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A specific screen for oligosaccharyltransferase mutations identifies the 9 kDa *OST5* protein required for optimal activity *in vivo* and *in vitro*

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The central reaction in the process of *N*-linked protein glycosylation in eukaryotic cells, the transfer of the oligosaccharide $Glc_3Man_9GlcNAc_2$ from the lipid dolicholpyrophosphate to selected asparagine residues, is catalyzed by the oligosaccharyltransferase (OTase). This enzyme consists of multiple subunits; however, purification of the complex has revealed different results with respect to its protein composition. To determine how many different loci are required for OTase activity *in vivo*, we performed a novel, specific screen for mutants with altered OTase activity. Based on the synthetic lethal phenotype of OTase mutants in combination with a deficiency of dolicholphosphoglucose biosynthesis which results in non-glucosylated lipid-linked oligosaccharide, we identified seven complementation groups with decreased OTase activity. Beside the known OTase loci, *STT3*, *OST1*, *WBP1*, *OST3*, *SWP1* and *OST2*, a novel locus, *OST5*, was identified. *OST5* is an intron-containing gene encoding a putative membrane protein of 9.5 kDa present in highly purified OTase preparations. *OST5* protein is not essential for growth but its depletion results in a reduced OTase activity. Suppression of an *ost1* mutation by overexpression of *OST5* indicates that this small membrane protein directly interacts with other OTase components, most likely with Ost1p. A strong genetic interaction with a *stt3* mutation implies a role in complex assembly.

Keywords: endoplasmic reticulum/glycosylation/membrane protein/oligosaccharyltransferase/*Saccharomyces cerevisiae*

Introduction

N-linked protein glycosylation is an essential and highly conserved process in eukaryotes. The core oligosaccharide $Glc_3Man_9GlcNAc_2$, assembled on the lipid carrier dolicholpyrophosphate, is transferred *en bloc* to selected asparagine residues of nascent polypeptide chains by the multimeric enzyme *N*-oligosaccharyltransferase (OTase) in the lumen of the endoplasmic reticulum (ER) (Kornfeld and Kornfeld, 1985; Silberstein and Gilmore, 1996). Amongst their diverse functions in eukaryotic species (Varki, 1993), *N*-linked glycans facilitate protein folding and oligomeric assembly in the ER and transport along the secretory pathway (Helenius, 1994; Fiedler and Simons, 1995). In all eukaryotes, the acceptor site for *N*-linked glycosylation fits the consensus sequon Asn–X–Ser/Thr, where X can be any amino acid except proline (Bause, 1984; Gavel and Van Heinje, 1990). This site is recognized most efficiently by the enzyme while the protein is in an unfolded state (Pless and Lennarz, 1977; Allen *et al.*, 1995; Holst *et al.*, 1996). Indeed, components of the OTase have been found not only to be abundant in the rough ER membrane but also to be associated intimately with the protein translocation machinery of the ER (Yu *et al.*, 1990).

Purified mammalian and avian OTase (Kelleher *et al.*, 1992; Kumar *et al.*, 1994; Breuer and Bause, 1995) consists of the subunits ribophorin I, ribophorin II and OST48. In *Saccharomyces cerevisiae*, different compositions of purified OTase were reported. Either a hexameric complex [Ost1p (62/64 kDa), Wbp1p (45 kDa), Swp1p (30 kDa), Ost2p (16 kDa), Ost3p (34 kDa) and a hitherto unidentified 9 kDa protein (Kelleher and Gilmore, 1994)] or a tetrameric complex [Ost1p, Wbp1p, Ost3p and Swp1p (Knauer and Lehle, 1994; Pathak *et al.*, 1995)] were found in highly purified OTase preparations. A high sequence similarity suggests that Ost1p and Swp1p are the yeast homologs of the ribophorins I and II, respectively, whereas Wbp1p is similar to OST48 (Silberstein *et al.*, 1992). Wbp1p was postulated to harbor the active site of the enzyme (Breuer and Bause, 1995; Pathak *et al.*, 1995). Genetic studies revealed that Ost1p (Silberstein *et al.*, 1995a), Wbp1p (te Heesen *et al.*, 1992), Swp1p (te Heesen *et al.*, 1993) and Ost2p (Silberstein *et al.*, 1995b) are essential components of the OTase complex *in vivo*. Loss of Ost3p reduces *in vivo* and *in vitro* OTase activity without causing a detectable alteration in growth rate (Karaoglu *et al.*, 1995). Recently, by genetic screens, two novel loci have been isolated, both affecting OTase activity *in vivo* while not being detected in the purified, active complex: *STT3* (Zufferey *et al.*, 1995) and *OST4* (Chi *et al.*, 1996). *STT3* encodes an essential 78 kDa transmembrane protein and seems to be required for the assembly of the OTase complex and/or recognition of the oligosaccharide substrate. *OST4* encodes a very small, hydrophobic polypeptide (3.4 kDa); its absence results in a strongly reduced cell growth and decreased OTase activity. These data suggest that OTase complexes lacking certain subunits may retain partial *in vitro* OTase activity.

To identify further components required for optimal *in vivo* transfer of core oligosaccharide onto protein, we have performed a genetic screen based on the synthetic phenotype of defects in oligosaccharide assembly and a decreased OTase activity (Stagljar *et al.*, 1994). This approach led us to the cloning of the *OST5* locus encoding

the small 9.5 kDa subunit of the yeast OTase complex. Its functional characterization is described.

Results

A synthetic lethal screen with Δalg5 is highly specific for mutations affecting oligosaccharyltransferase activity

In *S.cerevisiae*, lipid-linked $Glc_3Man_9GlcNAc_2$ is the preferred substrate of the OTase complex *in vivo* and *in vitro*. However, the yeast enzyme can transfer incompletely assembled oligosaccharide as well, albeit with a reduced efficiency (Trimble *et al.*, 1980; Sharma *et al.*, 1981; Ballou *et al.*, 1986). Due to this relaxed substrate specificity, mutations affecting late steps in the assembly pathway of the lipid-linked oligosaccharide (LLO) (*alg* mutations) do not result in a detectable growth phenotype but instead reduce glycosylation efficiency. In combination with the *wbp1* mutation which reduces OTase activity (te Heesen *et al.*, 1992), a synthetic lethal phenotype is observed (Stagljar *et al.*, 1994). This synthetic phenotype defines a specific tool to identify mutant strains with a reduced OTase activity. Therefore, we screened for mutations synthetically lethal with the *alg5* deletion. Δ*alg5* mutant strains lack Dol-P-Glc synthase activity and accumulate lipid-linked $Man_9GlcNAc_2$, a suboptimal substrate for OTase (Huffaker and Robbins, 1983; Runge *et al.*, 1984; te Heesen *et al.*, 1994). The terminal glucoses are crucial for efficient substrate recognition by the OTase, but are removed from the glycan shortly after transfer onto protein. Thus the altered structure of the LLO should not affect glycoprotein processing at later stages. To our knowledge, Dol-P-Glc synthase activity is required solely in the biosynthesis of the LLO. We therefore anticipated that a majority of mutations synthetically lethal with Δ*alg5* should reduce OTase activity.

To identify synthetic lethal mutations, the *ade2*/*ade3* red/white sectoring assay (Kranz and Holm, 1990; Wimmer *et al.*, 1992) was applied. Two Δ*alg5* strains, differing in the mating type and auxotrophic markers, but both harboring a plasmid carrying the *ADE3*, the *URA3* and the *ALG5* loci, were chemically mutagenized, and strains requiring the *ALG5* plasmid were identified by their uniform red colony color on YPD complete medium at 30°C. These strains were tested further for their inability to grow on medium containing 5-fluoro-orotic acid (5-FOA), an additional test for their plasmid requirement. Out of ~250 000 colonies screened, 109 mutant strains were isolated and classified into complementation groups. Five groups were found to be *ADE3* dependent (Wimmer *et al.*, 1992), whereas the remaining 11 groups all depended on an active *ALG5* locus. The latter were analyzed further for *N*-linked glycosylation defects by analysis of the vacuolar protein carboxypeptidase Y (CPY). CPY is a highly expressed peptidase containing four *N*-linked glycans (Hasilik and Tanner, 1978). It is an ideal reporter of glycosylation defects because its expression and transport are little affected by lack of *N*-glycans (Winther *et al.*, 1991). A decreased transfer of core glycans is visualized by glycoforms with an altered mobility in SDS–PAGE (te Heesen *et al.*, 1992). Mutants from seven different complementation groups were found to hypoglycosylate CPY, whereas four complementation groups did not have any defect in glycosylation. These groups are not discussed further in this report. Representative mutant strains of the seven glycosylation-deficient complementation groups were chosen and grown on YPD complete medium containing 1 M sorbitol at 15 or 23°C. Under these conditions, the *ALG5*-containing plasmid could be lost, resulting in highly temperature-sensitive, osmotically labile double mutant strains. In order to test whether or not known OTase loci were affected in the different complementation groups, the latter double mutants were transformed with YEp352-based plasmids comprising *STT3* (Zufferey *et al.*, 1995), *OST1* (Silberstein *et al.*, 1995a), *WBP1* (te Heesen *et al.*, 1992), *OST3* (Karaoglu *et al.*, 1995), *SWP1* (te Heesen *et al.*, 1993), *OST2* (Silberstein *et al.*, 1995b) and a low copy number plasmid containing *OST4* (Chi *et al.*, 1996) respectively. Transformants were tested for growth at 37°C on YPD complete medium. Based on this analysis, we were able to assign complementation groups to all known OTase subunits except *OST4* (Table I). In the cases of the *STT3*, *OST1*, *WBP1* and *OST3* complementation groups, the assignment of the mutant locus was confirmed by crossing representative strains from our screen to previously described mutants affected in the corresponding loci and analysis of CPY glycosylation in resulting diploids (data not shown). Our screen therefore yielded mutant strains defective in all known putative OTase loci with the exception of *OST4*. One complementation group containing mutants with a hypoglycosylation of CPY could not be assigned to known OTase loci. The novel locus defined by this group was termed *OST5*.

Table I. Mutation synthetically lethal with Δ*alg5* and hypoglycosylating secretory proteins

Locus	No. of alleles	Phenotype reference
STT3	50	Zufferey *et al.* (1995)
OST3	20	Karaoglu *et al.* (1995)
OST5	5	this work
WBP1	3	te Heesen *et al.* (1992)
OST1	2	Silberstein *et al.* (1995a)
SWP1	1	te Heesen *et al.* (1993)
OST2	1	Silberstein *et al.* (1995b)

The different mutant strains were classified into complementation groups based on the 5-FOA-sensitive phenotype. All mutations result in an underglycosylation of CPY. The loci defined by the different groups were identified by transformation of representative strains with plasmids encoding individual OTase subunits and tested for growth at 37°C.

The OST5 locus contains an intron and encodes the ζ-subunit of the OTase complex

The original *ost5-1* Δ*alg5* isolate was backcrossed to wild-type strain SS330 to generate the *ost5-1* Δ*alg5* strain YG511; we observed that the *ost5-1* Δ*alg5* double mutant is viable at 23°C but not at 30°C on YPD medium. The *OST5* locus was isolated by complementation of this temperature-sensitive phenotype using a genomic plasmid library. Approximately 10^4 transformants were tested for growth at 37°C. From seven transformants, complementing plasmids were isolated. Two harbored a plasmid containing the *ALG5* locus; five plasmids had inserts with common DNA sequences. These plasmids restored the growth of strain YG511 at 37°C and improved CPY glycosylation.

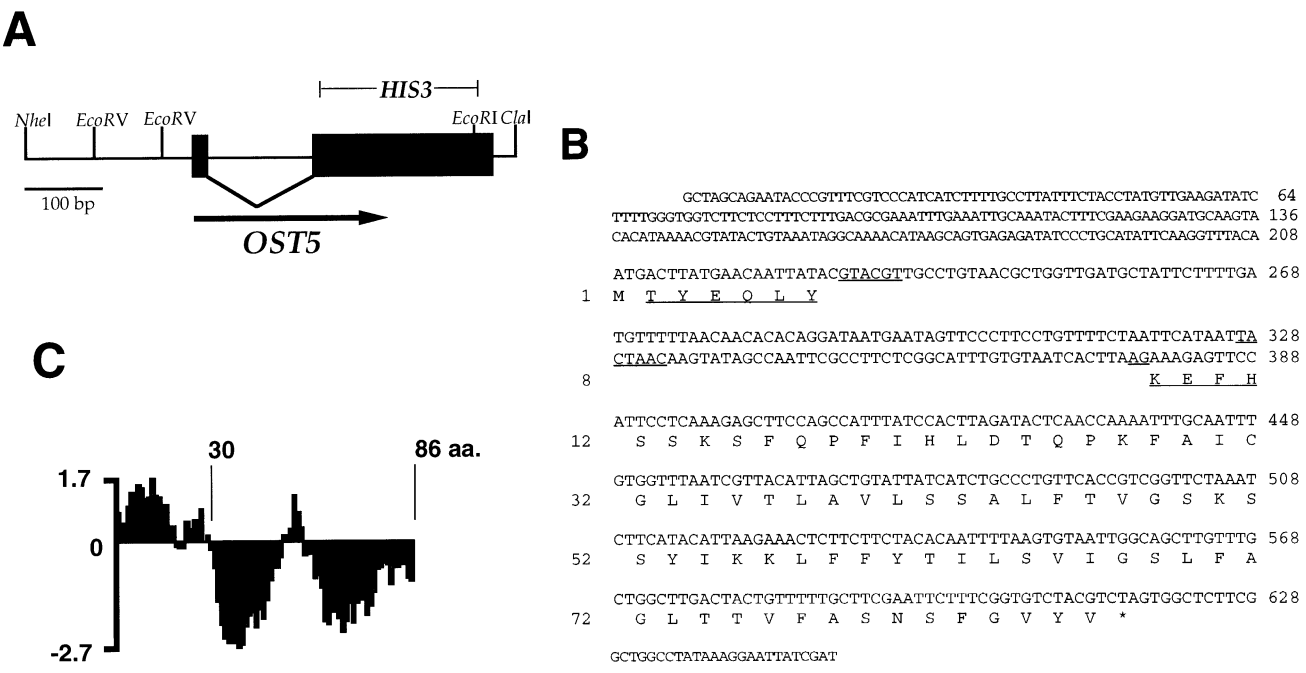


Fig. 1. (**A**) Physical map of the *OST5* locus. Deletion analysis mapped the locus to a 610 bp *Nhe*I–*Cla*I fragment. Selected restriction sites are shown. The coding sequence of the two exons is boxed. The region in exon 2 which has been replaced by a 1 kb *HIS3* cassette for gene disruption is indicated by a bar. (**B**) Sequence of the *OST5* locus and Ost5p. Numbering is for the DNA sequence (left) and the predicted amino acid sequence (right). In the DNA sequence (accesssion No. X97545), consensus elements of the intron are underlined. In the amino acid sequence, the part that has been confirmed by N-terminal protein sequencing is underlined. (**C**) Hydrophilicity plot of Ost5p according to the method of Kyte and Doolittle (1982), using a window of nine amino acids.

Deletion analysis located the complementing region on a 650 bp *Nhe*I–*Cla*I fragment which was sequenced (Figure 1A). Inspection of the sequence did not reveal any obvious large open reading frame. However, the presence of specific intron sequences (Parker and Guthrie, 1985; Woolford, 1989) prompted us to postulate an intron at position 230–378 (Figure 1B), the 3′ splice site not being clearly predictable. We confirmed experimentally the existence of a spliced transcript from this locus. RT–PCR with primers specific for the two postulated exons amplified an mRNA-derived fragment devoid of the intron sequence (as verified by sequencing of the PCR product, data not shown). The spliced mRNA encodes a polypeptide of 86 amino acids (Figure 1B) with a calculated mol. wt of 9.5 kDa. Hydropathy analysis (Figure 1C) using the method of Kyte and Doolittle (1982) revealed two highly hydrophobic segments accounting for two-thirds of the protein. The PREDICT PROTEIN algorithm (Rost *et al.*, 1995) identifies these two segments as transmembrane helices. The N-terminus is hydrophilic and lacks the characteristics of a cleavable signal sequence for ER translocation. A consensus site for asparagine-linked glycosylation is not present. Most probably Ost5p is a membrane protein which spans the membrane twice, with both the amino- and carboxy-terminus located on the cytoplasmic face of the membrane (Spiess, 1995). Importantly, the predicted N-terminal amino acid sequence of the protein derived from the spliced mRNA was also obtained experimentally from the N-terminus of the ζ-subunit of the purified yeast OTase complex (Kelleher and Gilmore, 1994). This experimental sequence (TYEQLYKEFH) starts with a threonine (position 2, Figure 1B) and encompasses the peptide sequence derived from the predicted exon 1–exon 2 junction (underlined, Figure 1B). The cytosolic enzyme methionine aminopeptidase can remove the initiator methionine preceding a threonine residue (Kendall *et al.*, 1990). This result confirms that *OST5* encodes an intron-containing mRNA and suggests that Ost5p is a component of the OTase complex.

Ost5p is required for N-linked glycosylation in vivo

To analyze Ost5p function, we first deleted one copy of the *OST5* coding sequence in a diploid strain and tested whether or not a haploid Δ*ost5* strain would be viable. After sporulation and ascus dissection, Δ*ost5* strains were obtained which did not show any growth defect on standard media. Crosses of the Δ*ost5* strain to a Δ*alg5* strain and subsequent tetrad analysis confirmed that the Δ*ost5* Δ*alg5* combination gives a synthetic temperature-sensitive phenotype at 30°C. Based on our screening procedure, we predicted a cumulative effect of Δ*alg5* and Δ*ost5* on protein glycosylation. A tetratype tetrad resulting from a cross of Δ*ost5* and Δ*alg5* was analyzed for CPY glycosylation (Figure 2A). Δ*alg5* shows a discrete spectrum of CPY molecules lacking no, one or two *N*-linked glycans (te Heesen *et al.*, 1994). Δ*ost5* leads to a rather mild underglycosylation of CPY. In striking constrast, the double mutant Δ*ost5* Δ*alg5* severely underglycosylates CPY. A significant proportion of protein carries no oligosaccharide, while the majority carries only one *N*-linked sugar. Similiar patterns of hypoglycosylation in Δ*ost5* Δ*alg5* strains were observed for two other glycoproteins, Wbp1p and Ost1p, both membrane proteins. Glycosylation of Ost1p in membrane preparations is shown (Figure 2B).

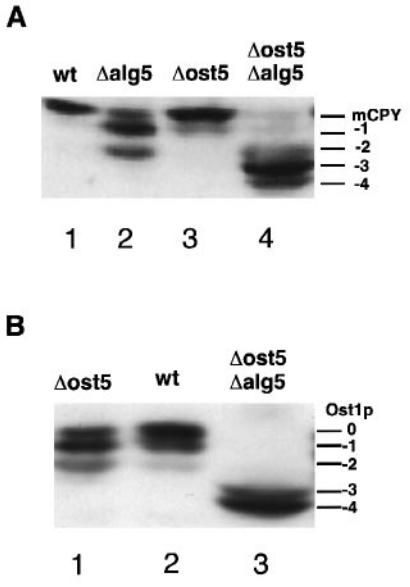


Fig. 2. Δ*ost5* affects glycosylation *in vivo*. (**A**) Analysis of CPY processing in a single tetrad from a cross of a Δ*ost5* and a Δ*alg5* strain by immunoblot. The position of mature CPY (mCPY) and the different glycoforms lacking one to four *N*-linked oligosaccharides (–1 to –4) are indicated. The relevant genotype of strains YG356 (lane 1), YG357 (lane2), YG358 (lane3) and YG359 (lane 4) is given. (**B**) Western analysis of Ost1p glycosylation in strains YG358 (lane 1), YG356 (lane2) and YG359 (lane3). Fifty μg of membrane protein per lane were analyzed. Ost1p appears in the wild-type in two glycoforms, lacking none or one out of four *N*-linked glycan. Glycoforms lacking more oligosaccharides are designated accordingly.

Wild-type yeast cells express two glycoforms of Ost1p (64 and 62 kDa) that contain four and three *N*-linked glycans (Silberstein *et al.*, 1995a). The double mutant Δ*ost5* Δ*alg5* nearly abolishes glycosylation whereas a Δ*ost5* strain shows a minor shift in the relative proportion of glycoforms. Similar results were obtained for Wbp1p glycosylation.

Overexpression of Ost5p suppresses an ost1 mutation in an ost1Δalg5 strain

Suppression of a mutant phenotype by overexpression of a different locus can indicate a direct interaction between the two corresponding proteins. In the case of the yeast OTase complex, suppression of the ts allele *wbp1-2* by overexpression of the physically interacting protein Swp1p has been observed (te Heesen *et al.*, 1992). The suppression acts on both the temperature sensitivity and the glycosylation defect of the *wbp1* mutant. The same suppressor activity was observed for Ost2p (Silberstein *et al.*, 1995b).

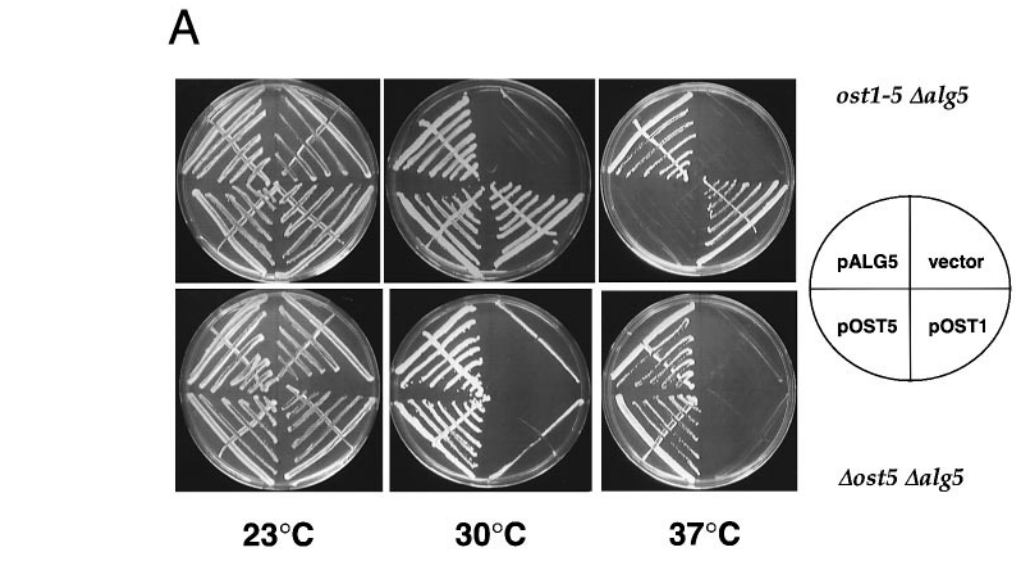
Taking advantage of the newly isolated mutations in the different subunits, we looked for suppression of their synthetic lethal phenotype in combination with the Δ*alg5* mutation. As for the *wbp1-2* mutation, we found that the temperature sensitivity of the novel *wbp1* Δ*alg5* strains was diminished by overexpression of Swp1p or Ost2p (data not shown). Interestingly, overexpression of Ost5p partially suppressed the ts defect of the *ost1-5* Δ*alg5* strains (Figure 3A), whereas the reverse experiment, suppression of the Δ*ost5* Δ*alg5* phenotype by overexpression of Ost1p, did not reveal positive results. Suppression of the *ost1-5* Δ*alg5* phenotype was observed only at 30°C but not at 37°C, distinguishing between suppression and complementation. The suppression of the growth phenotype is paralleled by an increased efficiency of *N*-linked glycosylation (Figure 3B and C). Overexpression of *OST5* improved the cumulative underglycosylation of CPY in the *ost1-5* Δ*alg5* double mutant (Figure 3C, lane 5). We also tested the effect of *OST5* overexpression in an *ost1-5* and Δ*alg5* single mutant (Figure 3B). The *ost1-5* mutation does not induce a detectable growth phenotype in strains with normal biosynthesis of the LLO (data not shown) and results in a mild hypoglycosylation of CPY (Figure 3B, lane 5). Probably due to the weak phenotype of *ost1-5*, we were not able to detect a significant increase in glycosylation efficiency in *ost1-5* cells overexpressing *OST5* (Figure 3B, lane 4). Overexpression of *OST5* did not suppress the temperature-sensitive phenotype of either *wbp1 alg5*, *stt3 alg5* or *ost3 alg5* double mutants and did not improve the hypoglycosylation in Δ*alg5* strains (Figure 3B, lane 8). We conclude that *OST5* is a specific high copy number suppressor of *ost1* mutations. The suppression of the temperature-sensitive phenotype of the *ost1-5* Δ*alg5* and the increased glycosylation efficiency in this strain are the most sensitive assays to visualize this suppression. We take these data as additional evidence that Ost5p is a subunit of the yeast OTase complex.

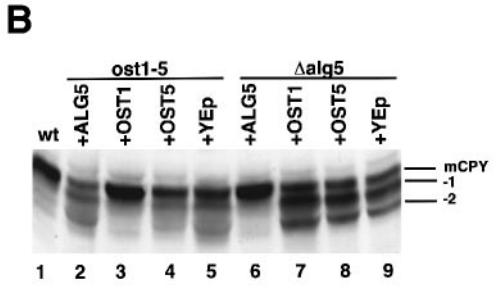
Ost5p is required for OTase activity in vitro

Assuming that Ost5p is an integral component of the enzyme complex, we assayed the effect of a deletion of Ost5p on OTase activity *in vitro*. Microsomal membranes were prepared from a wild-type and an isogenic Δ*ost5* strain. For both preparations, the enzymatic activity was found to be linear with time, excluding a defect in the stability of the complex during the time of incubation. Substrate dependence of the enzyme was assayed both for the peptide acceptor and the oligosaccharide donor. The activity of the mutant strain reaches about half of the wild-type value (V_{max}: 11.2 ± 0.9 versus 19 ± 0.9 pmol peptide/min/mg protein) under saturating conditions. The decreased catalytic efficiency is visualized by the increased slope of the Δ*ost5* curve in the Lineweaver–Burk plots, whereas the K_m values for both substrates were found to be essentially unchanged (Figure 4). Due to the inhomogeneity of the LLO substrate used in our OTase assay (Spiro *et al.*, 1976), the K_m value obtained for the LLO substrate is an estimate. With respect to *in vitro* OTase activity, a Δ*ost5* strain is comparable with a Δ*ost3* strain which also shows a 2-fold reduced OTase activity *in vitro* (Karaoglu *et al.*, 1995).

Genetic interaction between Δost5 and stt3-3

Ost3p and Ost5p are the only known components of the OTase complex whose deletion does not result in a growth defect. Depletion of Stt3p, Ost1p, Wbp1p, Swp1p and Ost2p is lethal to the cell; even loss of the 3.5 kDa Ost4p yields a drastically reduced growth rate and a temperature-sensitive phenotype (Chi *et al.*, 1996). We therefore tested whether Ost3p and Ost5p perform a redundant function. This seems not to be the case because a double knockout strain Δ*ost3* Δ*ost5* is viable, with a temperature-sensitive phenotype arising only at 37°C (Table II). We extended our studies of synthetic interactions to other mutations affecting OTase activity. A strong interaction was observed between *stt3-3* (Zufferey *et al.*, 1995) and





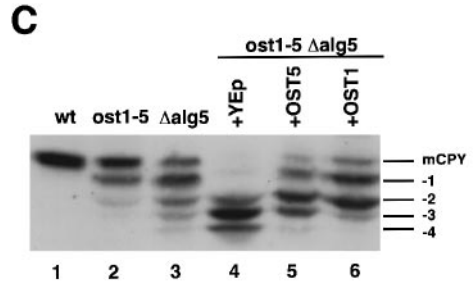


Fig. 3. High-copy number suppression of *ost1-5* by *OST5*. (**A**) Strains YG435 (*ost1-5 Δalg5)* and YG359 (*Δost5 Δalg5)* were transformed with plasmid YEp352 (vector) or YEp352 harboring either the *OST5* (pOST5), the *ALG5* (pALG5) or the *OST1* (pOST1) locus. Growth of the strains in the presence of the various plasmids was monitored on YPD + 1 M sorbitol medium at 23°C or on YPD lacking sorbitol at either 30 or 37°C. (**B**) Analysis of CPY processing in strains YG434 (*ost1-5*, lanes 2–5) and YG357 (*Δalg5*, lanes 6–9) transformed with vector YEp352 (YEp), pALG5, pOST1 or pOST5 by immunoblot. The positions of mature CPY (mCPY) and the different glycoforms lacking one or two *N*-linked oligosaccharides (–1 and –2) are indicated. Strain SS328 (wt) is shown for comparison. (**C**) Analysis of CPY processing in strain YG435 (*ost1-5 Δalg5*, lanes 4–6) transformed with the vector YEp352 (YEp), pOST5 or pOST1 by immunoblot. The positions of mCPY and the different glycoforms lacking one to all four *N*-linked oligosaccharides (–1 to –4) are indicated. Strains SS328 (wt), YG434 (*ost1-5*) and YG357 (*Δalg5*) are shown for comparison.

Δost5. The *stt3-3* allele confers only a mild underglycosylation of glycoproteins *in vivo*. Whereas both single mutants have no growth phenotype at all, the combination of the two is lethal under all conditions tested (Table II). On tetrad dissection plates, microcolonies arrested at the 10–20 cell stage were observed. Sporulation in the presence of a plasmid carrying the *STT3* locus abolished lethality (data not shown). To exclude a general sensitivity of the *stt3-3* allele to cumulative underglycosylation, we also tested the synthetic interaction between *Δost3* and *stt3-3*. A double mutant *Δost3 stt3-3* is viable and results solely in a temperature-sensitive phenotype at 37°C (Table II). Although loss of Ost5p or loss of Ost3p cause similar reductions in OTase activity *in vivo*, the interaction of these two proteins with the *STT3* protein must be quite different given the strikingly distinct phenotypes of the respective double mutants.

Discussion

We have described the identification and functional characterization of *OST5*, a novel *S.cerevisiae* gene encoding a subunit of the yeast OTase complex. The *OST5* locus was identified in a screen based on the lowered efficiency of *N*-linked glycosylation upon transfer of an unglucosylated core oligosaccharide to protein. We identified predominantly mutants with altered OTase activity (seven out of 11 complementation groups). In the remaining four groups (containing a total of 10 different mutant isolates), we expect to find mutant strains which do not tolerate general hypoglycosylation of proteins imposed by the *Δalg5* mutation. We did not analyze these groups in more detail. With respect to the OTase, our screen represents all loci previously reported to be required for OTase function *in vivo*, except for *OST4* (Chi *et al.*, 1996), encoding a small 3.4 kDa protein. The extremely small target size of

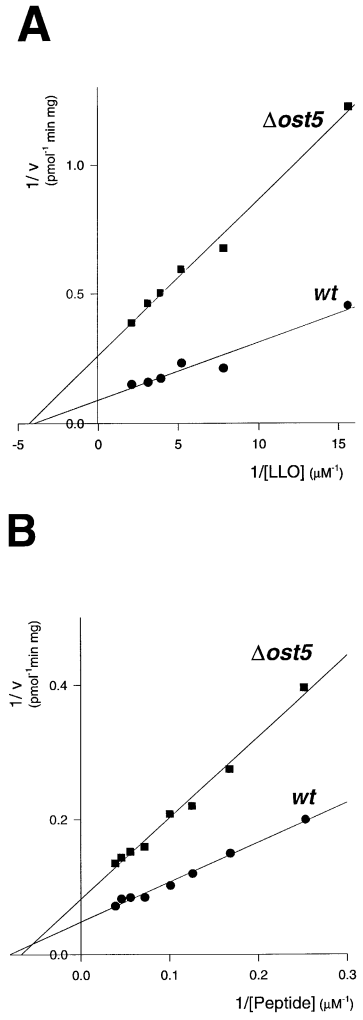


Fig. 4. *In vitro* OTase activity in extracts from a Δ*ost5* strain. The activity of isogenic strains YG508 (Δ*ost5*) and SS328 (wt) was assayed in solubilized microsomal membrane preparations using exogenous LLO and ^{125}I-labeled acceptor peptide. Two independent membrane preparations were tested for each strain. (**A**) Lineweaver–Burk diagram demonstrating substrate dependence of enzymatic activity for LLO. The peptide concentration was kept constant at 5 μM. Specific activities (1/*V*) are the mean values from triplicate measurements. The K_m value for the LLO substrate was 0.2 μM. (**B**) Lineweaver–Burk diagram demonstrating substrate dependence of enzymatic activity for the acceptor tripeptide. The LLO concentration was kept constant at 0.5 μM. The K_m value for the peptide substrate was 10 μM.

the *OST4* locus for mutagenesis is the likely reason for the absence of mutant *ost4* alleles in our screen. In summary, based on the composition of the purified yeast OTase complex and genetic screens, eight different loci have now been identified to be required for optimal OTase activity.

Analysis of the *OST5* coding sequence at the genomic and the transcript level revealed an intron-containing gene and predicted a gene product of 86 amino acids. In the yeast genome sequencing project, prediction of coding regions is confined to a minimal size of 100 amino acids. Thus, our functional approach was essential for the identification of such a small gene. Based on the finding that Ost5p is present in a purified enzyme preparation (Kelleher and Gilmore, 1994) and that its deletion results in a reduced OTase activity both *in vivo* and *in vitro*, we conclude that Ost5p is a component of the OTase complex. Ost5p is not required for the assembly of the LLO substrate (data not shown). Like all other OTase subunits, Ost5p is predicted to be an integral membrane protein and, like the other small subunits, *OST4* (3.5 kDa) and *OST2* (16 kDa), lacks significant hydrophilic domains. Based on the N-terminal sequence of the mature *OST5* protein, we predict a transmembrane protein with two membrane-spanning domains.

Table II. Synthetic interaction of mutations affecting OTase activity

	23°C	30°C	37°C
stt3-3	+	+	+
Δ*ost5*	+	+	+
Δ*ost3*	+	+	+
Δ*ost5* Δ*ost3*	+	+	−
stt3-3 Δ*ost3*	+	+	−
stt3-3 Δ*ost5*	−	−	−

Strains carrying the individual mutation were crossed, segregants were germinated on YPD + 1 M sorbitol medium at 23°C and tested for growth (+ or –) on YPD medium lacking sorbitol at the temperature indicated. *stt3-3* Δ*ost5* double mutants are not viable.

The requirement of OTase for Ost5p is not absolute. Absence of this protein does not result in a growth phenotype and only a minor hypoglycosylation of glycoproteins is observed. This contrasts sharply to the severe or lethal defect conferred by deletions of most of the other OTase loci, with the exception of *OST3*. What is the role of the Ost5p in the OTase complex? A first indication might come from our genetic analysis. The observed suppression of the *ost1* mutation by overexpression of Ost5p suggests a direct interaction between the two proteins. This suppression was *OST5* specific because overexpression of all other OTase components (except for *OST1*) was not effective. Conversely, overexpression of *OST5* did not rescue OTase mutations other than *ost1*. Clearly, Ost5p is not required for the stability of Ost1p in the complex because the level of Ost1p is normal in Ost5p-depleted strains (Figure 2B) and we did not observe an instability of the OTase *in vitro* activity in Ost5p-depleted extracts. In addition, our *in vitro* analysis suggests that loss of Ost5p from the OTase does not alter the affinity either for the LLO or for the peptide substrate. However, the specific suppression of *ost1-5* by overexpression of Ost5p suggests a direct interaction between the two proteins. It is possible that Ost5p supplies an environment in the membrane required for optimal activity of the essential Ost1p subunit. In addition, Ost5p seems to be required for optimal assembly of the OTase complex. This activity might be essential in an *stt3-3* background because, as we postulated previously (Zufferey *et al.*, 1995), Stt3p is required for the assembly of the OTase complex.

Biochemical and genetic data show that the OTase complex is composed of several hydrophobic membrane proteins. Some of them, as for example Ost4p and Ost5p, are very small. We note that the presence of small hydrophobic proteins is not uncommon in multi-subunit membrane protein complexes. Examples are the mammalian signal peptidase complex [SP12 (Kalies and Hartmann, 1996)], the translocation complexes of the ER [Sbh1p (Finke *et al.*, 1996)] and of mitochondria [Isp6p (Kassenbrock *et al.*, 1993)] in yeast, and the bacterial

Table III. Yeast strains used in this study

Strain	Genotype	Reference
SS328	MATα *ade2-101 ura3-52 his3Δ200 lys2-801*	Vijayraghavan *et al.* (1989)
SS330	MAT**a** *ade2-101 ura3-52 his3Δ200 tyr1*	Vijayraghavan *et al.* (1989)
YG175	MAT**a** *ade2-101 ura3-52 his3Δ200 tyr1 stt3-3*	Zufferey *et al.* (1995)
YG234	MATα *ade2-101 ura3-52 his3Δ200 tyr1 Δost3::HIS3 stt3-3*	this report
YG250	MAT**a** *ade2-101 ade3 ura3-52 his3Δ200 tyr1 leu2-112 Δalg5::HIS3 pCH1122AL65*	this report
YG251	MATα *ade2-101 ade3 ura3-52 his3Δ200 lys2 leu2-112 Δalg5::HIS3 pCH1122AL65*	this report
YG356	*ade2-101 ura3-52 his3Δ200 lys2-801 tyr1*	this report
YG357	*ade2-101 ura3-52 his3Δ200 lys2-801 tyr1 Δalg5::HIS3*	this report
YG358	*ade2-101 ura3-52 his3Δ200 lys2-801 Δost5::HIS3*	this report
YG359	*ade2-101 ura3-52 his3Δ200 lys2-801 Δalg5::HIS3 Δost5::HIS3*	this report
YG434	*ade2-101 ura3-52 his3Δ200 tyr1 ost1-5*	this report
YG435	*ade2-101 ura3-52 his3Δ200 lys2-801 Δalg5::HIS3 ost1-5*	this report
YG508	MATα *ade2-101 ura3-52 his3Δ200 lys2-801 Δost5::HIS3*	this report
YG511	MAT**a** *ade2-101 ade3 ura3-52 his3Δ200 lys2-801Δalg5::HIS3 ost5-1*	this report
YG513	MAT**a** *ade2-101 ura3-52 his3Δ200 Δost3::HIS3 Δost5::HIS3*	this report

cytochrome *c* oxidase [SU IV (Iwata *et al.*, 1995)]. These small subunits are dispensable for catalytic function of the respective protein complex. In the case of cytochrome *c* oxidase, the structure of the complex revealed that subunit IV is not involved in the formation of the catalytic center and it was suggested that this subunit is required for the stability of the complex (Iwata *et al.*, 1995). Most interestingly, in *S.cerevisiae*, the 8.5 kDa ER membrane protein Vma21p was found to be necessary for the assembly of the vacuolar ATPase in the ER (Hill and Stevens, 1994). It might be a general role of such small subunits to contribute to the assembly or stability of membrane protein complexes.

Materials and methods

Yeast strains and media

Yeast strains used are listed in Table III. Standard yeast media and genetic techniques (Guthrie and Fink, 1991) were used.

Isolation of mutants synthetically lethal with Δalg5

A liquid culture of either strain YG250 (MATa *ade2 ade3 tyr1 his3 leu2 ura3 Δalg5*) or YG251 (MATα *ade2 ade3 lys2 his3 leu2 ura3 Δalg5*) grown for 2 days to stationary phase at 30°C in minimal medium lacking uracil was mutagenized with ethylmethane sulfonate (Sigma) to 10–40% survival as described (Lawrence, 1991). Both strains harbor the pCH1122-ALG5 plasmid obtained by cloning the *ALG5* locus as a *Sal*I–*Bam*HI fragment (te Heesen *et al.*, 1994) into the *Nru*I–*Sal*I site of the URA3 ADE3 plasmid pCH1122 (Kranz and Holm, 1990). Cells were plated on YPD to yield a density of ~150 colonies per plate. Plates were incubated at 30°C for 3 days and then shifted to room temperature to permit full color development. Non-sectoring colonies were picked and retested twice for the non-sectoring phenotype when grown on YPD at 30°C. Finally, they were tested for growth on minimal medium containing 5-FOA (Boeke *et al.*, 1984) at 30°C. Strains which did not grow on 5-FOA-containing medium were classified into complementation groups. For complementation analysis, individual strains of the two mating types were mated on YPD, replica-plated onto minimal medium selective for the resulting diploid strains and, after growth of diploids, further replica-plated onto 5-FOA medium. Growth of the diploid cells on 5-FOA was taken as a complementing phenotype. To identify *ADE3*-dependent complementation groups, a plasmid shuffling procedure was performed. Representative strains of each complementation group were transformed with the *LEU2* plasmid pRS315 carrying the *ALG5* locus on a *Sal*I–*Bam*HI fragment. Mutants that regained the ability to sector on YPD medium and to grow on 5-FOA medium after transformation with pRS315-ALG5 were chosen for further analysis.

Isolation of the OST5 locus

A genomic library (Stagljar *et al.*, 1994) containing partially digested yeast chromosomal DNA of 4–8 kb size ligated into the vector YEp352 (Hill *et al.*, 1986) was transformed into the strain YG511 and transformants were selected at 23°C on minimal medium containing 1 M sorbitol but lacking uracil. Approximately 10^4 transformants were tested for growth at 37°C by replica plating. Plasmid DNA from positive colonies was recovered by extracting total yeast DNA (Guthrie and Fink, 1991) which was used to transform *Escherichia coli* strain DH5α to ampicillin resistance. Plasmids were tested by re-transformation into strain YG511, grouped by their restriction pattern and a representative plasmid insert was sequenced.

RT–PCR

Yeast total RNA was isolated according to Rose *et al.* (1990). Reverse transcription was done for 1 h at 45°C using Superscript™ polymerase (Gibco) and the specific primer 5′-CGTAGACACCGAAAGA-3′. The 25 μl reaction contained 6 μg of RNA, 20 U of RNase inhibitor (MBI), 100 pmol of primer, 400 μM of each dNTP and 150 U of Superscript™ reverse transcriptase in the commercial buffer. A 4 μl aliquot of the reaction mix served as the template in a subsequent PCR using the oligonucleotide 5′-CTTATGAACAATTATAC-3′ as the second primer. The PCR was performed in a 100 μl volume in the presence of 10 mM $MgCl_2$, 50 mM KCl, 0.1% Tween 20, 20 mM Tris–HCl pH 8.4, 50 pmol of each primer, 100 μM of each dNTP and 1 U of Supertaq DNA polymerase over 35 cycles (1 min 95°C, 1 min 41°C, 1.5 min 72°C). Gel analysis of the PCR reaction demonstrated a distinct band of 240 bp which was cloned into the vector YEp352 cleaved by *Ecl*136II and sequenced.

Inactivation of the OST5 locus

PCR amplification from a 1.8 kb *Bam*HI cassette carrying the *S.cerevisiae HIS3* gene (Struhl and Davis, 1981) with primers 5′-GAG CTT CCA GCC ATT TAT CCA CTT AGA TAC TCA ACC TCT TGG CCT CCT CTA G-3′ and 5′-CAG TAG TCA AGC CAG CAA ACA AGC TGC CAA TTA CAT CGT TCA GAA TGA CAC G-3′ generated a 1 kb *HIS3* cassette (Baudin *et al.*, 1993) flanked by 35 bp stretches homologous to the *OST5* coding sequence. The linear fragment was used to transform strain SS328 to His^+. Correct integration at the *OST5* locus was confirmed by whole cell PCR (Sathe *et al.*, 1991) using *OST5*- and *HIS3*-specific primers.

Protein immunoblots

Yeast cultures were grown overnight at the permissive temperature (23 or 30°C) in either supplemented minimal or YPD medium. Four $OD_{546\,nm}$ units of logarithmically growing cells were collected, suspended in 100 μl of lysis buffer [50 mM Tris–HCl, pH 7.5, 1% SDS, 200 mM phenylmethylsulfonyl fluoride (PMSF)] and broken by the glass beads method as described (Franzusoff *et al.*, 1991). The final extract was mixed with 30 μl of sample buffer (0.25 M Tris–HCl pH 6.8, 8% SDS, 20% β-mercaptoethanol, 50% glycerol, 0.1% bromphenol blue) and boiled for 5 min. Samples (20–30 μl) were applied to 8% SDS–PAGE and transferred onto nitrocellulose. Blots were probed for CPY with rabbit antiserum raised against deglycosylated CPY and visualized with peroxidase-labeled protein A using enhanced chemiluminescence (ECL, Amersham).

For detection of OST1p, ~50 μg of microsomal protein was loaded

per lane. Microsomal membranes were prepared as described (Silberstein *et al.*, 1995a) and the protein concentration was determined (Sailer and Weissmann, 1991).

Oligosaccharyltransferase assay

Microsomal membranes were prepared according to Knauer and Lehle (1994) from 4 l YPD cultures of log phase cells ($OD_{546\ nm}$ 1.6–2.3) grown at 30°C with the following modifications. The cells were pelleted, washed once in 50 mM Tris–HCl/1 mM $MgCl_2$/1 mM $MnCl_2$ (pH 7.4) and lysed in the same buffer containing 1 mM dithiothreitol (DTT) and protease inhibitors (1 mM PMSF, 2 μg/ml each of pepstatin A, leupeptin, chymostatin, antipain, aprotinin) under constant cooling on ice with the Bead-Beater (BioSpec, Oklahoma) by six 1 min pulses with a 1 min break in between. The lysate was centrifuged for 8 min in a Sorvall GS3 rotor at 2000 r.p.m. and the supernatant recentrifuged at 22 000 r.p.m. in a Beckman Ti45 rotor. The membrane pellet was washed once in the same buffer. The membranes were suspended in 3–4 ml of membrane buffer (50 mM Tris–HCl/1 mM $MgCl_2$/1 mM $MnCl_2$/1 mM DTT/35% glycerol (pH 7.5)/protease inhibitors as above) yielding a concentration of 20–40 mg/ml protein as determined by the Bio-Rad protein assay using bovine serum albumin as a standard. Extracts were frozen in liquid nitrogen and stored at –80°C. For use in the OTase assay, the membrane protein concentration was adjusted to 10 mg/ml with membrane buffer. LLOs were extracted from 340 g of bovine pancreas as described by Spiro *et al.* (Spiro *et al.*, 1976; Badet and Jeanloz, 1988). The LLOs were dissolved in 40 ml of chloroform/methanol/water 10:10:3 (by vol.), separated from insoluble material by centrifugation in a Sorvall HB-6 rotor at 10 000 r.p.m. at room temperature and stored at –20°C. The DEAE purification step was omitted. The assay of OTase was performed as follows (Das and Heath, 1980; Kelleher *et al.*, 1992); 15 μl of LLOs were dried in a Savant Speedvac and suspended in 75 μl of 50 mM Tris–HCl/25 mM NaCl/3 mM $MnCl_2$/140 mM sucrose/0.2% NP-40/1 mM DTT. Insoluble material was separated by centrifugation, followed by the addition of 10 μl of membrane buffer, 5 μl of iodinated peptide (500 pmol/5–7×10^6 c.p.m.) and 10 μl (100 μg) of membrane protein. After incubation at 23°C for 20 min, the reaction was stopped on ice by the addition of 100 μl of 2% NP-40. Glycosylated peptide was recovered as described below. As a control, membranes were inactivated prior to incubation by heating for 5 min to 95°C. Based on assays with limiting substrate, the concentration of LLO in the assay was estimated to be ~0.5 μM.

Radiolabeling of acceptor peptide and recovery of glycosylated peptide

The terminally acetylated and amidated tripeptide Nα-Ac-NYT-NH_2 (Wieland *et al.*, 1987) was purchased from Tana Laboratories, Houston and iodinated as described previously (Kelleher *et al.*, 1992) except that the amount of chloramine T was doubled. Recovery of glycosylated, ^{125}I-labeled peptide from the enzyme assay started by adding 1 ml of ice-cold wash buffer (50 mM Tris–HCl pH 7.4, 1 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.01% NP-40) to the assay mixture followed by 100 μl of concanavalin A–Sepharose beads (Pharmacia, freshly suspended 1:1 in wash buffer). The tubes were incubated for 20 min on a rotating wheel at 4°C and the beads subsequently were washed three times with 1 ml of buffer. The radioactivity retained was quantified with a Cobra™ (Packard) gamma counter.

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