesis and eukaryotic N-linked protein glycosylation has been noted before (Lechner and Wieland 1989). Our observation that the most highly conserved component of the eukaryotic oligosaccharyltransferase, Stt3p, shares structural and sequence homology with an archaeal protein supports the hypothesis that N-linked protein glycosylation, like protein translocation across membranes, is a conserved process in both prokaryotes and eukaryotes.

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CHAPTER 3

Blue native gel electrophoresis reveals two distinct oligosaccharyltransferase complexes in yeast and a defined role of the 3.4 kDa *OST4* subunit within the complex

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(Additional data are supplemented in an addendum p. 94)

Blue native gel electrophoresis reveals two distinct oligosaccharyltransferase complexes in yeast and a defined role of the 3.4 kDa *OST4* subunit within the complex

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oligosaccharyltransferase complexes, *Saccharomyces cerevisiae*, blue native gel

electrophoresis, subcomplexes, *OST6*, *OST4*

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ABSTRACT

In the central reaction of N-linked glycosylation, the oligosaccharyltransferase complex (OTase) catalyzes the transfer of a lipid-linked core-oligosaccharide onto asparagine residues of nascent polypeptide chains in the lumen of the endoplasmic reticulum. The *Saccharomyces cerevisiae* OTase has been shown to consist of at least eight subunits which are supposed to be grouped into three subcomplexes. In this study we analyzed this enzyme complex, applying the technique of blue native gel electrophoresis. Using available antibodies, six different subunits were detected in the wild-type complex, including Stt3p, Ost1p, Wbp1p, Swp1p, Ost3p, and additionally Ost6p, an Ost3p homologue, which we now demonstrate to represent an additional subunit of the OTase. Using *stt3*, *Δstt3*, *ost1*, *Δwbp*, *Δost3*, and *Δost4* mutant strains, we visualized defined subcomplexes on blue native gels. In combination with high copy number suppression studies we demonstrate that the small 3.4 kDa subunit Ost4p is required for incorporation of either Ost3p or Ost6p into the complex, resulting in two different OTase complexes *in vivo*.

INTRODUCTION

In the secretory pathway of eukaryotic cells, the transfer of the oligosaccharide $Glc_3Man_9GlcNAc_2$ to selected asparagine residues in nascent polypeptide chains is a highly conserved process. The oligosaccharide is assembled on the lipid carrier dolichylpyrophosphate and then transferred *en bloc* to asparagine residues in the sequon Asn-X-Ser/Thr, where X can be any amino acid except proline (1, 4, 9, 15, 26). This transfer is catalyzed by the enzyme complex oligosaccharyltransferase (OTase) (for review see (23)). Whereas purification of active OTase from ER membranes of higher

eukaryotes showed that OTase activity is associated with a heterotrimeric complex consisting of ribophorin I (66 kDa), ribophorin II (64 kDa), and OST48 (48 kDa), purified yeast oligosaccharyltransferase consists of at least six different subunits (12). The yeast oligosaccharyltransferase complex is composed of Ost1p, which is homologous to ribophorin I of higher eukaryotic cells (22), Wbp1p (OST48) (24, 27), Swp1p (ribophorin II) (12, 28), Ost2p (DAD1) (21), Ost3p (10) and Ost5p (17).

In addition to these six proteins, genetic screens have identified two other loci, *STT3* and *OST4*, which are required for full oligosaccharyltransferase activity *in vivo* (3, 30). *OST4* codes for a very small, 3.4 kDa hydrophobic protein. *OST4* deleted cells show a temperature-sensitive phenotype at 37°C and a marked hypoglycosylation of both soluble and membrane N-glycoproteins (3). Additional evidence suggests that Ost4p is a component of the OTase complex (11, 25). The *STT3* product is essential for vegetative growth of yeast cells and is highly conserved in eukaryotes. Depletion of the protein leads to reduced OTase activity *in vivo* (30). Recent experiments show that Stt3p is also a structural component of the yeast oligosaccharyltransferase complex (11, 25). Ost3p and Ost4p have been proposed to act together in a subcomplex with the OTase subunit Stt3p (11, 25). Furthermore, the overexpression of either Ost3p or Ost4p in *stt3* mutant strains restores the growth of the strain at 37°C and improves glycosylation of carboxypeptidase Y (CPY) (25).

A search in the available databases for homologues of the non-essential Ost3p revealed a hypothetical 37 kDa protein, now termed Ost6p, with 46% amino acid sequence homology and 21% sequence identity to Ost3p (13). The hydropathy plots of Ost3p and Ost6p are very similar, suggesting four potential transmembrane domains and a similar predicted arrangement of an N-terminal signal sequence (11). Until now, a definitive demonstration that this protein is in fact part of the OTase has not been provided.

It is also not clear whether all the proteins named above are always part of the OTase, as functional OTase complexes from *Saccharomyces cerevisiae* have been isolated which do

not contain all the components described above but retain transferase activity *in vitro* (14, 16). To address these questions, we analyzed the OTase complex by blue native polyacrylamide gel electrophoresis which allows separation of proteins and protein complexes under native conditions (18-20). Using this analytical method we detected defined subcomplexes accumulating in different OTase mutant strains. We were able to demonstrate that the *OST6* protein indeed is a component of the OTase complex. In addition, we showed that the very small 3.4 kDa subunit Ost4p is needed for the incorporation of either Ost3p or Ost6p into the complex and that two types of OTase complexes exist in yeast, containing, in addition to the set of shared proteins, either Ost3p or Ost6p.

MATERIALS AND METHODS

Yeast strains and media

Yeast strains are listed in Table I. Standard yeast media and genetic techniques were used (8).

Preparation of microsomal membranes and solubilization of membrane proteins

Microsomal membranes were prepared as described (17) with the following modification: cells were grown as 2 l cultures and the membranes were finally resuspended in 1 ml of membrane buffer (50 mM Tris/HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 35% glycerol) containing 1 mM DTT, 1 mM PMSF, and protease inhibitors (aprotinin, antipain, chymostatin, leupeptin, and pepstatin, 2 μg/ml each). To 100 μl of membrane suspension in membrane buffer were added 300 μl TM-buffer (50 mM Tris/HCl (pH 7.4), 0.2 M mannitol, 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$)

containing 1 mM DTT, 1 mM PMSF, and protease inhibitors (aprotinin, antipain, chymostatin, leupeptin, and pepstatin, 2 µg/ml each). DNA was digested with 0.2 mg/ml DNAse I (3000 U/mg, Fluka) for 45 min at 25°C on a thermoshaker. Glycerol was added to a final concentration of 10% and the protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin as a standard. Protein concentration was adjusted to 7 µg/µl with GTM-buffer (TM-buffer containing 10% glycerol) containing 1 mM DTT, 1 mM PMSF, and the above described protease inhibitors. Membrane proteins were then solubilized by the addition of digitonin (1.5% final concentration) (Sigma) and 6-aminocaproic acid (750 mM final concentration) (Fluka). Incubation was for 45 min at 4°C with shaking. Unsoluble material was removed by centrifugation for 30 min at 40000 rpm in a Kontron TFT 80.4 rotor at 4°C. Protein concentration of the supernatant was determined, samples frozen in liquid nitrogen and stored at -80°C.

Blue native polyacrylamide gel electrophoresis

Blue native electrophoresis was carried out in the Protean II cell from Bio-Rad (gel dimensions: 20 x 15 x 0.15 cm). The gels consisted of a separating gel with a 5-12% acrylamide gradient and a stacking gel (4% acrylamide). Buffers and gel compositions were as described (20) except that Tris-Base was used in all buffers instead of Bistris and that the pH was adjusted to pH 7.5 (4°C) instead of pH 7.0 (4°C). Protein concentration of the solubilized membrane protein samples was adjusted to 1 µg/µl with GTM-buffer containing 750 mM 6-aminocaproic acid, 1.5% digitonin, 1 mM DTT, 1 mM PMSF, and the above described protease inhibitors. Sample buffer (100 mM Tris-HCl (pH 7.5 (4°C)), 500 mM 6-aminocaproic acid, 5% Serva blue G) was added (15% of the original sample volume), gently mixed and the sample was loaded on the gel. The electrophoresis was at 4°C with the current limited to 25 mA and the voltage limited to 380 V for 15 h, and an additional hour with the current limited to 25 mA and the voltage limited to 500 V. After 6 running-hours, the cathode buffer (50 mM Tricine, 15 mM Tris/HCl, 0.02% Serva blue G, pH7.5(4°C)) was removed and the electrophoresis was continued with a

cathode buffer containing no Serva blue G (50 mM Tricine, 15 mM Tris/HCl, pH7.5(4°C)).

Electroblotting of blue native gels and immunodetection

Gels were soaked for 5 min in transfer buffer (25 mM Tris-Base, 200 mM Glycine, 0.1 % SDS, 20% methanol) and proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a semi dry blotter from Kem-En-Tec (Copenhagen, Denmark) with a constant current of 1 mA/cm^2 for 135 min. Removal of Coomassie dye from the nitrocellulose was achieved by soaking the blots for 2 x 7 min in 50% methanol, 10% acetic acid and subsequent washing in PBST (136 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 0.1% Tween 20 (v/v)). Blots were then air dryed and incubated with appropriate antisera. Antibody-binding was visualized with peroxidase-labeled protein A using enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, England).

Isolation of *OST4* and *OST6*

The *OST4* locus was isolated as a high copy number suppressor of the temperature-sensitive phenotype of the *stt3-6* mutation (25), and the *OST6* locus was isolated as a high copy number suppressor of the temperature-sensitive phenotype of the *Δost4* strain YG493. The YEp352-bound library described by Fleischmann *et al.* (1991) was used (6).

Western blot analysis of CPY

Western blot analysis of CPY has been described (2).

RESULTS

The yeast oligosaccharyltransferase complex can be visualized by blue native gel electrophoresis

Blue native electrophoresis is a powerful technique for the isolation and characterization of native protein complexes from biological membranes (20). In order to analyze the native form of the oligosaccharyltransferase in wild-type and mutant yeast strains, we adapted this recently described method to the isolation of this enzyme complex. Several detergents and detergent concentrations were tested for the solubilization of the complex (data not shown), and digitonin in a final concentration of 1.5% in combination with 750 mM 6-aminocaproic acid was chosen. Solubilized membranes of wild-type yeast cells were supplemented with Serva blue G and subsequently separated on polyacrylamide gels using a 5-12% acrylamide gradient. After transfer to nitrocellulose, specific proteins were revealed by immunodetection with defined antibodies. Antibodies against the OTase subunits Ost1p, Wbp1p, Swp1p, and Ost3p detected a complex running at a position estimated to be about 500 kDa (Fig. 1) by comparing the mobility of the complex with those of thyroglobulin and apoferritin. This complex was termed wild-type OTase complex I. Antibodies against the largest OTase subunit Stt3p detected a protein complex running at the same position, confirming the recently published results showing that Stt3p is a component of the yeast OTase (11, 25). In addition, antibodies against Ost6p (13) revealed a complex running at the same position as well, indicating that Ost6p is indeed a component of the OTase complex. We also detected a protein complex with a slower mobility than complex I. The composition of this complex, termed complex I_0, was not analyzed in more detail. Blots were also incubated with antibodies directed against a component of the protein translocation machinery, Sec61p (Fig. 1, lane 9), as well as antibodies against Gas1p, a glycophospholipid-anchored surface glycoprotein (data not shown). Both these antibodies revealed proteins running at a position different from the complexes detected by

antibodies against OTase subunits, indicating that complex I and I_0 are not unspecific aggregates of proteins. Using a yeast strain expressing the *STT3* protein tagged at its C-terminus with the IgG-binding domain of *Staphylococcus aureus* protein A, we confirmed our previous results that this tagged protein is a component of the oligosaccharyltransferase complex (25). In addition, this complex was destroyed upon addition of SDS (Fig. 1, lanes 7 and 8), demonstrating the detergent-sensitivity of the complex. These data suggested that complex I represented the native oligosaccharyltransferase complex.

To demonstrate that this protein complex was functionally linked with oligosaccharyltransferase activity, we analyzed complex composition in mutant cells carrying a conditional allele of the *STT3* locus. The *stt3-7* mutation results in a temperature-sensitive phenotype when grown at 37°C and a strong hypoglycosylation of glycoproteins as visualized by analysis of CPY (25). In *stt3-7* cells grown for 9 hours at 37°C, the level of complex I was strongly reduced as compared to wild-type (Fig. 2, lane 2). However, when the *stt3-7* cells carried the *OST3* or the *OST4* locus on a multicopy plasmid, which has been shown to restore growth at 37°C and improve glycosylation of CPY (25), complex I was again detected (Fig. 2, lanes 3 and 4). These observations provided a functional link between oligosaccharyltransferase activity and complex I, and suggested that complex I represented the active oligosaccharyltransferase enzyme.

Using antibodies against Wbp1p, a novel complex running faster than complex I was seen in *stt3-7* cells. This complex remained present in the cells complemented with pOST3 or pOST4 (Fig. 2, lanes 2-4). The appearance of this complex (complex IV, see below) also suggested that the assembly of the OTase complex was altered in *stt3-7* cells. Therefore, we analyzed the OTase complexes in strains containing mutations in different OTase subunits.

Analysis of Stt3p-depleted as well as *ost1-4* mutant cells revealed a complex containing Wbp1p and Swp1p (complex IV)

We first analyzed the consequence of depleting the *STT3* protein, an essential component of the OTase complex, on OTase composition *in vivo*. We used a yeast strain with a deletion in the *STT3* locus but containing a hybrid gene in which the *STT3* coding sequence was placed under the control of the galactose-inducible and glucose-repressible GAL1 promotor (30). This strain was grown in galactose medium to mid-log phase, washed and shifted to glucose medium. Cells were harvested 11 hours after the shift, membranes prepared and proteins separated by blue native gel electrophoresis as described. Antibodies against four different OTase subunits were used for immunodetection, namely αOst1p, αSwp1p, αStt3p, and αWbp1p (Fig. 3). When cells containing the galactose inducible *STT3* allele were grown in galactose-containing medium, all four antibodies used revealed an OTase complex migrating at the same position as wild-type complex I (Fig. 3, lanes 2). The somewhat weaker signal derived from galactose-grown cells compared to wild-type control cells (lane 1) was attributed to a lower amount of protein loaded. Since the GAL1 promotor leads to an overexpression of the *STT3* gene, we concluded that overexpression of *STT3* did not affect the composition or the assembly of the OTase complex. Furthermore, these results indicated that overexpressed *STT3* protein was rapidly degraded because we saw neither free Stt3p (Fig. 3C) nor a higher amount of assembled complex I in these preparations.

In cells in which the expression of the *STT3* gene was repressed, complex I was not detected. Instead, a complex running at a position of about 120 kDa was seen using both the αSwp1p and αWbp1p antibodies (Fig. 3B and D, lanes 3). As expected, αStt3p antibodies gave no signal in Stt3p-depleted extracts. The αOst1p antibody revealed a signal at a position of less than 100 kDa; experiments with denatured extracts as well as with a yeast strain expressing the *OST1* protein tagged with protein A suggested that this broad band represented free Ost1p (data not shown). We concluded that in Stt3p-depleted cells a subcomplex containing at least Wbp1p and Swp1p but not Ost1p accumulated.

This subcomplex was designated complex IV. It was also detected with αWbp1p antibodies in *stt3-7* mutant cells grown at non-permissive temperature (Fig. 2A, lane 2); free Ost1p also accumulated in these cells (Fig. 2B, lane 2). To exclude the possibility that the accumulation of complex IV in Stt3p-depleted cells was an unspecific effect due to the induced glycosylation deficiency and growth arrest, we analyzed a diploid yeast strain heterozygous for a *stt3* deletion. In contrast to wild-type cells, this phenotypically normal strain revealed both fully assembled OTase complex I as well as significant levels of complex IV (Fig. 4, lane 3). We concluded that stable complex IV accumulated when phenotypically normal cells had reduced amounts of Stt3p.

We also detected the presence of subcomplex IV in a strain with a mutation in the *OST1* locus (Fig. 3B and D, lanes 6). *ost1-4* mutant cells show a reduced growth rate at permissive temperatures and a 75% reduction of OTase activity *in vitro* (22) . *ost1-4* cells grown in complete medium at 30°C revealed a complex running at the same position as seen in preparations of Stt3p-depleted cells (complex IV) when detection was performed by αSwp1p as well as αWbp1p antibodies (Fig. 3B and D, lanes 6). As in the case of Stt3p-depleted cells, complex IV of *ost1-4* cells did not appear to contain mutant Ost1p, since no signal was seen with αOst1p antibodies at the position of complex IV (Fig. 3A, lane 6). Additionally, αStt3p antibodies did not detect a complex IV in *ost1-4* cells, suggesting that this protein is also not a part of the complex (Fig. 3C, lane 6). It was significant that a complex running with identical mobility and having the same protein composition appeared under three different conditions, namely by non-permissive growth of *stt3-7* cells, depletion of wild-type Stt3p and in *ost1-4* strains. We concluded that the absence or instability of Stt3p, or a mutation in the *OST1* locus led to the formation and accumulation of the highly stable subcomplex IV of the OTase.

Wbp1p-depleted cells accumulated free Ost1p and an Ost1p-containing subcomplex

The effects of depleting the OTase component Wbp1p were tested in the same way as was done for Stt3p. A strain containing a deletion of the endogenous *WBP1* gene and a *GAL1-WBP1* gene (27) was grown in galactose medium, washed and shifted to glucose medium. Cells were harvested 11 hours after the shift, membranes were prepared and proteins analyzed by blue native gel electrophoresis. In preparations from cells grown in galactose medium, immunodetection with αOst1p, αSwp1p, αStt3p, and αWbp1p antibodies showed the accumulation of wild-type complex I (Fig. 3, lanes 4). Additionally, αWbp1p antibodies but not αOst1p, αSwp1p, or αStt3p antibodies detected the accumulation of a complex running slightly slower than complex IV (Fig. 3D, lane 4). Since this small complex was only detected in Wbp1p overexpressing cells and no other OTase subunit was observed in this complex, we concluded that it represented a protein complex specific for Wbp1p overexpressing cells probably due to an interaction of *WBP1* protein with other proteins.

As was seen in cells depleted of Stt3p, cells lacking Wbp1p also lost complex I (Fig. 3, lanes 5). Antibodies against Ost1p revealed free Ost1p as well as the presence of a subcomplex running at a position between wild-type complex I and complex IV. This complex, present at low levels was termed complex III. It evidently did not contain Swp1p or Wbp1p, as antibodies against these proteins gave no signal in glucose-grown *GAL1-WBP1* cells. However, antibodies against Stt3p detected low levels of protein at the position of complex III (Fig. 3C, lane 5).

Ost4p was required for the formation of complex I by recruiting either Ost3p or Ost6p, leading to two distinct fully assembled complexes, one containing Ost3p and one containing Ost6p

The 3.4 kDa *OST4* gene product is likely to play an important role in N-glycosylation in *S. cerevisiae*, since *OST4* deleted cells show a temperature-sensitive

phenotype at 37°C and a marked hypoglycosylation of N-glycoproteins (3). In a screen for high copy suppression of the Δost4 temperature-sensitive phenotype, we recovered plasmids encoding both OST3 (25) and OST6. Deletion of the OST6 locus was reported to affect OTase activity (13). pOST3 gave a strong suppression of the temperature-sensitivity, while pOST6 suppressed to a lesser extent (Fig. 5A). Correspondingly, the overexpression of OST3 in the Δost4 strain yielded nearly normal glycosylation of CPY, while overexpression of OST6 did not improve the hypoglycosylation in the Δost4 strain (Fig. 5B, lane 6-9). Cells containing a deletion of OST3 are fully viable, and show only modest changes in glycosylation compared to wild-type cells (10)(Figure 5B, lane 2). While both Δost3 and Δost4 strains are viable, the strain containing both of these mutations was inviable (data not shown). This synthetic lethality, in addition to the temperature suppression results, is further evidence of a functional interaction between Ost3p and Ost4p.

We therefore examined the oligosaccharyltransferase complex in Δost4 strains and Δost4 strains overexpressing Ost3p or Ost6p. Due to the observed interaction between Ost4p and Ost3p, a parallel set of Δost3 strains containing the vector, pOST3, pOST4, or pOST6 was also examined. Membranes from each of these strains were digitonin-solubilized and proteins were subjected to blue native gel electrophoresis. Using αOst1p antibodies, very low levels of complex I were detected in Δost4 cells, but instead, a complex that migrated faster was revealed (Fig. 6A, lane 1). A normal complex I was restored in the Δost4 cells that were complemented with pOST4 (Fig. 6A, lane 3). These results argued that Ost4p was required for the formation of complex I. The faster migrating complex, termed complex II, was partially converted to complex I by overexpression of Ost3p in Δost4 cells (Fig. 6A, lane 2) and to a lesser extent by overexpression of Ost6p (Fig. 6A, lane 4). These results were consistent with the strong suppression of the temperature-sensitivity (Fig. 5A) and hypoglycosylation phenotypes (Fig. 5B) observed when Ost3p was overexpressed, and with the weaker suppression seen by overexpression of Ost6p in a Δost4 strain (Fig. 5). Complex II was also detected

in *Δost4* cells and those overexpressing either Ost3p or Ost6p using antibodies directed against Stt3p, Wbp1p, or Swp1p (data not shown), indicating that complex II contained at least Ost1p, Stt3p, Wbp1p, and Swp1p.

Complexes I and II were also detected in the set of *Δost3* strains, using the αOst1p antibody. In this case, the *Δost3* strain contained both complexes, and the overexpression of *OST4* did not change the composition of the complexes (Fig. 6B, lanes 2 and 4). Complex II could be fully converted to complex I when *Δost3* was complemented by pOST3; a complete conversion to complex I was also observed by overexpression of Ost6p (Fig. 6B, lanes 3 and 5).

When αOst6p antibodies were used to analyze the OTase complexes in the set of *Δost3* and *Δost4* strains, only complex I was detected, indicating that complex II did not contain Ost6p. However, this antibody revealed a heterogeneity in the composition of complex I. Complex I from wild-type cells yielded a signal with αOst6p antibodies, indicating that at least a fraction of wild-type complex I did contain Ost6p (Fig. 6C, lane 1). The complementation of *Δost4* with pOST4 led to a complex I that contained Ost6p (Fig. 6C, lane 8), while the complex I that was detected in *Δost4* strains transformed with pOST3 (Fig. 6A, lane 2) did not produce a signal with αOst6p antibodies, and thus lacked Ost6p (Fig. 6C, lane 7). The small amount of complex I seen after overexpression of Ost6p in the *Δost4* strain also contained Ost6p (data not shown). These results suggested that there were two forms of complex I, one containing and one lacking Ost6p.

This heterogeneity was also seen in the *Δost3* strains. The complex I seen in *Δost3* cells and *Δost3* cells overexpressing *OST4* contained Ost6p (Fig. 6C, lanes 2 and 4). In contrast, the complex I formed in *Δost3* cells complemented by pOST3 did not contain Ost6p (Fig. 6C, lane 3). Since the *Δost3*/vector and *Δost3*/pOST3 strains differed only by overexpression of Ost3p and yet show different forms of complex I, this result argued that the overexpression of Ost3p precluded Ost6p from taking part in complex I. We therefore suggested that complex I consisted of one form containing Ost3p and lacking Ost6p (termed complex Ia) and a second form containing Ost6p and lacking Ost3p

(complex Ib). Ost4p was required for the integration of either of these proteins into complex I, since complex I was not detected in *Δost4* cells. However, since overexpression of either Ost3p or Ost6p in a *Δost4* strain resulted in complex I, we concluded that there are parallel pathways that led either to the formation of complex Ia or complex Ib.

DISCUSSION

In the recent years, the oligosaccharyltransferase was shown to be composed of several different subunits, not only in yeast but also in higher eukaryotes (for review see (23)). In order to analyze the OTase complexes accumulating in different OTase mutants we took advantage of a novel technique, blue native gel electrophoresis, which allows the visualisation of proteins and protein complexes in their native form. The phenotype of the different mutant strains and the analysis of the corresponding OTase complex by blue native gel electrophoresis led us to conclude that a "native" OTase complex can indeed be resolved by this technique. Our results revealed defined subcomplexes accumulating in different OTase mutant strains (see table II). We demonstrated that the Ost3p homologue Ost6p indeed represents an additional subunit of the yeast OTase. Our results also revealed that the very small subunit Ost4p was required for the integration of either Ost3p or Ost6p into the complex resulting in two different fully assembled OTase complexes.

Digitonin in a final concentration of 1.5% was found to be a suitable solvent to solubilize the enzyme for analysis by blue native gel electrophoresis. The enzyme complexes were found to be highly stable, since harsh conditions as freezing and thawing serveral times, or incubation for 1 hour at room temperature or for several hours on ice, did not alter complex composition. Thus, blue native gel electrophoresis represented an

additional tool to explore the composition, function, and assembly of the oligosaccharyltransferase.

In wild-type extracts, two complexes were detected by antibodies against different OTase subunits. The faster running, more abundant complex was termed complex I, the slower running complex I_0. Complex I and I_0 were detected by six different antisera, namely αStt3p-, αOst1p-, αWbp1p-, αOst3p-, αSwp1p-, and αOst6p-antiserum (Fig. 1). Ost1p, Wbp1p, Ost3p, and Swp1p are components of purified yeast OTase complex (12). The results of our experiments using αStt3p-antibodies as well as a protein A-tagged version of Stt3p confirmed the recently published results that also Stt3p is a component of the yeast OTase (11, 25). A direct link to OTase function was provided by the analysis of the *stt3-7* mutant cells. The *stt3-7* mutation results in a hypoglycosylation of proteins *in vivo*, a strongly reduced OTase activity as measured *in vitro*, and a temperature-sensitive phenotype (25). In *stt3-7* cells grown at non-permissive 37°C no significant amounts of wild-type complex I was detected but instead complex IV accumulated (see below). Importantly, the phenotypic high copy number *stt3-7* suppressors pOST3 and pOST4 (25) were also observed to restore significant amounts of complex I. We concluded that complex I represented fully active oligosaccharyltransferase. The addition of other proteins, whose identity remains to be determined, appears to yield complex I_0.

We have used a set of large proteins as molecular size markers in blue native gel electrophoresis. Such size markers were reported to allow a reliable assessment of the molecular masses of the protein import complex of yeast mitochondria (5). Based on these markers, the mobility of complex I suggested a mass of around 500 kDa. However, the expected molecular mass of the OTase consisting of Stt3p, Ost1p, Wbp1p, Ost3p, Swp1p, Ost2p, Ost5p, and Ost4p (11, 12, 25) is 280 kDa. It is possible that active OTase represents a dimer. However, since we do not know all the parameters that influence mobility of protein complexes in blue native gel electrophoresis, we believe that it is premature to estimate the molecular mass of the OTase complex band on its relative

mobility in this gel system. Instead, we used size markers to compare the results of individual gels and not to deduce the molecular mass of a given complex.

Using antibodies against Ost6p, we demonstrated that this protein was also a component of oligosaccharyltransferase complex I and thus represented an additional subunit of the yeast oligosaccharyltransferase. Our results confirmed the previous observation that Ost6p is involved in the N-linked glycosylation process (13). The presence of the *OST6* protein in fully assembled OTase complex I was surprising, because this subunit was not detected either in genetic screens (30) or in highly purified OTase preparations (12). Interestingly, a 31kDa-band not identical to the 34kDa-Ost3p-band and speculated to represent the Ost3p homologue YML019W (Ost6p) has been found in immunoprecipitates from strains expressing HA-tagged Stt3p (11). The result that Ost6p was a high copy number suppressor of an *ost4* deletion (Fig. 5A) is in agreement with this observation.

Both depletion of Stt3p and a shift of *stt3-7* cells to non-permissive temperature resulted in the appearance of a novel complex that contained both Wbp1p and Swp1p but not Ost1p (complex IV). This complex was also detected in *ost1-4* mutant cells, suggesting that this stable complex required both Stt3p and Ost1p for further processing into mature complex I. Complex IV did not contain either Ost3p or Ost6p, and we speculate that it was composed of Wbp1p, Swp1p, and Ost2p. Based on genetic (25) and biochemical data (11), it has been suggested that these three proteins form a protein complex *in vivo*. Recently, Fu *et al.* demonstrated direct physical interactions between the mammalian Wbp1p homologue OST48 and the Swp1p homologue ribophorin II as well as between OST48 and the Ost2p homologue DAD1 using the yeast two hybrid system (7).

Not only are Stt3p and Ost1p required to form a stable association with the core complex IV, but they might also themselves form a subcomplex. We detected a complex III in Wbp1p-depleted cells with antibodies against Stt3p and Ost1p but not against Swp1p or Ost3p. However, Ost1p seemed to be more abundant in this complex than

Stt3p (compare Fig. 3A and C, lanes 5), indicating that the complex III band possibly represented two different aggregates in which both Ost1p and Stt3p assembled with unknown proteins.

The fact that depletion of Wbp1p, which is one of the complex IV components, results in the degradation of Swp1p (14) (Fig. 3B) and in the appearance of low levels of complex III, and that depletion of either Stt3p, Ost4p, or Ost3p yielded stable subcomplexes suggested that the formation of complex IV was a prerequisite for the assembly of the complete OTase. Is complex IV sufficient for OTase activity? Although *ost1-4* cells are able to grow at 30°C, immunodetection by αStt3p antibodies revealed that these cells contain, in addition to large amounts of complex IV, trace amounts of complex I (Fig. 3C, lane 6) which possibly allowed for growth. In *stt3-7* cells grown at non-permissive 37°C as well as in Stt3p-depleted cells, complex IV accumulated and no complex I was detected. Since both of these conditions lead to lethality of the strains, we therefore concluded that complex IV is enzymatically not active *in vivo*.

Strains containing a deletion of *OST4* exclusively accumulated another stable subcomplex, complex II, while those deleted for *OST3* accumulated both complex I and II. Complex II contained Wbp1p, Swp1p, Stt3p, Ost1p, and (possibly) Ost2p. Immunodetection with αOst6p antibodies revealed that Ost6p was also not present in complex II. Due to the lack of specific antibodies, we do not know whether or not Ost4p was also part of complex II accumulating in Δost3 cells (Fig. 6B, lane 2). Recently, both genetic and biochemical evidences were presented that the very small 3.4 kDa protein Ost4p is a structural component of the mature OTase (11, 25). We defined complex II as an OTase complex with or without Ost4p but lacking both Ost3p and Ost6p. Our results explain recently published results which show that only trace amounts of OTase subunits were recovered in non-denaturing immunoprecipitates from a Δost4 strain that expresses HA-tagged Ost3p; additionally, OTase complexes isolated from a Δost4 mutant that expresses HA-tagged Stt3p by immunoprecipitation lack Ost3p (Karaoglu et al., 1997). Is complex II an enzymatically active complex? Δost4 cells are viable at 23°C and

accumulated complex II. However, we do not know whether or not small amounts of complex I lacking Ost4p are present in Δost4 cells. This low level of complex I might account for the low level of OTase activity in Δost4 cells (3, 25) (Fig. 5A). At 37°C, the non-permissive temperature of Δost4 cells, we also observed complex II in these cells (data not shown). This suggests that complex II is not thermolabile and that the general hypoglycosylation of glycoproteins is responsible for the temperature-sensitive phenotype observed in Δost4 cells.

Δost3 cells accumulate both complex I and complex II. The surprising observation that cells lacking a component of the oligosaccharyltransferase were able to produce "wild-type size" oligosaccharyltransferase led us to conclude that two distinct oligosaccharyltransferase complexes existed in yeast. These complexes differed by one component, Ost3p or Ost6p. We termed the oligosaccharyltransferase complex containing Ost3p complex Ia, the one containing Ost6p complex Ib. Cells lacking Ost3p therefore had complex Ib (with Ost6p) and complex II (lacking both Ost3p and Ost6p) (Fig. 6). Complex II in the Δost3 strain was chased into complex Ib by overexpression of OST6. Similarly, overexpression of OST3 yielded only complex Ia and not complex Ib. Ost3p and Ost6p share sequence similarity and the similar hydropathy profiles suggest a similar topology of the two proteins in the membrane (13). Also the observation that most but not all of the cellular pool of Wbp1p, Swp1p, or Ost1p copurified with epitope-tagged Ost3p was taken as a hint for the existance of a small pool of OTase complexes that lack Ost3p (11). The presence of a 31 kDa-band speculated to represent YML019W (Ost6p) in purified OTase preparations from strains expressing HA-tagged Stt3p but not from strains expressing tagged Ost3p (11) supports our suggestion that two distinct OTase complexes exist in yeast and that the Ost3p containing OTase does not contain Ost6p. It will be very interesting to investigate the roles of the different OTase complexes Ia and Ib in the process of N-linked glycosylation. It was previously shown that cells lacking OST3 show a biased underglycosylation of membrane proteins (10). This leads to the speculation that the two different OTase complexes may show different affinities to soluble and membrane

glycoproteins respectively. Another possibility would be that one OTase is responsible for cotranslocational glycosylation and so would be located in close proximity to the translocon, whereas the other one would catalyze posttranslocational glycosylation. However, the two homologeous genes *OST3* and *OST6* may be a relict from an ancient gene duplication event during evolution and therefore have redundant functions.

Our results suggested that both Ost3p and Ost6p are assembled at a late time point in the assembly pathway of the OTase, because we observe only fully assembled OTase complex (Ia or Ib) containing either of these subunits. Compatible with this observation is the hypothesis that Ost3p is a peripheraly associated component of the OTase complex (11). For the association of both Ost3p and Ost6p, the small *OST4* subunit seemed to be required. We therefore propose a possible function of the Ost4p subunit to be an assembly factor for integration of Ost3p and Ost6p into the complex. However, Ost4p may only be needed for the overall stability of the fully assembled OTase complex to help Ost3p and Ost6p remaining incorporated in the complex. Whether this assembly / stability function of the *OST4* protein is its single function, or if Ost4p plays an additional, maybe catalytical role in OTase function remains to be determined. The function of the very small, hydrophobic *OST4* protein in the assembly of the OTase complex strongly suggests that at least a part of this process involves the transmembrane domains of specific OTase components. Such interactions seem to be crucial for the assembly or the stability of a funcional OTase complex.

In the past few years, much progress has been made in characterising the multimeric membrane complex oligosaccharyltransferase in yeast. Here, we confirmed the existence of an additional, ninth subunit of this complex. We demonstrated the existence of two distinct OTase complexes in yeast *in vivo*, whose functional significance will be an exciting line of future investigation. Additionally, we suggested a possible function of the smallest OTase component Ost4p. However, not much is known about the function of the other subunits of the complex. To define the function of the individual components in the transfer reaction of the oligosaccharide from the lipid carrier dicholpyrophosphate to

asparagine residues of polypeptides, specific mutants with altered OTase activity but full competence in OTase assembly will be needed. Blue native gel electrophoresis will provide a tool for these studies.

ACKNOWLEDGEMENTS

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Table I. Yeast strains used in this study

Strain	Genotype	Reference
SS328	MATα ade2-101 ura3-52 his3Δ200 lys2-801	(29)
45-C3	MATa ade2-101 tyr1 his3Δ200 Δwbp1::HIS3 URA3:GAL1-WBP1	(27)
RGY117	MATa ura3-52 leu2-3,112 lys2-801 Δost1::LEU2 pRS316(ost1-4)	(22)
YG156	MATα/a Δstt3::HIS3/STT3 ura3-52/ura3-52 lys2-801/LYS2 tyr1/TYR1 his3Δ200/his3Δ200	(30)
YG157	MATα ade2-101 lys2-801 URA3:GAL1-STT3 Δstt3::HIS3 his3Δ200	(30)
YG191	MATα ade2-101 ura3-52 his3Δ200 Δost3::HIS3	this study
YG393	MATα ade2-101 ura3-52 his3Δ200 tyr1 stt3-6	(25)
YG469	MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δstt3::STT3-ProtA	(25)
YG493	MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δost4::kanMX4	(25)
YG535	MATa ade2-101 ura3-52 his3Δ200 leu2 lys2-801 stt3-7	(25)

Table II. Characterisation of selected oligosaccharytransferase mutations

subunit	MW (kDa)	mutation	high copy number suppression	phenotype of the mutation / suppressed mutation				references
				growth	glycosylation of CPY	OTase activity	complex/ subcomplex accumulating	
STT3	78	Δstt3		lethal	strongly reduced	strongly reduced	IV	(30) this study
		stt3-7		temperature sensitive	strongly reduced	strongly reduced	IV	(25) this study
		stt3-7	pOST3	improved[1]	improved[1]	ND	I, IV	(25) this study
		stt3-7	pOST4	improved[1]	improved[1]	ND	I, IV	(25) this study
OST1	65	ost1-4		temperature sensitive	reduced	reduced	IV	(22) this study
WBP1	45	Δwbp1		lethal	strongly reduced	strongly reduced	(III)	(27) this study
OST3	34	Δost3		normal	slightly reduced	reduced	Ib, II	(10)
		Δost3	pOST4	normal	not improved[1]	ND	Ia, II	this study
		Δost3	pOST6	normal	not improved[1]	ND	Ib, II	this study
OST6	31	Δost6		normal	normal	slightly reduced	Ia	(13) D.Bodmer, unpublished
SWP1	30	Δswp1		lethal	strongly reduced	strongly reduced	ND	(28)
OST2	16			lethal	strongly reduced	strongly reduced	ND	(21)
OST5	9	Δost5		normal	slightly reduced	reduced	ND	(17)
OST4	3.4	Δost4		temperature sensitive	strongly reduced	strongly reduced	II	(3) this study
		Δost4	pOST3	improved[1]	improved[1]	ND	Ia, II	(25) this study
		Δost4	pOST6	slightly improved[1]	not improved[1]	ND	Ib, II	this study

ND, not determined
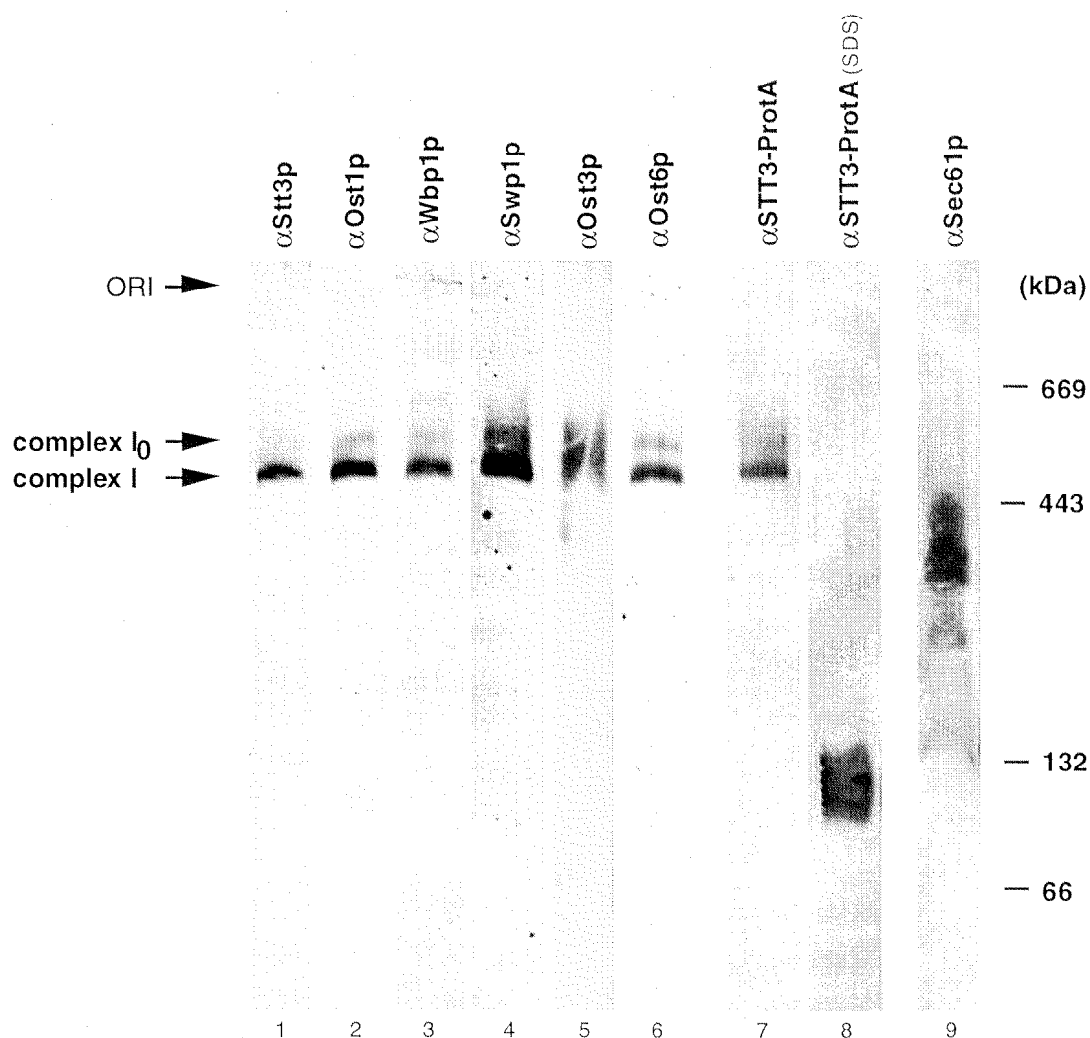[1] in relation to the strain without plasmid

Fig. 1 The wild-type yeast oligosaccharyltransferase complex (OTase) analyzed by blue native gel electrophoresis.

SS328 wild-type (lanes 1-6, 9) and YG469 cells (*STT3-ProtA*, lanes 7 and 8) were grown at 30°C. Membranes were prepared and proteins were solubilized with 1.5% digitonin or 2% SDS (lane 8). 40 µg of solubilized membrane proteins were analyzed by blue native gel electrophoresis using a 5-12% acrylamide gradient-gel. Proteins were blotted onto nitrocellulose and detected by specific antibodies (indicated above each lane) against different OTase subunits (Stt3p, Ost1p, Wbp1p, Swp1p, Ost3p, and Ost6p) (lanes 1-6), or PAP (a soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase) (lanes 7 and 8), or against the translocon component Sec61p (lane 9). The positions of marker proteins (albumin, momomeric, 66 kDa, and dimeric form, 132 kDa; apoferritin, 443 kDa; and thyroglobulin, 669 kDa) and the origin of the separating gel (ORI) are indicated. Wild type OTase complexes I and I_0 are indicated by arrows.
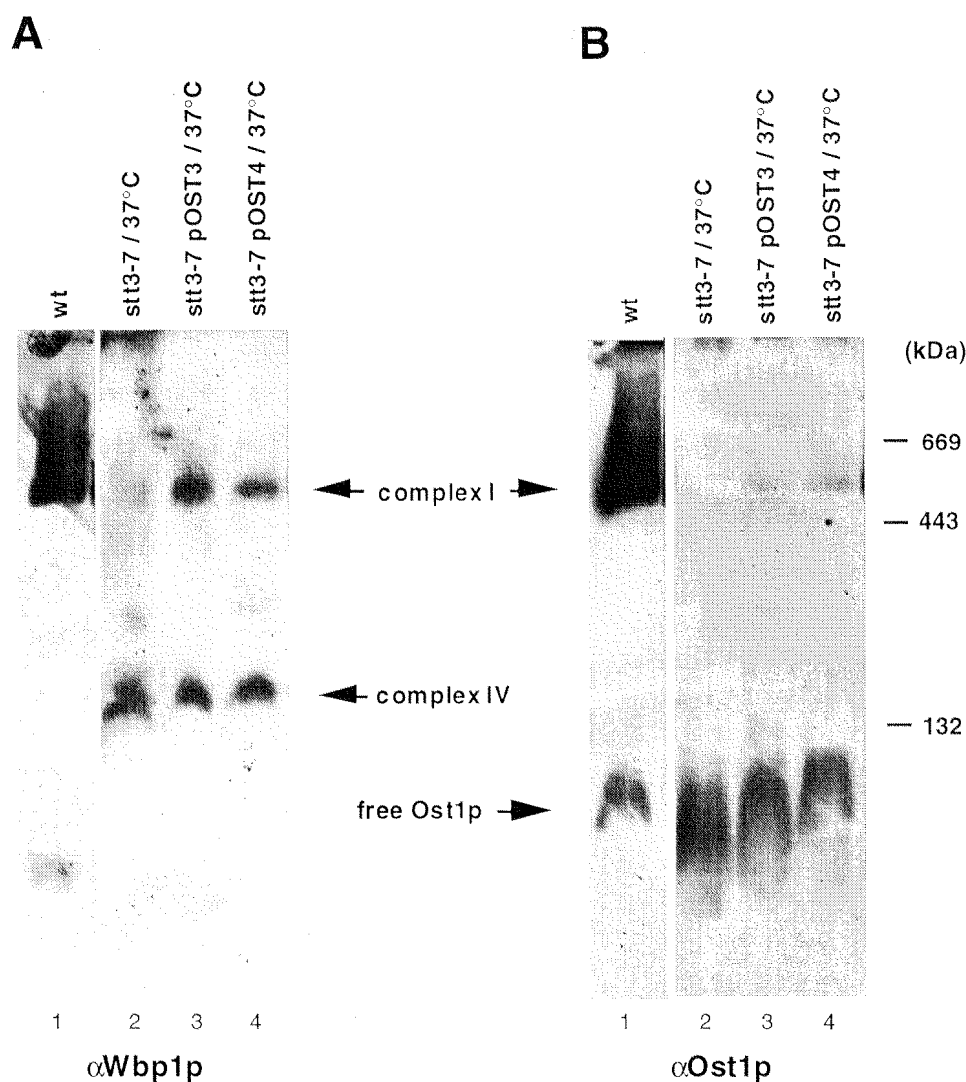
Fig. 2 Analysis of OTase complexes in wild-type and *stt3-7* mutant cells.
YG535 *stt3-7* cells (lane 2) and *stt3-7* cells harboring the high copy number plasmids pOST3 or pOST4 (lane 3 and 4) were grown in liquid medium at 23°C to mid-log phase and shifted to 37°C. Cells were harvested 9 hours after the shift, membranes were prepared and proteins were solubilized with 1.5% digitonin. Solubilized proteins (40 µg per lane) were analyzed by blue native gel electrophoresis using a 5-12% acrylamide gradient gel. Proteins were blotted onto nitrocellulose and detected by antibodies against Wbp1p (A) or Ost1p (B). SS328 wild-type cells grown at 30°C (wt, lane1) served as a control. The positions of complex I, subcomplex IV, as well as free Ost1p are indicated by arrows. Marker proteins are as in the legend to Figure 1.
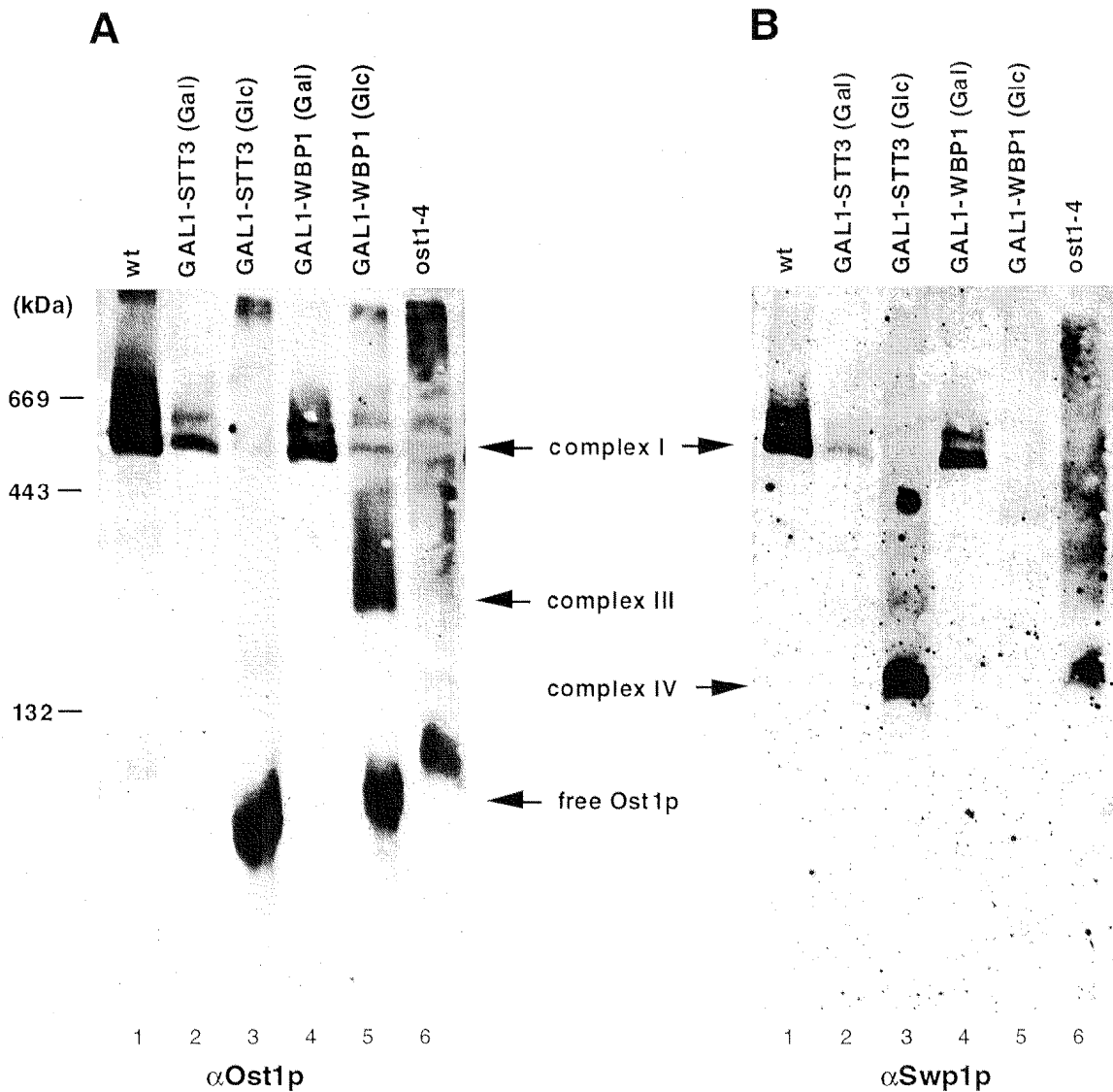
Fig. 3 Analysis of OTase complexes in Stt3p- and Wbp1p-depleted and *ost1-4* mutant cells.
SS328 wild-type cells (lane 1) and the *ost1-4* mutant strain RGY117 (lane 6) were grown in complete medium at 30°C. Strain YG157 in which the *STT3* gene was placed under the control of the galactose-inducible and glucose-repressible GAL1 promotor, and strain 45-C3, where *WBP1* expression was controlled by the GAL1 promotor, were grown at 30°C in galactose-containing medium (lanes 2 and 4) or glucose-containing medium for 11 hours (lanes 3 and 5). Membranes were prepared, proteins solubilized with 1.5% digitonin and analyzed by blue native gel electrophoresis. Immunodetection was performed using antibodies against Ost1p (**A**), Swp1p (**B**), Stt3p (**C**), and Wbp1p (**D**). The relevant genotype of the strains is indicated above each lane. Oligosaccharyltransferase complexes I, III, and IV as well as free Ost1p are indicated by arrows. Marker proteins are as in the legend to Figure 1.
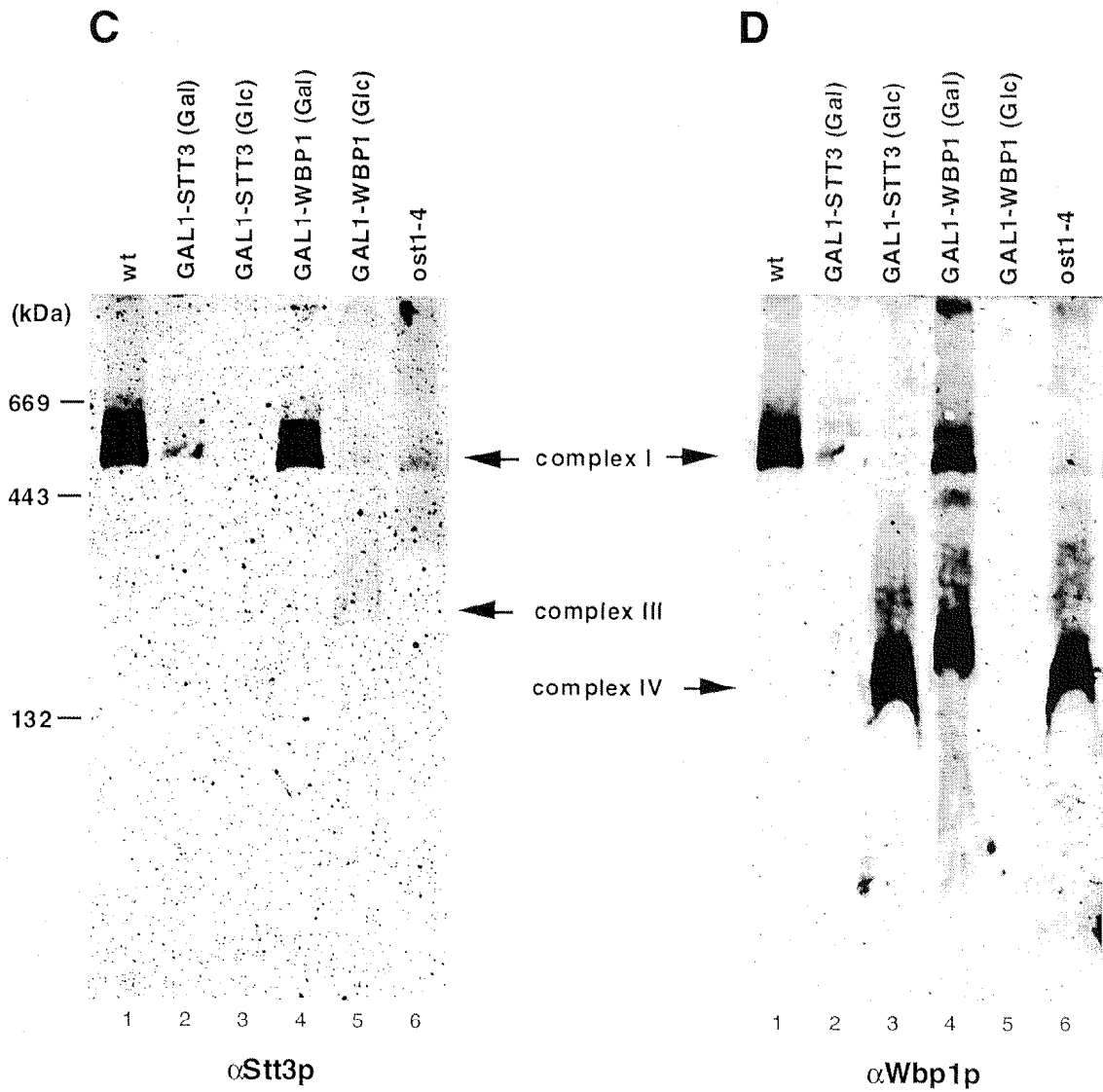
C

wt
GAL1-STT3 (Gal)
GAL1-STT3 (Glc)
GAL1-WBP1 (Gal)
GAL1-WBP1 (Glc)
ost1-4

(kDa)

669 —

443 —

132 —

1   2   3   4   5   6

αStt3p

D

wt
GAL1-STT3 (Gal)
GAL1-STT3 (Glc)
GAL1-WBP1 (Gal)
GAL1-WBP1 (Glc)
ost1-4

← complex I →

← complex III

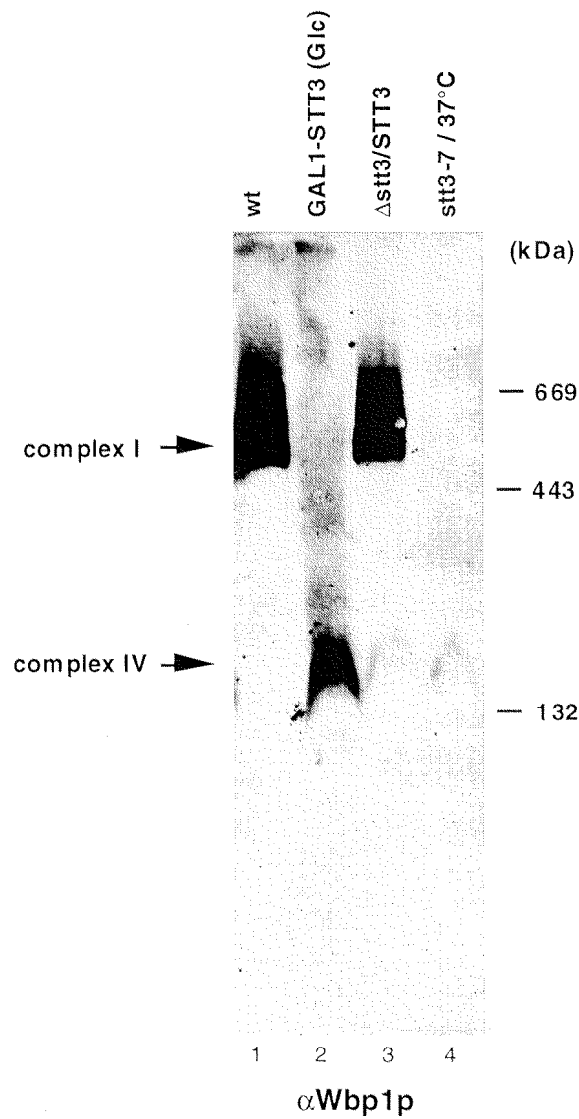complex IV →

1   2   3   4   5   6

αWbp1p

Fig. 3 (continued)

88

Fig. 4 Analysis of OTase complexes in a Δstt3 heterozygous diploid strain.
Membranes from the wild-type strain SS328 (lane 1), strain YG157 grown for 11 hours in glucose-medium (lane 2), the heterozygous Δstt3 strain YG156 (lane 3), and the temperature-sensitive stt3-7 mutant strain YG535 grown for 9 hours at 37°C (lane 4) were solubilized with 1.5% digitonin and proteins separated by blue native gel electrophoresis. Immunodecoration was performed using αWbp1p antibodies. Oligosaccharyltransferase complex I and complex IV are indicated by arrows. Marker proteins are as in the legend to Figure 1.
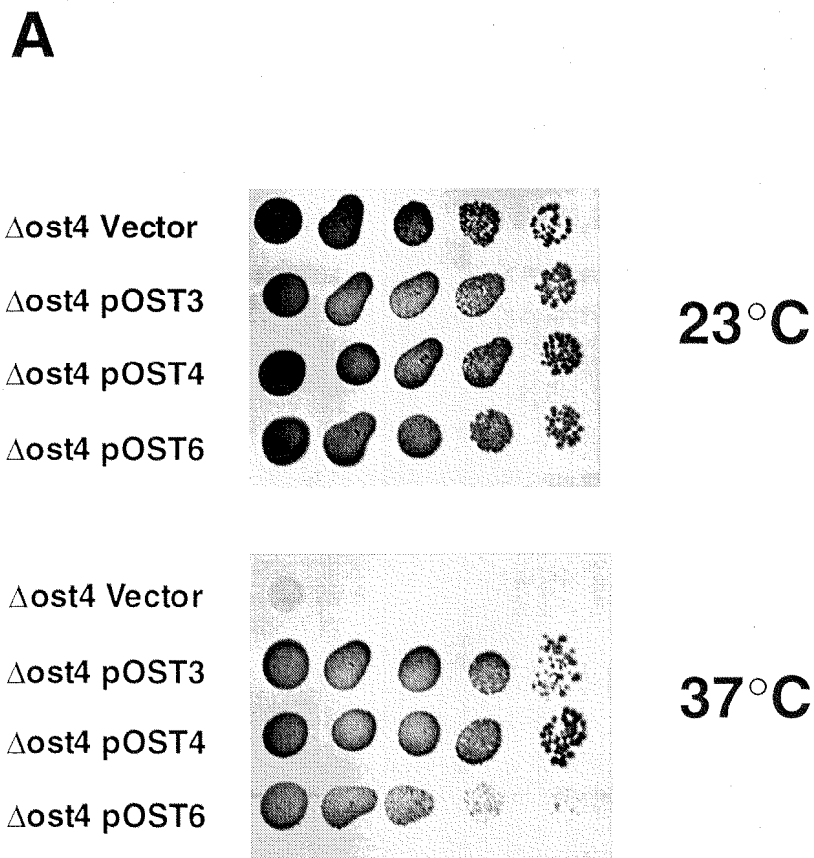
A

Δost4 Vector

Δost4 pOST3

Δost4 pOST4   **23°C**

Δost4 pOST6

Δost4 Vector

Δost4 pOST3

Δost4 pOST4   **37°C**

Δost4 pOST6

Fig. 5 *OST3* and *OST6* are high copy number suppressors of an *OST4* deletion
(A) YG493 (*Δost4*) cells harboring either the vector or the plasmid pOST3, pOST4, or pOST6 were grown at 23°C to mid-log phase in liquid minimal medium lacking uracil. Serial 1:10 dilutions starting at 5 x 10^5 cells were spotted onto plates containing minimal medium lacking uracil. The plates were incubated for 4 days at 23°C or 37°C and photographed.

(B) YG191(*Δost3*) and YG493 (*Δost4*) cells harboring either the vector, the plasmid pOST3, pOST4, or pOST6 were grown at 23°C to mid-log phase in liquid minimal medium lacking uracil. Wild-type SS328 cells were grown at 23°C to mid-log phase in complete medium. Protein extracts were prepared and Western blot analysis was performed using CPY-specific antibodies. The position of mature CPY (mCPY) and the glycoforms lacking one (-1), two (-2), three (-3), or four (-4) oligosaccharide units are indicated. The relevant genotypes of the different strains and the high copy number plasmids present are indicated.
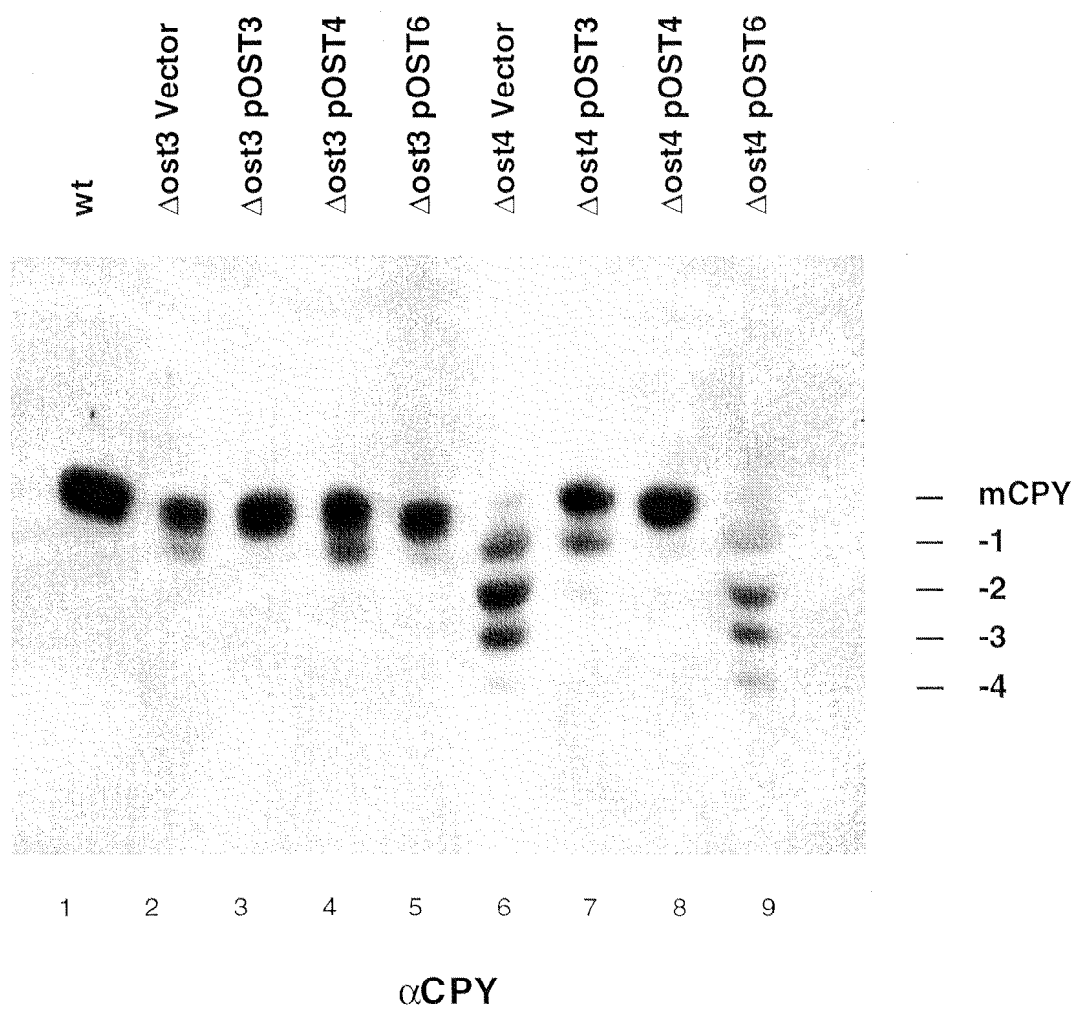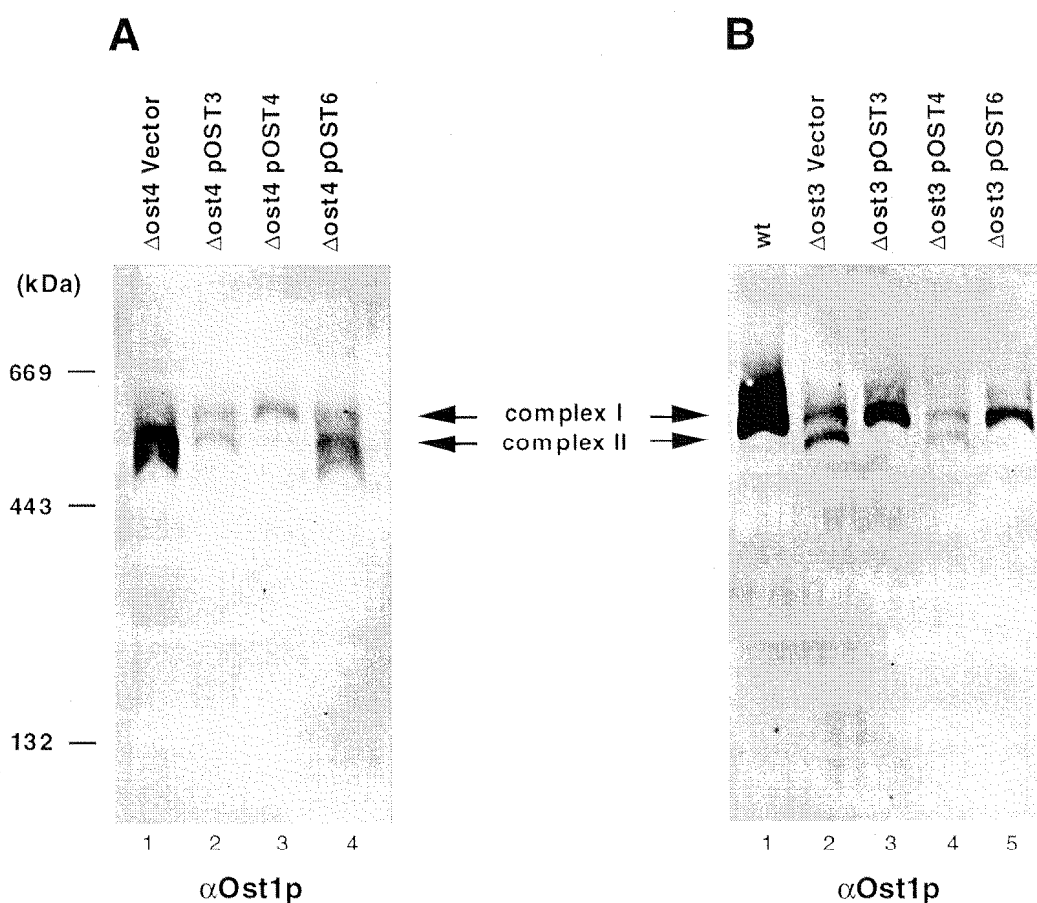
B

Fig. 5 (continued)

Fig. 6 Analysis of OTase complexes in *Δost3* **and** *Δost4* **cells**

SS328 wild-type cells (wt) were cultured in complete medium at 30°C to mid-log phase. YG191 *(Δost3)* and YG493 *(Δost4)* cells harboring either the vector or the high copy number plasmid pOST3, pOST4, or pOST6 were grown in liquid minimal medium lacking uracil at 30°C *(Δost3)* or at 23°C *(Δost4)* to mid-log phase. Cells were harvested, membranes prepared and proteins solubilized in 1.5% digitonin. The samples were analyzed by blue native gel electrophoresis in a 5-12% gradient gel, proteins were blotted onto nitrocellulose and detected with αOst1p antibodies (A and B), or αOst6p antibodies (C). The relevant genotypes of the different strains and the high copy number plasmids present are indicated above each lane. Oligosaccharyltransferase complexes I and II are indicated by arrows. Marker proteins are as in the legend to Figure 1.
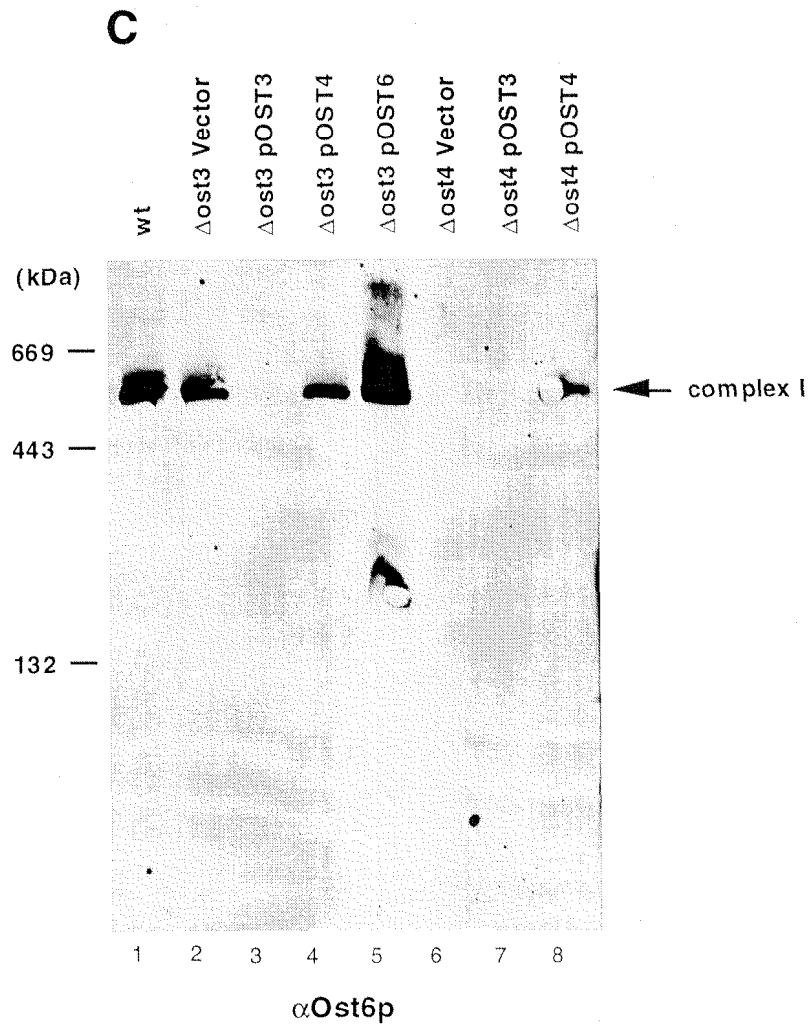
C

Fig. 6 (continued)

Addendum

Assembly of the yeast oligosaccharyltransferase complex *in vivo*

Based on the analysis of the OTase complex formation in wild-type and mutant cells, we were able to postulate a pathway for the assembly of the membrane-bound enzyme complex (Fig. 7). The smallest complex we were able to detect was complex IV. Based on biochemical and genetic data, we proposed that this complex contains Wbp1p, Swp1p, and Ost2p and was required for the assembly of other components. In a next step, Stt3p and Ost1p assembled onto complex IV to yield complex II. *stt3* and *ost1* mutant cells accumulated complex IV and we were not able to detect a complex containing either Stt3p or Ost1p in combination with complex IV components. Based on the observation that *OST5* is a high copy number suppressor of a specific *ost1* mutation (17) we postulated that Ost5p might be involved in the formation of complex II. However, we did not analyze the role of Ost5p experimentally. Ost4p was essential to complete the assembly of complex Ia and complex Ib, because Ost3p and Ost6p were not incorporated in the absence of Ost4p.

This model of OTase assembly was based on the analysis of stable subcomplexes accumulating in specific mutant strains with deficiencies in defined OTase components. Whether these subcomplexes represent actual intermediates in the biosynthesis of the complex remains to be determined, but several arguments support this assumption. It is likely that the assembly of such a complicated enzyme complex is a stepwise and directional process and not a one step event. Intermediates in this pathway are therefore required to be stable in the ER membrane, at least for a limited time. Subcomplex stability is also required to allow their detection in mutant cells. The observation that subcomplexes accumulating in mutant cells can be chased into completely assembled OTase also supports the assumption that they are intermediates in the assembly pathway: complex IV accumulating in *stt3-7* cells at non-permissive conditions was forced into fully assembled complex I by overexpression of *OST3* and *OST4*. Within the framework

of our hypothesis, the equilibrium between complex IV and complex I was shifted towards the fully assembled complex due to the increased level of either Ost3p or Ost4p which were part of complex I. Similarly, complex II in *Δost4* cells was chased into complex I by overexpressing either Ost3p or Ost6p.
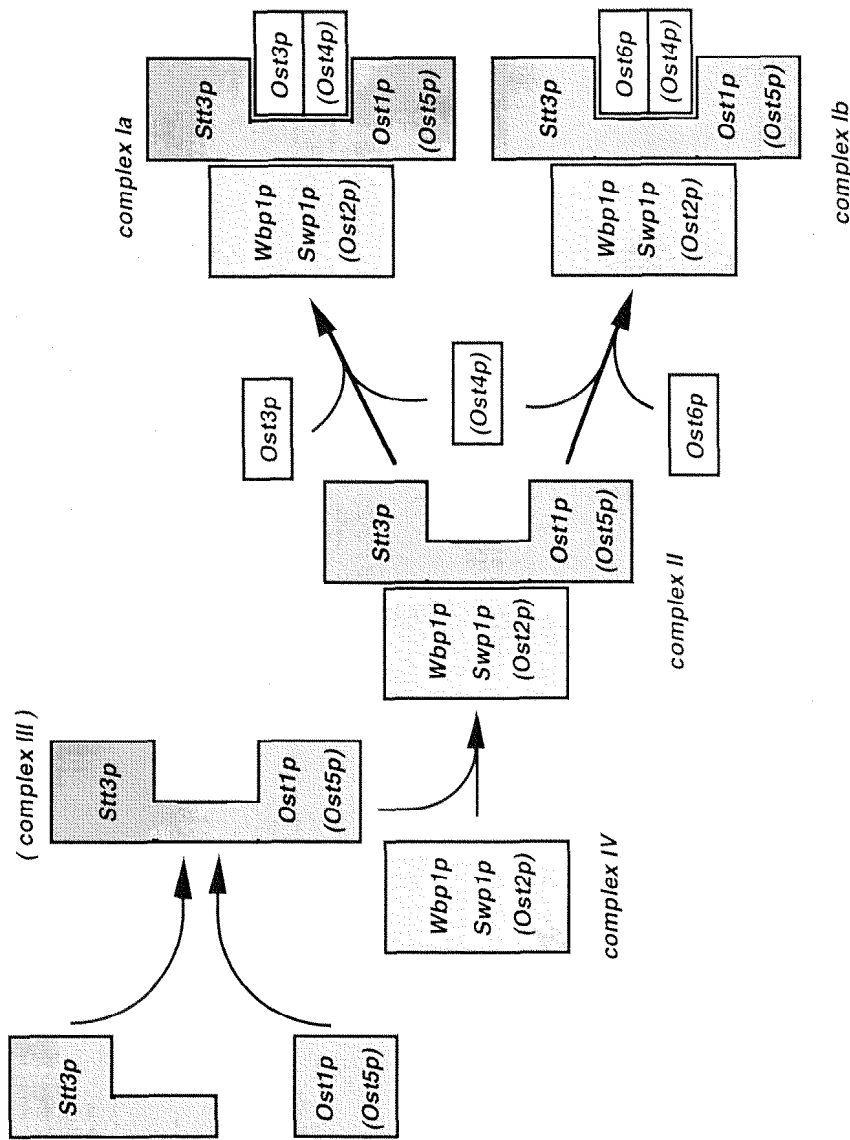
Fig. 7 A model for the *in vivo* assembly pathway of the yeast oligosaccharyltransferase complex.

The different components of the yeast oligosaccharyltransferase are shown in the various subcomplexes. Components that were not experimentally demonstrated to be present in the different complexes are shown in parenthesis. The different complexes identified by blue native gel electrophoresis are indicated by their corresponding number. Complex III is set in parenthesis because its existence was not unambiguously shown. The organization of the different subunits within the complex does not represent experimental findings but was chosen due to artistic reasons.

CHAPTER 4

Mutations in yeast oligosacchayltransferase complex assembly can be suppressed by inhibition of protein degradation

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to be submitted

Mutations in yeast oligosacchayl transferase complex assembly can be suppressed by inhibition of protein degradation

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suppression, degradation, assembly,

Saccharomyces cerevisiae

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ABSTRACT

In the process of N-linked glycosylation, the oligosaccharyltransferase (OTase) complex consisting of nine subunits catalyzes the transfer of a dolichylpyrophosphate-linked oligosaccharide onto selected asparagine residues of polypeptide chains in the lumen of the endoplasmic reticulum. Using the technique of blue native gel electrophoresis, it was recently shown that mutations in OTase subunits result in the accumulation of subcomplexes lacking defined subunits, suggesting a failure in complex assembly. Here, we showed that mutations in the Stt3p resulted in the degradation of non-assembled OTase components. Suppression analysis revealed different mechanisms of suppressing the mutant phenotype. Most importantly, suppression correlated with an increase in the steady-state levels of defined complex components. This could be achieved by overexpression of other OTase subunits. Furthermore, non-assembled subunits could be stabilized by directly influencing protein degradation by either overexpression or deletion of *UBC6*, a locus involved in the ubiquitin-dependent proteasomal pathway, or by deleting the *MNS1* locus that is involved in the ER-located N-linked oligosaccharide trimming. Mns1p was suggested to play a key role in targeting malfolded proteins for degradation.

INTRODUCTION

N-linked protein glycosylation in eukaryotes can be divided into three different processes: the assembly of the lipid-linked oligosaccharide (LLO) at the membrane of the endoplasmic reticulum, the transfer of the oligosaccharide to selected asparagine residues of the polypeptide chain by the oligosaccharyltransferase (OTase), and the modification of the protein-bound oligosaccharide in the ER and Golgi-apparatus.

The biosynthesis of the lipid-linked oligosaccharide begins with the addition of a N-acetylglucosamine residue to dolichylphosphate at the cytoplasmic side of the ER membrane (for review see (Burda and Aebi *in press*)). In *Saccharomyces cerevisiae*, this GlcNAc-PP-Dol synthase activity is encoded by the *ALG7* locus and is sensitive to the inhibitor tunicamycin (Rine *et al*. 1983). The oligosaccharide is build up further on the cytoplasmic side and, after a translocation across the ER membrane, on the lumenal side of the ER membrane. The completely assembled $GlcNAc_2Man_9Glc_3$ oligosaccharide is transferred *en bloc* to selected asparagine residues in the sequon Asn-X-Ser/Thr of polypeptide chains where X can be any amino acid except proline (Cummings 1992; Herscovics and Orlean 1993; Kornfeld and Kornfeld 1985; Tanner and Lehle 1987). This transfer is catalyzed by the enzyme complex oligosaccharyltransferase (for review see (Silberstein and Gilmore 1996)). Highly purified yeast oligosaccharyltransferase consists of at least six different subunits (Kelleher and Gilmore 1994). This complex is composed of Ost1p, which is homologous to ribophorin I of higher eukaryotic cells (Silberstein *et al*. 1995b), Wbp1p (OST48) (Silberstein *et al*. 1992; te Heesen *et al*. 1992), Swp1p (ribophorin II)

(Kelleher and Gilmore 1994; te Heesen *et al.* 1993), Ost2p (DAD1) (Silberstein *et al.* 1995a), Ost3p (Karaoglu *et al.* 1995), and Ost5p (Reiss *et al.* 1997).

Genetic screens have identified two other loci, *STT3* and *OST4*, that are required for full oligosaccharyltransferase activity *in vivo* (Chi *et al.* 1996; Zufferey *et al.* 1995). The *STT3* product is highly conserved in eukaryotes and is essential for vegetative growth of yeast cells. Depletion of Stt3p leads to a loss of OTase activity *in vivo* (Zufferey *et al.* 1995). Further experiments showed that Stt3p is indeed a structural component of the yeast oligosaccharyltransferase complex (Karaoglu *et al.* 1997; Spirig *et al.* 1997). Ost4p is a small hydrophobic protein of only 3.4 kDa. *OST4* deleted cells have a temperature-sensitive phenotype at 37° and a marked hypoglycosylation of N-glycoproteins (Chi, Roos and Dean 1996). There is genetic and biochemical evidence that Ost4p is a structural component of the mature OTase complex (Karaoglu, Kelleher and Gilmore 1997; Spirig *et al.* 1997). Recently, the Ost3p homologue Ost6p has been identified to be required for oligosaccharyltransferase activity *in vivo* (Knauer and Lehle 1997; Spirig *et al.* 1997) and it has been shown that it represents an additional subunit of the OTase complex (Spirig, Bodmer and Aebi *submitted*). Furthermore, it has been demonstrated that two different oligosaccharyltransferase complexes exist in yeast. The presence of Ost3p is characteristic for the more abundant complex, and Ost3p is replaced by Ost6p in the second complex. For the integration of both Ost3p and Ost6p into the complex, the small subunit Ost4p is required (Spirig, Bodmer and Aebi *submitted*).

After the transfer to the protein, the N-linked oligosaccharide (NLO) is subjected to trimming reactions involving glucosidase I, glucosidase II, and mannosidase I (Herscovics and Orlean 1993; Moremen *et al.* 1994; Roth 1995). It was suggested that

the relatively slow action of the ER mannosidase I sets the time frame for glycoproteins to fold correctly (Jakob *et al.* 1998). While properly folded glycoproteins can exit the ER, improperly folded glycoproteins are retained in the ER and are eventually degraded. In many cases, degradation occurs via the ubiquitin-proteasome pathway (Bonifacino 1996; Jentsch and Schlenker 1996; Kopito 1997; Sommer and Wolf 1997; Varshavsky 1997)

In this report, we describe that the phenotype of a mutant OTase can be rescued in different ways. High copy number suppression and searches for a synthetic phenotype are powerful tools for studying fundamental processes in yeast cells (Hartmann and Roth 1973; Jin *et al.* 1995; Kopski and Huffaker 1997; Reiss *et al.* 1997; Sommer and Jentsch 1993; Vallen *et al.* 1994). Suppressor analysis can yield insight into arrays of problems not readily subjected to more classic genetic experimentation. We show that the temperature-sensitive phenotype of a mutant OTase can be rescued either by influencing the lipid-linked oligosaccharide (LLO) biosynthesis, by overexpression of defined OTase subunits or other membrane proteins, by altering the protein degradation pathway, or by influencing the ER-localized N-linked oligosaccharide trimming.

MATERIALS AND METHODS

Yeast strains and media: Yeast strains are listed in Table 1. Standard yeast media and genetic techniques were used (Shermann 1991). G418-resistant strains were grown on yeast extract/ peptone/ dextrose (YPD) plates containing 200 mg/l G418 (Geneticin, GIBCO-BRL). Plates containing tunicamycin (Sigma) were adjusted to a final concentration of 3.0 μg/ml.

Isolation of *UBC6*, *ALG7* and *YET1*: The *UBC6* and the *ALG7* loci were isolated as high copy number suppressors of the temperature-sensitive phenotype of the Δost4 strain YG493. The *YET1* locus was isolated as a high copy number suppressor of the temperature-sensitive phenotype of the strain YG393 (*stt3-6*). The Yep352-bound library described by Fleischmann *et al.* (1991) was used.

Disruption of the *UBC6* locus: A 0.14 kb *BspEI-XbaI* fragment from the *UBC6* coding region of pUBC6 was replaced by a 1.76 kb *BamHI HIS3* cassette (Struhl and Davis 1981). The resulting plasmid was digested with *NarI* and *SacI*. The linear fragment was used to transform the wild-type strain SS328 to His$^+$. Correct replacement of the *UBC6* locus by the *HIS3* cassette was confirmed by whole cell PCR using *UBC6*- and *HIS3*-specific primers (Sathe *et al.* 1991).

Disruption of the *YET1* locus: The *YET1* gene was disrupted according to the PCR-based gene disruption method using the KanMX4 module (Wach *et al.* 1994). A linear DNA fragment was amplified from pFA6a-KanMX4 as template. The primers contain

17- and 18-bp sequences derived from pFA6a-KanMX4 at their 3' end and a 42-nucleotide sequence at the 5' end derived form *YET1*. The linear fragment was used to transform the wild-type diploid strain SS328 x SS330. Geneticin resistent transformants were colony purified, sporulated and subjected to tetrad dissection. Correct replacement of the *YET1* locus by the KanMX4 cassette was confirmed by whole cell PCR using *YET1*-and KanMX4-specific primers (Sathe *et al.* 1991), and the resulting strains were tested for temperature sensitivity, hygromycine sensitivity, and a hypoglycosylation phenotype.

Disruption of the YMR040W locus: PCR amplification from a plasmid carrying the *HIS3* gene gererated a 1kb *HIS3* cassette (Baudin *et al.* 1993) flanked by 35 bp stretches homologous to the YMR040W coding sequence. The linear fragment was used to transform the diploid strain heterozygous for an *YET1* deletion. His[+] transformants were colony purified, sporulated and subjected to tetrad dissection. Correct replacement of the YMR040W locus by the *HIS3* cassette was confirmed by whole cell PCR using YMR040W- and *HIS3*-specific primers, and the resulting singel and double knockout strains were tested for temperature sensitivity, hygromycine sensitivity, and a hypoglycosylation phenotype.

Western blot analysis of Ost3p, Ost6p, Stt3p, Hexokinase and CPY: Western blot analysis was performed as described for CPY by Burda *et al.* (Burda *et al.* 1996).

RESULTS

Identification of high copy number suppressors of *Δost4* and *stt3* mutations: Many different genetic techniques are available to identify gene products involved in cellular processes of the yeast *Saccharomyces cerevisiae*. These include, among others, second-site reversion analysis, synthetic lethal screens, and high copy number suppressor screens (Phizicky and Fields 1995). High copy number suppression screens can identify genes which encode proteins that serve a related function or interact with the originally mutated gene (Bender and Pringle 1989; Spirig *et al.* 1997). In some cases, suppression analysis can also uncover new relationships between gene products not previously predicted (Sommer and Jentsch 1993).

We performed two screens to identify high copy number suppressors of mutations affecting oligosaccharyltransferase activity. In the first screen, we searched for high copy number suppressors able to rescue the temperature-sensitive phenotype of an *OST4* deletion. *OST4* deleted cells grow poorly at 23° and show a strong hypoglycosylation of glycoproteins. The null mutant is inviable at 37° (Chi, Roos and Dean 1996). A yeast genomic DNA library bound to YEp352 (Fleischmann *et al.* 1991) was transformed into *Δost4* cells. Starting from about 50'000 Ura$^+$ transformants, 213 transformants were able to grow at 37°. After several rounds of retesting, 15 plasmids were found to be able to suppress the temperature-sensitive phenotype of the *OST4* deletion (Table 2A). PCR analysis using *OST4-*and *OST3*-specific primers, respectively, revealed nine plasmids containing the *OST4* locus and one plasmid containing the *OST3* locus (plasmid classes 1 and 2). Ost3p represents a non-essential, hydrophobic 34 kDa subunit of the yeast oligosaccharyltransferase complex (Karaoglu, Kelleher and

Gilmore 1995). It was shown previously that *OST3* is a high copy number suppressors of an *OST4* deletion (Spirig, Bodmer and Aebi *submitted*; Spirig *et al.* 1997). The genes of plasmid class 3-7 were identified by sequencing the ends of each insert and comparing the sequence with known sequences in the database. Interestingly, each plasmid contained at least one gene coding for a potential transmembrane protein (Table 2A). Plasmid 3 contained the *OST6* locus, a 31 kDa transmembrane protein showing similarity to Ost3p. Ost6p was recently shown to be part of the wild-type oligosaccharyltransferase complex (Knauer and Lehle 1997; Spirig, Bodmer and Aebi *submitted*). Plasmid 4 contained, among other open reading frames, the *ALG7* locus encoding the *N*-acetylglucosamine-1-P transferase (GPT) that catalyzes the initial step in the biosynthesis of the lipid-linked oligosaccharide in the endoplasmic reticulum. Plasmid 5 contained the *UBC6* locus coding for an 28.4 kDa integral ER-membrane protein that was shown to fuction as an ubiquitin-conjugating enzyme in the ubiquitin-proteasome protein degradation pathway (Sommer and Jentsch 1993). To demonstrate that suppression was conferred by *ALG7*, *OST6*, and *UBC6* respectively, these genes were cloned into the high copy vector Yep352 and the resulting plasmids were transformed into the *Δost4* strain. All the three plasmids were found to retain suppression activity. Plasmids 6 and 7 were not analyzed further.

In the second screen, we used cells harbouring a mutation in the *STT3* locus, encoding the largest OTase subunit. The *stt3-6* allele confers a temperature-sensitive phenotype at 30° and a reduced *in vitro* oligosaccharyltransferase activity, resulting in hypoglycosylation of glycoproteins (Spirig *et al.* 1997). The Yep352 bound genomic library was transformed into cells carrying the *stt3-6* mutation. Out of 80'000 Ura[+] transformants, more than 300 transformants were able to grow at 37°. 44 of them were

analyzed further and after retesting, 28 plasmids were able to rescue the temperature-sensitive phenotype of the *stt3-6* mutation (Table 2B). These plasmids were grouped into nine classes according to PCR and restriction digest analysis. PCR analysis using *STT3*- and *OST4*-specific primers, respectively, revealed two plasmids harbouring the *STT3* locus (plasmid class 1) and 12 plasmids containing the *OST4* locus (plasmid class 2). It was shown previously that *OST3* and *OST4* are high copy number suppressors of a *stt3* mutation (Spirig, Bodmer and Aebi *submitted*; Spirig *et al*. 1997). However, no plasmids containing the *OST3* locus were identified in this screen. The genes of at least one member of plasmid classes 3-9 were identified by sequencing the terminal regions of each insert and comparing the sequence with known sequences in the yeast database (Table 2B). Also the plasmids isolated in this screen all contained at least one open reading frame encoding a potential membrane protein. The plasmid of class 3 contained the *YET1* locus coding for a putative 23.4 kDa ER-transmembrane protein showing similarity to the murine B-cell receptor associated proteins BAP29 and BAP31 (Adachi *et al*. 1996; Kim *et al*. 1994). To show that the suppression was conferred by *YET1*, this gene was cloned into Yep352, the resulting plasmid transformed into *stt3-6* and indeed, the plasmid was found to retain suppression activity. The plasmid classes 4 to 9 were not analyzed further. The high copy number suppressors *ALG7*, *OST3* and *OST4*, *YET1*, and *UBC6* were analyzed in more detail and are discussed below.

Overexpression of Alg7p rescued the temperature-sensitive phenotype of the *OST4* deletion: The *ALG7* locus was isolated as a weak high copy number suppressor of the temperature-sensitive phenotype of an *OST4* deletion (Figure 1). The *ALG7* locus encodes an UDP-GlcNAc:dolichyl-phosphate GlcNAc-1-phosphate transferase (GPT)

that catalyzes the initial step in the biosynthesis of the lipid-linked oligosaccharide, namely the addition of a N-acetylglucosamine residue to dolichylphosphate on the cytoplasmic side of the ER membrane (Lehrman 1991). To test whether other ER glycosyltransferases involved in the assembly of the lipid-linked oligosaccharide were able to suppress the *OST4* deletion phenotype, we transformed other high copy plasmids carrying either *ALG3* (Aebi *et al.* 1996), *ALG5* (te Heesen *et al.* 1994), *ALG6* (Reiss *et al.* 1996), *ALG8* (Stagljar *et al.* 1994), *ALG9* (Burda, te Heesen and Aebi 1996), or *ALG10* (Nikawa and Hosaki 1995) into the *Δost4* strain. However, only overexpression of Alg7p was able to partially suppress the temperature-sensitive phenotype of the *OST4* deletion (Figure 1; for *ALG5, ALG6, ALG10:* data not shown). To demonstrate that the *Δost4* cells overexpressing Alg7p indeed had increased levels of functional *ALG7* enzyme, we tested these cells for increased resistance towards tunicamycin. Tunicamycin is a competitive inhibitor of GPT encoded by the *ALG7* locus, and GPT overexpressing cells have an increased resistance towards this drug (Hartog and Bishop 1987). *Δost4* cells were hypersensitive towards this drug, most probably due to the combined effect of reduced lipid-linked oligosaccharide biosynthesis (tunicamycin) and reduced OTase activity (*Δost4*). Importantly, the multicopy plasmid carrying the *ALG7* locus suppressed this sensitivity and these cells were even more resistant than *OST4* wild-type cells (*Δost4*+pOST4) (Figure 2). We concluded that overexpression of the *ALG7* protein resulted in an increased level of functional GPT enzyme. The fact that the *ALG7* protein was the only one of the *ALG* loci tested so far that suppressed the temperature-sensitive phenotype of an *OST4* deletion indicated a specific role of this enzyme in the biosynthetic pathway of the lipid-linked oligosaccharide. We speculated that the overexpression of the enzyme catalyzing the first step in LLO biosynthesis

resulted in an increased level of the lipid-linked core oligosaccharide and subsequently an improved glycosylation in *Δost4* cells.

Overexpression of Ost3p and Ost4p rescued the temperature-sensitive phenotype of a *stt3* mutation and correlated with increased steady state-levels of the mutant *stt3* protein: Plasmids carrying the *OST4* locus were the most frequently isolated high copy number suppressors of the *stt3-6* mutation (Table 2b). It has previously been shown that different conditional *stt3* alleles can be suppressed by overexpression of either *OST3* or *OST4* (Spirig *et al.* 1997) (Figure 3A). Overexpression of specific subunits can result in an increased level of fully assembled OTase complex (Spirig, Bodmer and Aebi *submitted*). It was argued that the high level of one subunit of the OTase complex can shift the equilibrium between free complex components and fully assembled complex towards the latter, resulting in an elevated level of OTase complex. This hypothesis implied that this type of suppression resulted in an increased steady-state level of the mutant OTase subunit. Therefore, we analyzed the steady-state levels of a subset of OTase subunits, namely Ost3p, Ost6p, and Stt3p in *stt3* cells. We also tested the glycosylation pattern of CPY in these cells. Indeed, the steady-state levels of mutant Stt3p, Ost3p and Ost6p as well as CPY glycosylation were significantly reduced as compared with the wild-type cells (Figure 3B). We have shown previously that Stt3p is required for the incorporation of both Ost3p and Ost6p into the complex (Spirig, Bodmer and Aebi *submitted*). We postulated that the *stt3-7* mutation resulted in an impaired assembly of the OTase complex and a subsequent degradation of the non-incorporated subunits, leading to a reduced glycosylation capacity *in vivo*. Overexpression of either Ost3p or Ost4p not only resulted in a suppression of the

temperature-sensitive phenotype (Figure 3A) but also in an increased steady-state level of both Stt3p and Ost3p. Ost6p was only detectable in *stt3-7* cells overexpressing Ost4p (Figure 3B). We concluded that overexpression of specific OTase subunits resulted in increased steady-state levels of other OTase subunits. Stabilisation of mutant OTase subunits led to a suppression of the temperature-sensitive phenotype and an increased glycosylation of glycoproteins.

YET1 is a high copy number suppressor of a temperature-sensitive *stt3* mutation and increases the steady-state level of defined OTase subunits in these cells: We isolated the *YET1* locus as a multicopy suppressor of the temperature-sensitive *stt3-6* mutation. *YET1* stands for yeast ER membrane protein twenty-four kDa (N. Fatal, personal communication). This gene encodes a putative ER-transmembrane protein with 206 amino-acid residues and an estimated molecular weight of 23.4 kDa. The gene has two homologues in yeast (*YDL072c, YMR040w*) as well as a human (*BAP31*) and two murine homologues (*BAP29, BAP31*) (Figure 4A and 4B). The primary sequence similarity of these proteins is spread out over the whole sequence, ranging from 53% amino-acid similarity between the yeast homologues Yet1p and Ymr040wp, to 30% similarity between Yet1p and the murine homologue BAP29 (Figure 4B) as analyzed by the Jotun and Hein method. Hydrophobicity analysis of these proteins revealed a very similar overall structure, suggesting three transmembrane domains as well as a C-terminal hydrophilic extension. In addition, all proteins, except the yeast *YDL072c* protein, have a KKXX ER-retrieval signal, suggesting ER-localisation of these proteins (Gaynor *et al.* 1994). Transformation of the Yep352 plasmid containing *YET1* into *stt3-4, stt3-5,* and *stt3-7* cells revealed that overexpression of Yet1p also suppressed these

stt3 alleles (data not shown). Suppression of the temperature-sensitive phenotype of the *stt3-7* mutation was observed at 30° but not at 37° (Figure 3A). Interestingly, expression of Yet1p from a multicopy plasmid also resulted in an improved glycosylation efficiency in *stt3-7* cells as shown for the vacuolar glycoprotein CPY (Figure 3B), however to a lesser extent than did overexpression of either Ost3p or Ost4p. We asked whether overexpression of Yet1p also influenced the steady-state level of Ost3p, Ost6p, and Stt3p as was observed for the multicopy suppressors *OST3* and *OST4*. Indeed, *stt3-7* cells overexpressing Yet1p showed increased steady-state levels of these proteins as compared to *stt3-7* cells harboring the vector (Figure 3B).

Disruption of the *YET1* locus did not result in a growth phenotype and the *Δyet1* deletion did not alter the efficiency of N-linked protein glycosylation (data not shown). Also no specific phenotype of the *Δyet1* mutation was observed when present in *stt3-4*, *stt3-6*, *stt3-7*, *Δost3*, and *wbp1-1* strains. We also constructed a deletion of the *YET1* homologue *YMR040w*, but also no phenotype could be attributed to the double mutant. Also triple mutant strains carrying in addition to the *YET1* and the *YMR040w* deletion the *stt3-7*, *Δost4*, *wbp1-1*, or the BiP mutation *kar2-203* showed no specific phenotype (data not shown). We concluded that Yet1p had no direct function in oligosaccharyltransferase activity, but its overexpression stabilized the mutant *stt3-7* protein, resulting in a suppression of the temperature-sensitive phenotype.

The temperature-sensitive *OST4* deletion and the *stt3-7* mutation were rescued either by overexpression of *UBC6* or by the *UBC6* deletion: It was previously reported that overexpression of *UBC6* and a deletion of this locus both suppressed a *sec61* mutation (Sommer and Jentsch 1993). The *UBC6* protein is a highly conserved

integral ER-membrane protein with a molecular weight of 28.4 kDa. It functions as an

ubiquitin-conjugating enzyme, the catalytic activity facing the cytosol. *UBC6* null

mutants are viable and grow at wild-type rates (Sommer and Jentsch 1993). We isolated

the *UBC6* locus in the screen for loci able to suppress the temperature-sensitive

phenotype of *Δost4*. However, high copy expression of Ubc6p also resulted in a weak

suppression of the temperature-sensitive *stt3-7* mutation (Figure 5A). We therefore

checked whether a disruption of this gene could also suppress the temperature-sensitive

phenotype of the *stt3-7* mutation. The disruption of this non-essential gene indeed

rescued the temperature-sensitive phenotype of *stt3-7* mutant cells (Figure 5A). It is

well-known that the ubiquitin-proteasomal pathway plays a major role in degrading

mutated or incorrectly folded proteins. We therfore asked if the improvement of growth

of the *stt3-7* mutant cells by either overexpressing Ubc6p or deleting *UBC6* correlated

with an increased steady-state level of the OTase subunits Ost3p, Ost6p, and Stt3p in

these cells. Indeed, the steady-state levels were increased by either overexpressing

Ubc6p or deleting the *UBC6* locus (Figure 5B). In addition, glycosylation efficiency

was slightly improved in *stt3-7* cells by either overexpression of *UBC6* or the presence

of *Δubc6* (Figure 5B). We concluded that the *UBC6* protein is involved in the

degradation of the mutant *stt3-7* protein. Reduction of this degradation resulted in an

increased level of this protein and therfore a partial suppression of the temperature-

sensitive phenotype.

Degradation of the mutant form of the glycoprotein Stt3p was controlled by

oligosaccharide trimming: In *Saccharomyces cerevisiae*, the transfer of the fully

assembled oligosaccharide onto the polypeptide chain is immediately followed by

trimming reactions catalyzed by ER-localized glycosidases. The removal of the terminal

α1,2 glucose and the α1,3 glucose by glucosidase I and glucosidase II respectively,

occurrs rapidly, whereas mannose cleavage by mannosidase I (Mns1p) is slow, setting

the time frame for the glycoprotein to fold correctly (Jakob *et al*. 1998). Studies on

misfolded carboxypeptidase Y (CPY*) showed that the degradation of this mutated

soluble protein is dependent on the specific mannosidase trimming step, as CPY* was

stabilized in a *Δmns1* background (Jakob *et al*. 1998; Knop *et al*. 1996). As shown

above, deleting one component of the ubiquitin-dependent proteasomal pathway

(Ubc6p) suppressed the temperature-sensitive phenotype of a *stt3* mutation. We

therefore asked whether the degradation of the mutated form of the Stt3p, a

glycoprotein with multiple transmembrane domains (Zufferey *et al*. 1995), was also

controlled by oligosaccharide trimming and would result in a suppression of the

temperature-sensitive phenotype. We crossed the temperature-sensitive *stt3-7* strain

with a strain carrying a deletion of the *MNS1* locus and tested the resulting double

mutant *stt3-7 Δmns1* for growth at elevated temperatures. Indeed, a deletion of the

MNS1 locus was able to partially suppress the growth phenotype of *stt3-7* mutant cells

at 30° (Figure 6A). Interestingly, the temperature-sensitive phenotype of *Δost4* cells

was not rescued in a *Δmns1* background (data not shown). We determined the steady-

levels of Stt3p, Ost3p, and Ost6p in the *stt3-7* mutant cells as well as in *stt3-7* cells with

a disruption of the *MNS1* locus. A small but reproducible increase in *stt3-7* protein

levels was observed, whereas no detectable improvement of *OST3* protein and *OST6*

protein levels was seen in *stt3-7 Δmns1* cells as compared to the *stt3-7* cells (Figure

6B). This slight increase of the steady-state level of the mutant *stt3* protein correlated

with a slight improvement of the glycosylation pattern of CPY (Figure 6B). Our results

demonstrated that the trimming of the oligosaccharide structure specifically affected the degradation of the mutant *stt3-7* membrane protein.

DISCUSSION

The OTase complex is located in the ER membrane and is composed of several membrane proteins. This membrane-bound complex served as a model system to study suppressors that are able to rescue the temperature-sensitive phenotype of a mutant enzyme complex.

We performed two high copy number suppressor screens to isolate genes whose overexpression rescued the temperature-sensitive phenotype of a mutant OTase complex. In the first screen we used yeast cells with a deletion of the *OST4* locus, in the second screen cells carrying a mutation in the Stt3p subunit were used. Both mutations result in an incompletely assembled OTase complex. Using the blue native gel electrophoresis technique, a powerful tool to analyze protein and protein complexes under native conditions and according to their molecular weight, we showed previously that in *OST4* deleted cells as well as in *stt3-7* cells, defined OTase subcomplexes accumulated which could partially be shifted to wild type OTase complex upon overexpressing of either Ost3p or Ost6p (Schägger and von Jagow 1991; Spirig, Bodmer and Aebi *submitted*). In this study, we showed that in *stt3-7* cells, Ost3p and Ost6p as well as the mutant *stt3* protein was degraded. The finding that a mutation in an OTase subunit led to degradation of other subunits has also recently been reported as a

result of a mutation in the *DAD1* subunit of the mammalian OTase complex (Sanjay *et al.* 1998).

Three specific categories of high-copy suppressors able to rescue the temperature-sensitive phenotype induced by an incomplete assembled OTase complex were analyzed. In a first category, we found the *ALG7* locus most probably affecting the substrate level of the OTase. A second category of high-copy suppressors included genes encoding subunits of the OTase complex itself: *OST3* and *OST4* as high copy suppressors of different *stt3* alleles (Spirig *et al.* 1997; this study), and *OST3* as a high-copy number suppressor of *Δost4* (Spirig *et al.* 1997). A third category of high copy number suppressors was found to affect stabilization and/or degradation of OTase subunits.

The *ALG7* locus was isolated as a high copy number suppressor of the temperature-sensitive phenotype of an *OST4* deletion. The *ALG7* protein catalyzes the first step in the biosynthetic pathway of the lipid-linked oligosaccharide, namely the addition of an N-acetylglucosamine residue to dolichylphosphate on the cytosolic side of the ER membrane (Lehrman 1991). This GlcNAc-PP-Dol synthase activity can be inhibited competitively by tunicamycin (Hartog and Bishop 1987). Interestingly, Alg7p was the only tested ER glycosyltransferase involved in the assembly of the lipid-linked oligosaccharide able to suppress the temperature-sensitive phenotype of an OTase deficient in Ost4p (Figure 1), suggesting a defined role of this enzyme in the biosynthetic pathway of the lipid-linked oligosaccharide. It is possible that the *ALG7* protein, as the first enzyme in the pathway, plays a key role in the regulation of the lipid-linked oligosaccharide biosynthesis: insufficient quantities of GlcNAc-PP-Dol will

diminish the overall extent of lipid-linked oligosaccharide biosynthesis resulting in a reduced N-linked glycosylation capacity. There are datas indicating that Alg7p is indeed regulated in order to fulfill the cellular requirements for glycoprotein biosynthesis. The enzyme is activated by dolichol-P-mannose, ensuring that sufficient Dol-P-Man is available to form the complete oligosaccharide structure containing nine mannose residues. In addition, the Alg7p activity is modulated by the phospholipid composition of the membrane (Lehrman 1991). Our finding that overexpression of Alg7p could rescue the temperature-sensitive phenotype of an *OST4* deletion might be explained by an expanded pool of lipid-linked oligosaccharide in these cells overcoming the deficient OTase complex. This suggests that feedback-inhibition is not or incompletely functional in this pathway.

We showed that overexpression of either Ost3p or Ost4p in *stt3-7* cells resulted in an increased steady-state level of both Stt3p and Ost3p as compared to *stt3-7* cells harbouring the vector (Figure 3B). While the steady-state levels of Ost6p are increased in *stt3-7* cells overexpressing Ost4p as compared to *stt3-7* cells harbouring the vector, in *stt3-7* cells overexpressing Ost3p the steady-state levels of Ost6p are not increased (Figure 3B). We postulate that overexpression of either Ost3p or Ost4p allows the assembly of the mutant *stt3* protein into the complex by shifting the assembly equilibrium towards fully assembled complex, resulting in increased steady-state levels of the mutant protein, that otherwhise, if not incorporated into the complex, would be degraded. This hypothesis is in good agreement with the finding that the OTase subcomplex accumulating in *stt3-7* cells, harbouring Wbp1p and Swp1p but not Stt3p, Ost3p, and Ost6p, was chased into wild-type complex upon overexpression of either

Ost3p or Ost4p (Spirig, Bodmer and Aebi *submitted*). The finding that in *stt3-7* cells overproducing Ost3p, the steady-state level of the *OST6* protein was lower than in *stt3-7* cells overproducing Ost4p confirmed our recent finding that two different OTase complexes exist in the yeast *Saccharomyces cerevisiae*. These two OTase complexes share a set of proteins, but differ in the presence of either Ost3p (OTase complex Ia) or Ost6p (OTase complex Ib) (Spirig, Bodmer and Aebi *submitted*). We speculated that overexpression of Ost3p could chase the *OST6* protein out of the OTase complex, resulting in the accumulation of only complex Ia. The free Ost6p was subsequently degraded. Overexpression of specific OTase subunits was able to suppress the temperature-sensitive phenotype of *stt3* and *Δost4* mutants. We postulated that this overexpression shifted the equilibrium of complex formation towards the fully assembled, functional complex. Besides the OTase complex , there are other membrane-bound multi-protein complexes in which one subunuit can act as a high-copy number suppressor to rescue the temperature-sensitive phenotype of a mutation in another subunit: the small *SOM1* gene is a suppressor of a mutation in the *IMP1* locus which is a subunit of the mitochondrial inner membrane peptidase complex of *Saccharomyces cerevisiae* (Esser *et al.* 1996). In addition, the temperature-sensitive phenotype of a yeast strain harbouring a mutation in the *SEC61* locus whose gene product is a subunit of the membrane-bound translocation pore is suppressed by overexpression of Sss1p, another component of the translocation pore, and the mutated Sec61p was stabilized by Sss1p overproduction (Esnault *et al.* 1994).

In line with this hypothesis, we isolated suppressors that resulted in increased steady-state levels of both mutant and wild-type OTase components, however, these genes were found not to be necessary for OTase activity *in vivo*.

The *YET1* gene was isolated in a high copy number suppressor screen for the temperature-sensitive phenotype of the *stt3-6* mutation. The gene encodes an ER transmembrane protein with a molecular weight of 23.4 kDa. For this gene, two homologues in yeast (*YDL072c, YMR040w*) as well as a human (*BAP31*) and two murine homologues (*BAP29, BAP31*) can be found in the database. The hydrophobicity plots of the corresponding proteins show a similar overall structure (Figure 4A). The Yet1p has 30% sequence similarity to the murine BAP29 (Figure 4B). Overexpression of the *YET1* protein resulted in increased steady-state levels of Ost3p, Ost6p, and Stt3p (Figure 3B). Several crosses between a *YET1* knockout strain, the *YET1* homologue *YMR040w* deleted strain, and strains mutated in different OTase subunits showed no synthetic interaction, indicating that this protein is not a part of the mature OTase itself. One possible explanation for these findings is that the *YET1* protein may act as an assembly factor required for the incorporation of OTase subunits into the complex, although it itself is not part of the mature complex. On the other hand, Yet1p may stabilize free OTase subunits and prevent their degradation.

A direct role of protein degradation in the assembly of functional OTase complex was provided by the fact that the *UBC6* gene was isolated as a high copy number suppressor of the temperature-sensitive phenotype of a disrupted *OST4* gene. Overexpression of Ubc6p was also able to rescue the temperature-sensitive phenotype of the *stt3-7* mutation (Figure 5A), indicating that the rescue of the temperature-sensitive phenotype by overexpressing Ubc6p was not restricted to an OTase complex lacking Ost4p. The *UBC6* gene encodes an ubiquitin-conjugating enzyme localized in the ER membrane, the catalytic domain facing the cytosol (Sommer and Jentsch 1993). Overexpression of

Ubc6p as well as a disruption of the *UBC6* locus in *stt3-7* cells increased the steady-state levels of Ost3p, Ost6p, and Stt3p, as compared to *stt3-7* cells (Figure 5B). Moreover, in *stt3-7 Δubc6* cells, there was a slight improvement of the glycosylation of CPY as compared to *stt3-7* cells (Figure 5B). We suggested that unassembled subunits are degraded via the ubiquitin-dependent proteasomal pathway. By inhibiting this degradation pathway, steady-state levels of free subunits increased and the assembly equilibrium was therefore shifted towards fully assembled OTase complex.

A similar result was reported previously on the suppression of a temperature-sensitive *sec61* mutation by either overproducing Ubc6p or by deleting the *UBC6* locus (Sommer and Jentsch 1993). Like Stt3p, Sec61p is a component of a protein complex, the translocon, located in the ER membrane (Deshaies and Schekman 1987). The puzzling finding that either overproduction of Ubc6p or deleting the *UBC6* locus suppressed the mutant phenotype was explained by the need of some ubiquitin-conjugating enzymes to form a complex with specific substrate recognition (E3) proteins in order to be active on certain substrates. Overproduction of Ubc6p therefore sequestered a limiting E3 from the substrate, leading to a reduced ubiquitination capacity of substrates (Sommer and Jentsch 1993). Our finding that by either overexpressing Ubc6p or deleting the *UBC6* locus, increased levels of the glycoprotein Stt3p were detected, showed that ER-membrane glycoproteins are degraded by the ubiquitin-dependent proteasomal pathway.

Work from this laboratory indicated that the degradation of malfolded carboxypeptidase Y, a soluble glycoprotein, was regulated by the trimming of the N-linked oligosaccharide (Jakob *et al.* 1998). It was suggested that the $Man_8GlcNAc_2$ oligosaccharide represents a signal for degradation of malfolded proteins. We showed

that the deletion of the *MNS1* locus inactivating α-1,2 mannosidase and resulting in a Man$_9$GlcNAc$_2$ oligosaccharide structure, also resulted in a partial suppression of the *stt3-7* mutation (Figure 6A). However, the temperature-sensitive phenotype of *Δost4* cells was not rescued in a *Δmns1* background (data not shown). Interestingly, the steady-state levels of the glycoprotein Stt3p in *stt3-7 Δmns1* cells was slightly increased as compared to *stt3-7* cells, whereas the steady-state levels of the two unglycosylated proteins Ost3p and Ost6p was not affected (Figure 6B). Furthermore, a slight improvement of CPY glycosylation could be detected in *stt3-7 Δmns1* cells as compared to *stt3-7* cells, indicating an improved oligosaccharyltransferase activity *in vivo* (Figure 6B). We concluded that the degradation of the mutant form of the oligosaccharyltransferase component Stt3p, a glycoprotein with multiple transmembrane domains, was controlled by oligosaccharide trimming. Based on the observation that, in contrast to the *Δubc6* deletion, the *Δmns1* mutation did not suppress the *Δost4* deletion nor did it affect the steady-state levels of both Ost3p and Ost6p, lead us to conclude that degradation of ER membrane proteins occurred via the ubiquitin-dependent proteasomal pathway. In the case of membrane glycoproteins, this degradation might be regulated by the trimming of the protein bound oligosaccharide. That this suppression of the *stt3-7* mutation is not an unspecific effect is shown by the finding that the temperature-sensitive phenotype of *Δost4* cells was not suppressed in a *Δmns1* background (data not shown). We suggest that the degradation of ER membrane glycoproteins is initiated by the same signal as the degradation of soluble, malfolded glycoproteins.

Our results obtained from the analysis of high-copy number suppressors of mutations in oliosaccharyltransferase subunits has revealed different mechanisms of suppression. Most importantly, suppression of mutations affecting the assembly of the oligosaccharyltransferase complex was achieved by increasing the steady-state level of complex components, either by overexpression of one of these components or by affecting the degradation of specific components. This resulted in an elevated level of a fully assembled OTase complex. Therefore, expression- and degradation-rates of single subunits determined the level of a fully assembled OTase complex. Our collection of different mutants and suppressors will make it possible to study the assembly of this complex in more detail.

ACKNOWLEDGEMENTS

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Table 1. Yeast strains used in this study

Strain	Genotype	Reference
SS328	MATα ade2-101 ura3-52 his3Δ200 lys2-801	(Vijayraghavan et al. 1989)
SS328xSS330	MATa/α ade2-101/ ade2-101 ura3-52/ ura3-52 his3Δ200/ his3Δ200 tyr1/+ lys2-801/+	(Vijayraghavan et al. 1989)
YG393	MATα ade2-101 ura3-52 his3Δ200 tyr1 stt3-6	(Spirig et al. 1997)
YG493	MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δost4::kanMX4	(Spirig et al. 1997)
YG519	MATa ade2-101 ura3-52 his3Δ200 tyr1 Δyet1::kanMX4	this study
YG520	MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δyet1::kanMX4 ΔS52889::HIS3	this study
YG521	MATα ade2-101 ura3-52 his3Δ200 lys2-801 ΔS52889::HIS3	this study
YG543	MATa ade2-101 ura3-52 his3Δ200 lys2-801 leu2 stt3-7	this study
YG746	MATa ade2-101 ura3-52 his3Δ200 tyr1 Δmns1::kanMX4	(Jakob et al. 1998)
YG885	MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δubc6::HIS3	this study
YG894	MATa ade2-101 ura3-52 his3Δ200 lys2-801 leu2 stt3-7Δubc6::HIS3	this study
YG950	MATα ade2-101 ura3-52 his3Δ200 tyr1 stt3-7 Δmns1::kanMX4	this study
YG951	MATa ade2-101 ura3-52 his3Δ200 tyr1 lys2-801 stt3-7 Δmns1::kanMX4	this study
YG957	MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δmns1::kanMX4 Δost4::kanMX4	this study
YG958	MATa ade2-101 ura3-52 his3Δ200 tyr1 Δmns1::kanMX4 Δost4::kanMX4	this study

Table 2. Isolation of plasmids suppressing the temperature-sensitive phenotype of

Δost4 (A) or *stt3-6* cells (B).

High copy number plasmids from a Yep352-bound yeast genomic library that rescued

the *Δost4* or *stt3-6* mutation, respectively, were grouped into plasmid classes based on

PCR analysis *(STT3, OST3, and OST4)* or restriction enzyme mapping. The isolated

plasmids were transformed back into the original mutant strain in order to test for

suppression and the insert of at least one plasmid of each class that was not analyzed by

PCR was sequenced. *UBC6, ALG7,* and *OST6* of screen a and the *YET1* locus of screen

b were shown to be the loci responsible for suppression by cloning into high copy

expression vector Yep352 and transforming the resulting plasmids into *Δost4* or *stt3-6*

cells, respectively. ORFs only partially present on the appropriate plasmid are indicated.

A

Plasmid class	Locus responsible	number of independent transformants
1	*OST4*	9
2	*OST3*	1
3	*OST6*	1
4	*ALG7*	1
5	*UBC6*	1
6	YPL131w *(RPL1)* (incomplete) YPL130w YPL129w *(ANC1)* YPL128c *(TBF1)* (incomplete)	1
7	YOR151c *(RPB2)* (incomplete) YOR152c YOR153w *(PDR5)* (incomplete)	1

B

Plasmid class	Locus responsible	number of independent transformants
1	*STT3*	2
2	*OST4*	12
3	YKL065c *(YET1)*	3
4	YBR105c YBR106w YBR 107c (incomplete)	1
5	D1298 D1293 YL41A *VMA1*	1
6	YHR120w *(MSH1)* YHR121w YHR122w YHR123w *(EPT1)* (incomplete)	3
7	YBR069c (VAP1) YBR070c (SAT2) YBR068c (incomplete)	3
8	YGL124c YGL125w (incomplete)	2
9	YJL154c *(VPS35)* (incomplete) YJL153c *(INO1)* (incomplete)	1

WT

*Δ*ost4 Vector

*Δ*ost4 pALG3

*Δ*ost4 pALG7

*Δ*ost4 pALG8

*Δ*ost4 pALG9

23°

WT

*Δ*ost4 Vector

*Δ*ost4 pALG3

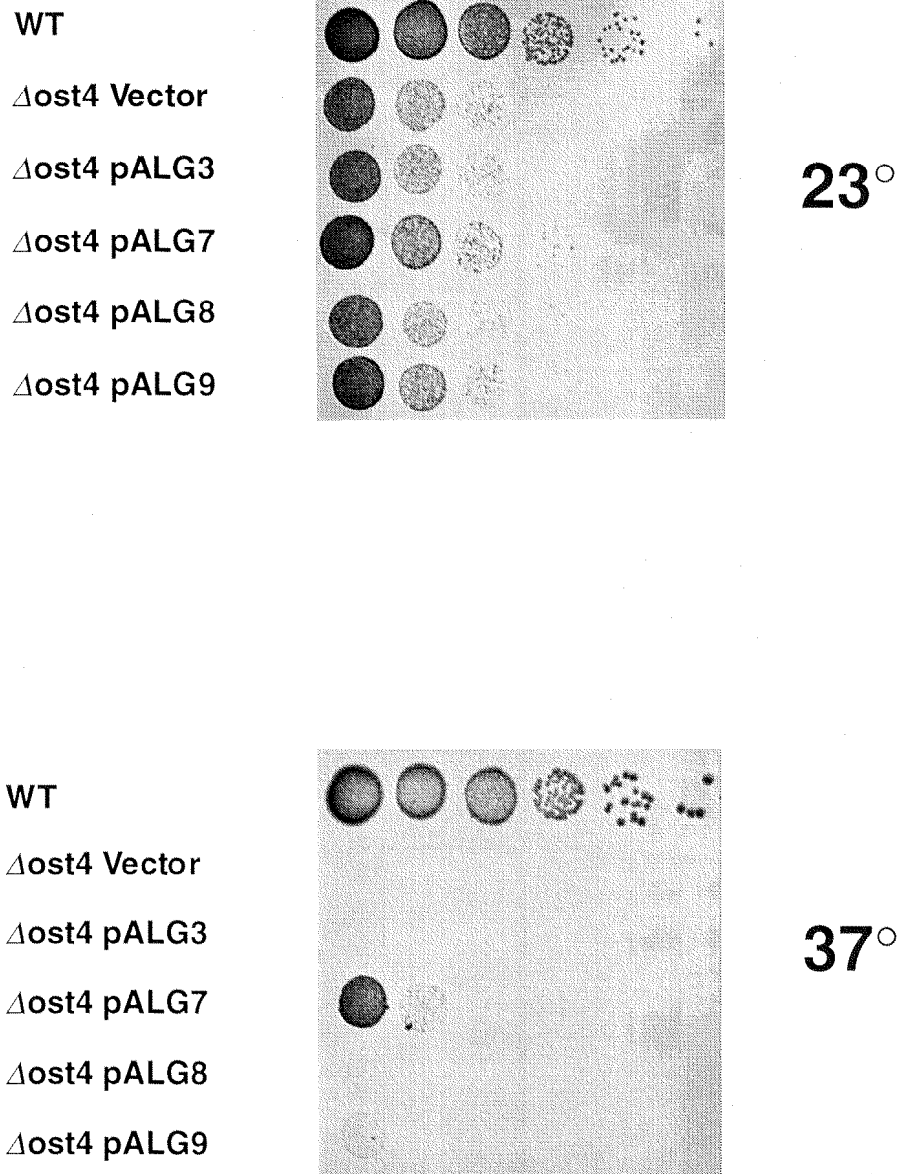*Δ*ost4 pALG7

*Δ*ost4 pALG8

*Δ*ost4 pALG9

37°

FIGURE 1. - *ALG7* **was a high copy number suppressor of an** *OST4* **deletion.**
SS328 wild-type cells *(WT)* and YG493 *(Δost4)* cells harboring either the vector or the plasmid pALG3, pALG7, pALG8, or pALG9 were grown at 23° to mid-log phase in complete liquid medium or in liquid minimal medium lacking uracil, respectively. Serial dilutions starting at 5 x 10^5 cells were spotted onto YPD plates, incubated for 3 days at 23° or 37° and photographed.
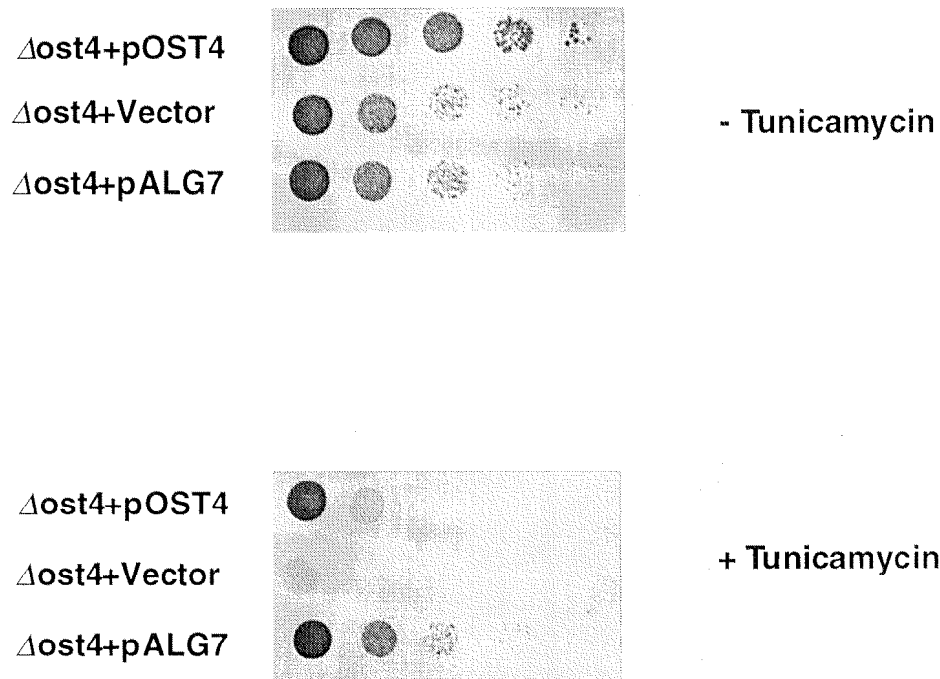
Δost4+pOST4

Δost4+Vector - Tunicamycin

Δost4+pALG7

Δost4+pOST4

Δost4+Vector + Tunicamycin

Δost4+pALG7

FIGURE 2. - *Δost4* cells harboring *ALG7* on a high copy number plasmid grew on plates containing the inhibitor tunicamycin.

YG493 *(Δost4)* cells harboring either the vector (Yep352), or the high copy number plasmid pOST4 or pALG7 were grown at 23° to mid-log phase in liquid minimal medium lacking uracil. Serial dilutions starting at 5 x 10^5 cells were spotted onto plates containing complete medium (YPD) supplemented with tunicamycin in a final concentration of 3.0 mg/ml. The plates were incubated for 4 days at 23° and photographed.
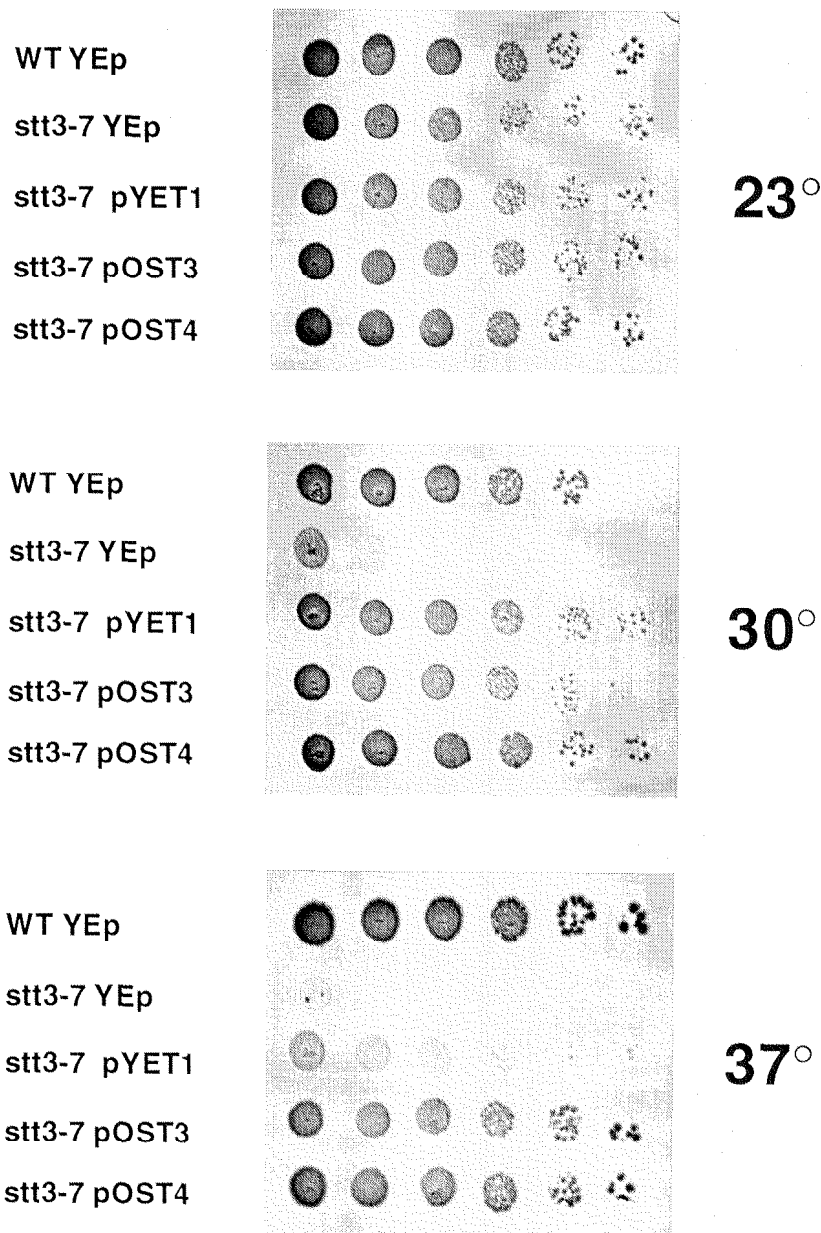
A

WT YEp

stt3-7 YEp

stt3-7 pYET1

stt3-7 pOST3

stt3-7 pOST4

23°

WT YEp

stt3-7 YEp

stt3-7 pYET1

stt3-7 pOST3

stt3-7 pOST4

30°

WT YEp

stt3-7 YEp

stt3-7 pYET1

stt3-7 pOST3

stt3-7 pOST4

37°

FIGURE 3. - High copy number suppression of the temperature sensitive *stt3-7*
mutation by *OST3, OST4,* **and** *YET1.*

(A)   growth of *stt3-7* mutant cells harboring YEp352, pYET1, pOST3, or pOST4.
stt3-7 cells were transformed with the 2m plasmids Yep352, pYET1, pOST3, or
pOST4, and the resulting uracil prototrophs were cultured to mid-log phase in minimal
medium lacking uracil. Serial dilutions starting at 5×10^5 cells were spotted onto plates
containing minimal medium lacking uracil and incubated for 3 days at 23°, 30°, or 37°
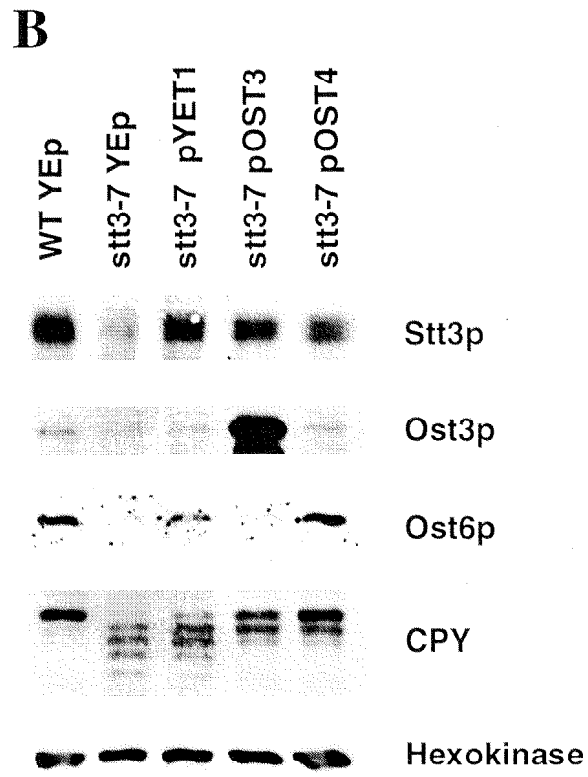and photographed. SS328 +Yep352 is included in each set for comparison.

FIGURE 3. - (continued)

(B)   Steady state level of Stt3p, Ost3p, and Ost6p, and CPY underglycosylation phenotype in *stt3-7* mutant cells harboring pYET1, pOST3, or pOST4. The transformants described in A were grown in minimal medium lacking uracil at 23° to mid-log phase. Protein extracts were prepared and equal amounts of protein were analysed by western blotting using specific antibodies against Stt3p, Ost3p, Ost6p, and CPY. Antibodies against Hexokinase served as a control to visualize the amount of protein loaded.
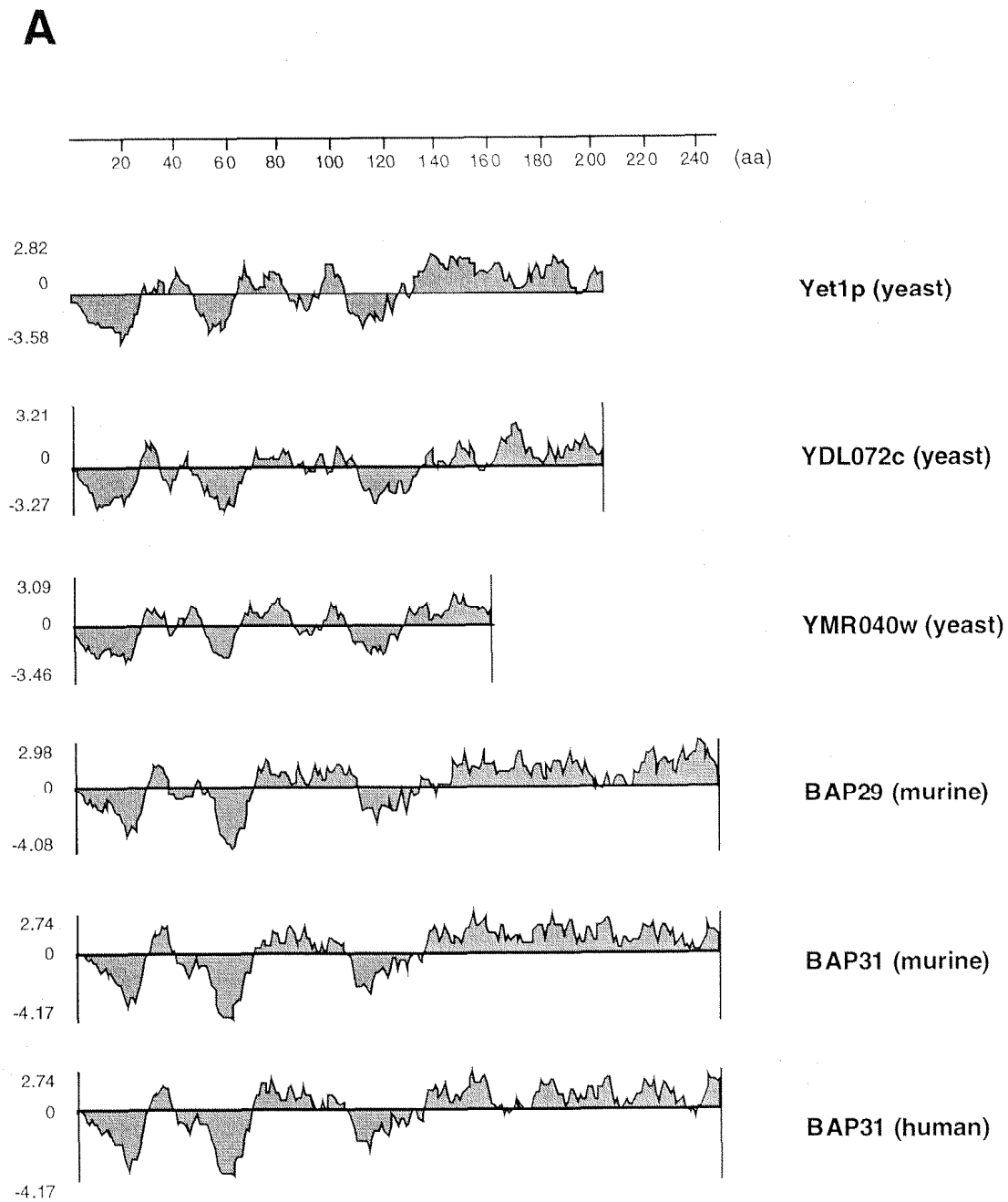
A

FIGURE 4. - Analysis of the *S. cerevisiae* *YET1* protein.

(A)   Hydropathy plots, calculated according to Kyte and Doolittle (1982) using a window of 19 residues, of Yet1p, its two yeast homologues YDL072c and YMR040w, the murine homologues BAP29 and BAP31, and the human homologue BAP31.

B

```
1    M S L Y F T T L F L L L T V E V V M L F I F V L P L P F R I   Yet1p  (S.cerevisiae)
1    M T I Q W A A V A S F L Y A E I G L I L L F C L P F I P P Q   BAP29  (M.musculus)

31   R R G - - - I F S T Y N Q L T A - - K Q Q I K T I I F I T G   Yet1p  (S.cerevisiae)
31   R W Q K I F S F S V W G K I A S F W N K A F L T I T I - - -   BAP29  (M.musculus)

56   C L V G L L F I D S W K R S Q I R - - V S L Y H N D N S G S   Yet1p  (S.cerevisiae)
58   - L L I I L F L D A - - V R E V R K Y S S T N V V E K N S A   BAP29  (M.musculus)

84   I G S S A V T P I Q A L A S R A Y N Q R N M Y I S G F I L Y   Yet1p  (S.cerevisiae)
85   I R P S A F E H T Q M K L F R - - S Q R N L Y I S G F S L F   BAP29  (M.musculus)

114  F S I C I P T V M S I V K R L V K Y Q G - - - - - - - - - -   Yet1p  (S.cerevisiae)
113  F W L V L R R L V T L I T Q L A K E I A N K G V L K I Q A E   BAP29  (M.musculus)

134  - - - - - - - - L I N E Q E K Q K L N K P S S N S K K D S N   Yet1p  (S.cerevisiae)
143  N T N K A A K K F M E E N E K L K L G L R N D N A E E H L L   BAP29  (M.musculus)

156  E A D S T K L Q E - - - - - - - E L R K K Q I S L E G L Q K   Yet1p  (S.cerevisiae)
173  E A E N K K L I E S K E N L K T E L K K A S D A L L K A Q N   BAP29  (M.musculus)

179  Q V K N - - - - - - - L E K Y F D E - - K N Q P G N V A A A   Yet1p  (S.cerevisiae)
203  D V M T M K I Q S E R L S K E Y D R L L K E H S E L Q N R L   BAP29  (M.musculus)

200  E A S K K G N                                               Yet1p  (S.cerevisiae)
233  E K E K K K G L                                             BAP29  (M.musculus)
```

FIGURE 4. - (continued)

(B)   Primary Yet1p sequence (*S.cerevisiae*) in comparison with the sequence of the murine homologue BAP29 (*M.musculus*). The MEGALIGN program (DNASTAR) was used. Identical amino acids are highlighted.
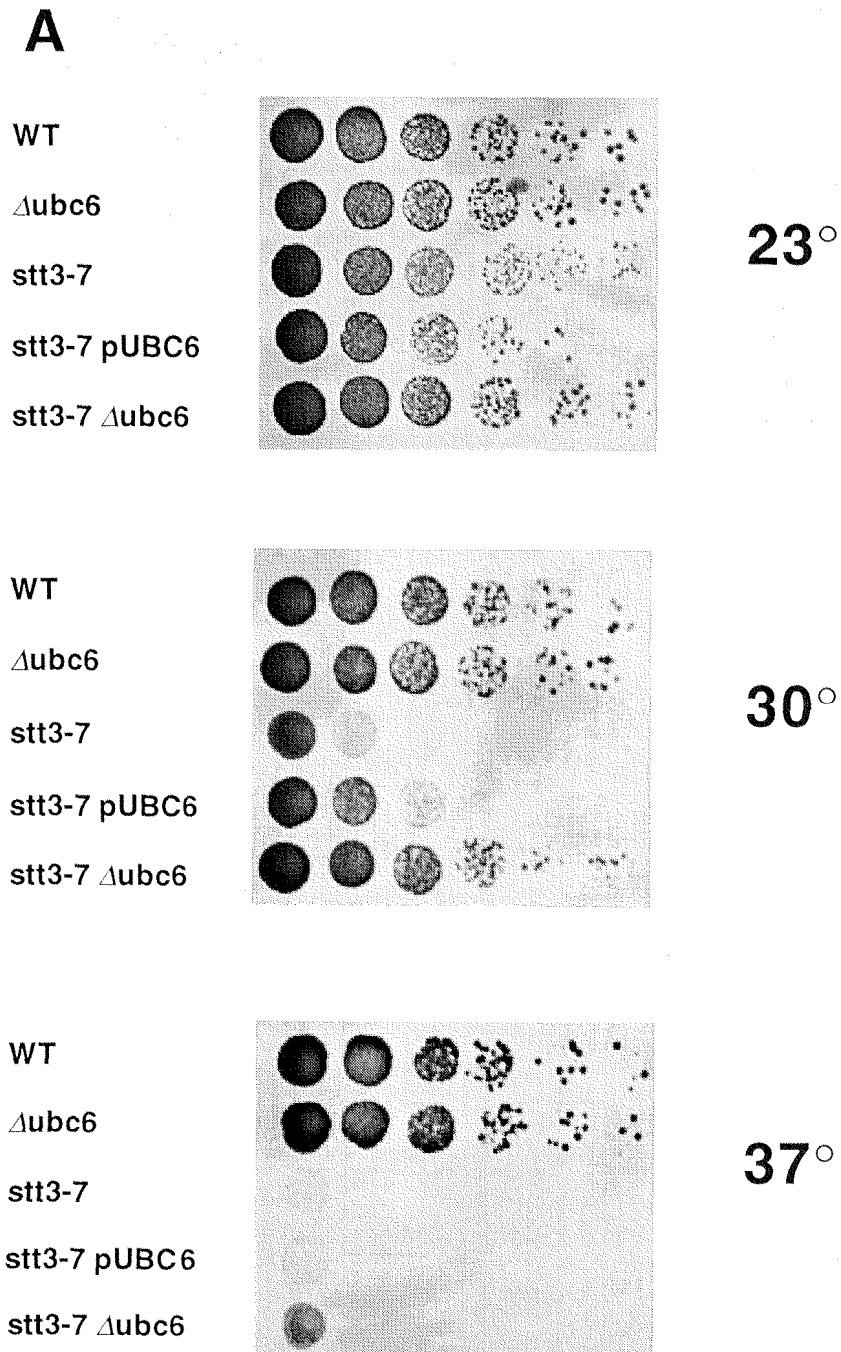
A

WT

Δubc6

stt3-7

stt3-7 pUBC6

stt3-7 Δubc6

23°

WT

Δubc6

stt3-7

stt3-7 pUBC6

stt3-7 Δubc6

30°

WT

Δubc6

stt3-7

stt3-7 pUBC6

stt3-7 Δubc6

37°

FIGURE 5. - The temperature sensitive *stt3-7* mutation was suppressed either by the high copy number suppressor *UBC6* or the *UBC6* deletion.

(A) SS328 wild-type cells *(WT)*, YG885 *(Δubc6)* cells, YG543 *(stt3-7)* cells and YG894 *(stt3-7 Δubc6)* cells were grown at 23° to mid-log phase in complete medium. YG543 *(stt3-7)* cells harboring the plasmid pUBC6 were grown at 23° to mid-log phase in liquid minimal medium lacking uracil. Serial dilutions starting at 5×10^5 cells were spotted onto plates containing complete medium (YPD). The plates were incubated for 3 days at the temperature indicated and photographed.
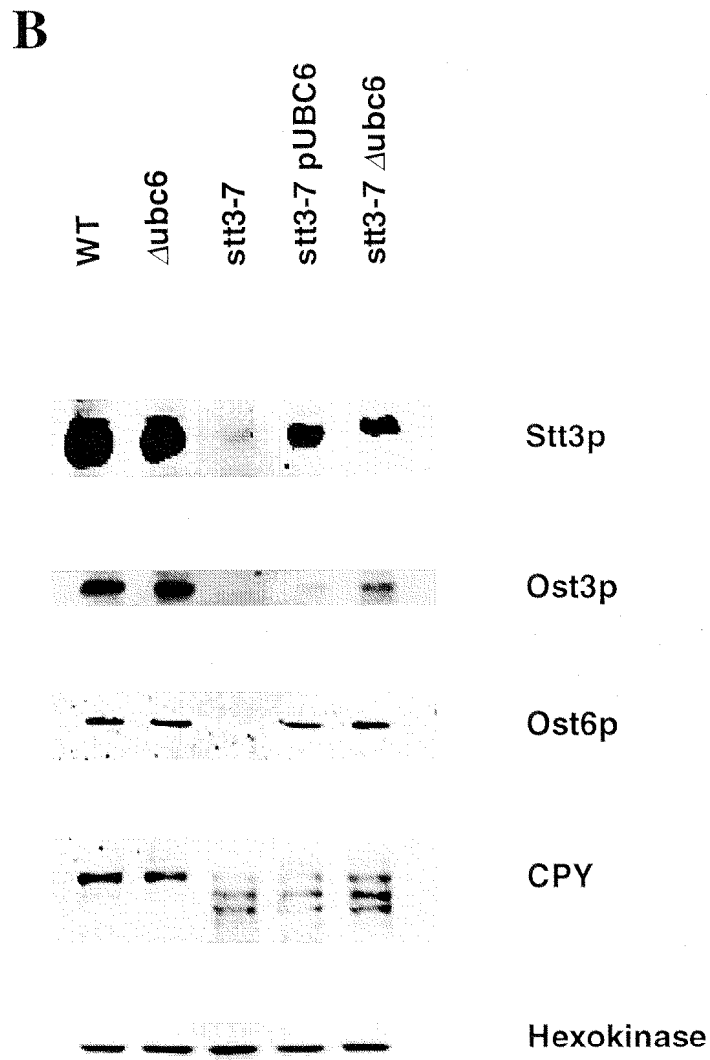
B

FIGURE 5. - (continued)

(B) Steady state level of Stt3p, Ost3p, Ost6p, and CPY underlycosylation phenotype in SS328 wild-type cells *(WT)*, in YG885 *(Δubc6)* cells, in YG543 *(stt3-7)* cells, in YG543 *(stt3-7)* cells harboring the plasmid pUBC6 and in YG894 *(stt3-7 Δubc6)* cells. These cells were grown in liquid complete or in liquid minimal medium lacking uracil *(stt3-7 pUBC6)* to mid-log phase. Protein extracts were prepared and equal amounts of protein were analysed by western blotting using specific antibodies against Stt3p, Ost3p, Ost6p, and CPY. Antibodies against Hexokinase served as a control to visualize the amount of protein loaded.
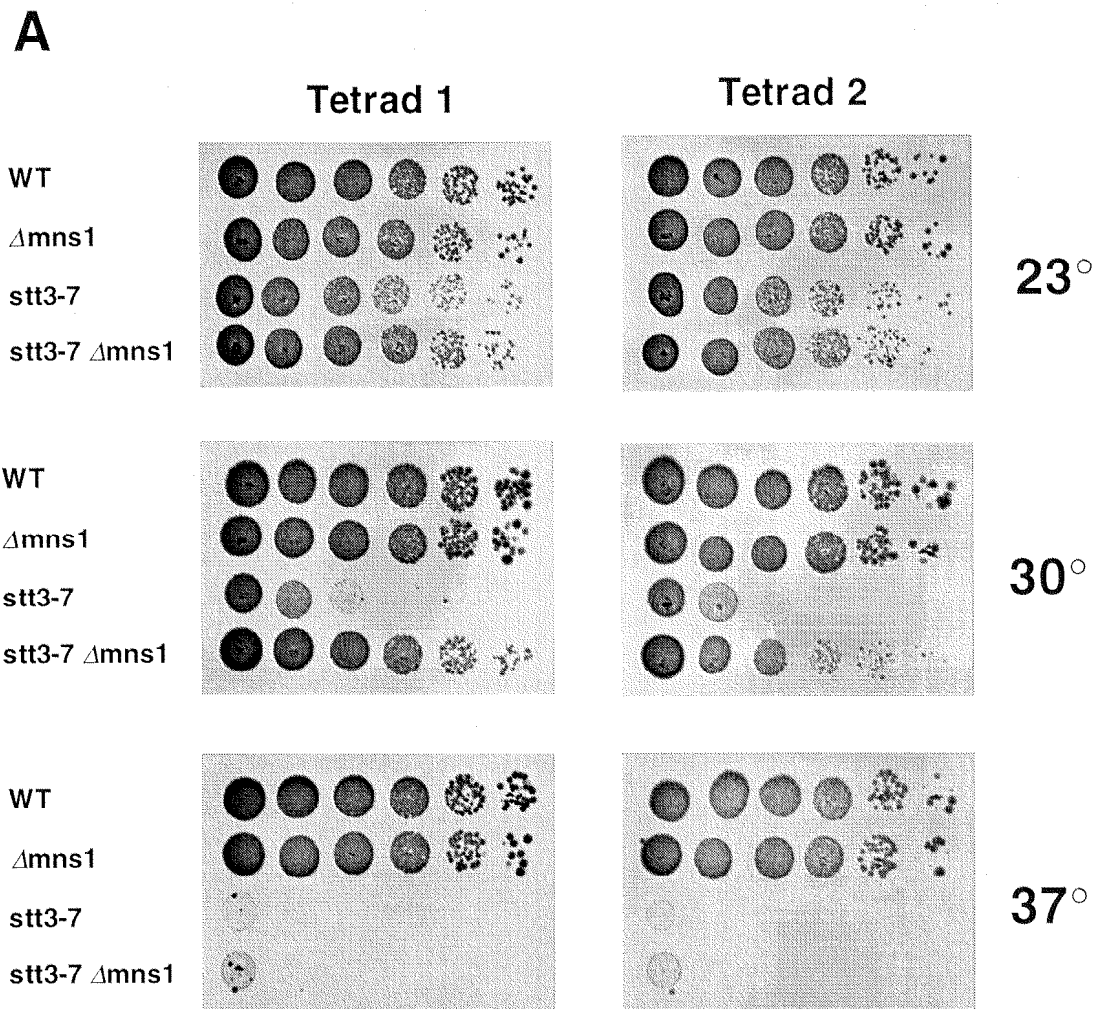
A

FIGURE 6. - Effect of a *mns1* deletion on the mutant phenotype of *stt3-7*.

(A) The *mns1* deletion rescued the temperature sensitive phenotype of a *stt3-7* mutation at 30°. The four segregants of two individual tetrads (tetrad 1 and tetrad 2) derived from a *stt3-7 Δmns1* diploid strain were grown in complete medium to mid-log phase. Serial 1:10 dilutions starting at 5 x 10^5 cells were spotted onto agar plates containing complete medium. Plates were incubated for 3 days at 23°, 30°, or 37° and photographed.

B

WT   Δmns1   stt3-7   stt3-7 Δmns1
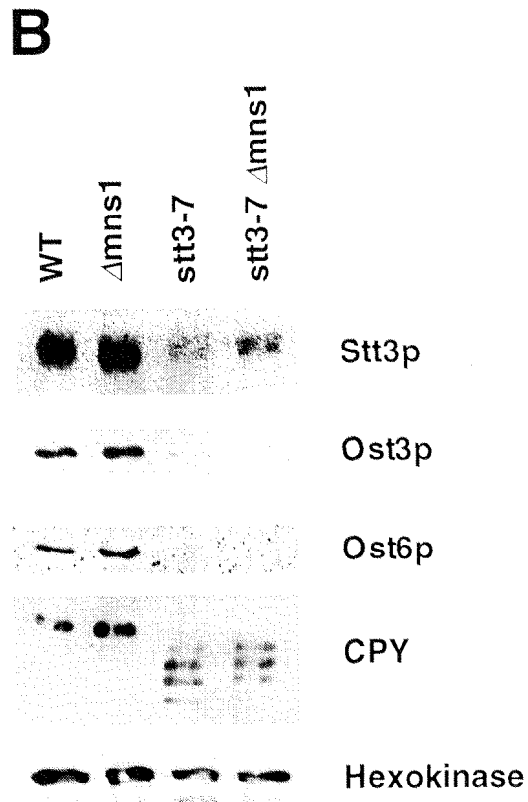
Stt3p

Ost3p

Ost6p

CPY

Hexokinase

FIGURE 6. - (continued)

(B) Steady state level of Stt3p, Ost3p, Ost6p, and CPY underglycosylation phenotype of the four segregants of tetrad 1 (described in A). Cells were grown in complete medium at 23° to mid-log phase. Protein extracts were prepared and equal amounts of protein were analysed by western blotting using specific antibodies against Stt3p, Ost3p, Ost6p, and CPY. Antibodies against Hexokinase served as a control to visualize the amount of protein loaded.

Curriculum Vitae

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1975-1983	Primary education in Montlingen ('Primarschule') and Oberriet ('Sekundarschule')
1983 - 1987	Secundary education, 'Kantonsschule' Heerbrugg School leaving examination: Matura type B
1988 - 1994	Studies of Biology at the University of Zurich and at the Swiss Federal Institute of Technology (ETH) in Zurich Diploma in Natural Sciences (dipl. Natw. ETH)
1995 - 1998	PhD thesis under the guidance of Prof. Dr. Markus Aebi and research assistant at the Institute of Microbiology of the ETH in Zurich