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The 3.4-kDa Ost4 protein is required for the assembly of two distinct oligosaccharyltransferase complexes in yeast

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In the central reaction of N-linked glycosylation, the oligosaccharyltransferase (OTase) complex catalyzes the transfer of a lipid-linked core oligosaccharide onto asparagine residues of nascent polypeptide chains in the lumen of the endoplasmic reticulum (ER). The *Saccharomyces cerevisiae* OTase has been shown to consist of at least eight subunits. 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 (wt) complex, including Stt3p, Ost1p, Wbp1p, Swp1p, Ost3p, and Ost6p. We demonstrate that the small 3.4-kDa subunit Ost4p is required for the incorporation of either Ost3p or Ost6p into the complex, resulting in two, functionally distinct OTase complexes *in vivo*. Ost3p and Ost6p are not absolutely required for OTase activity, but modulate the affinity of the enzyme toward different protein substrates.

Key words: blue native gel electrophoresis/N-linked protein glycosylation/oligosaccharyltransferase/*Saccharomyces cerevisiae*

Introduction

In the secretory pathway of eukaryotic cells, the transfer of the oligosaccharide Glc₃Man₉GlcNAc₂ 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 sequence Asn-X-Ser/Thr, where X can be any amino acid except proline (Tanner and Lehle, 1987; Cummings, 1992; Herscovics and Orlean, 1993; Karaoglu, *et al.*, 1997; Spirig, *et al.*, 1997; Burda and Aebi, 1999; Kornfeld *et al.*, 1999). This transfer is catalyzed by the enzyme complex oligosaccharyltransferase (OTase) (for review see Silberstein and Gilmore, 1996; Knauer and Lehle, 1999; Dempski and Imperiali, 2002; Yan and Lennarz, 2005b). Whereas the purification of active OTase from endoplasmic reticulum (ER) membranes of higher eukaryotes showed that OTase activity is associated with a protein complex consisting of STT3-A/B, ribophorin I, ribophorin

II, OST48, and DAD1 (Kelleher and Gilmore, 1997; Kelleher *et al.*, 2003), purified yeast OTase consists of at least six different subunits (Kelleher and Gilmore, 1994). The yeast OTase 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) (te Heesen *et al.*, 1993; Kelleher and Gilmore, 1994), Ost2p (DAD1) (Silberstein *et al.*, 1995a), Ost3p (Karaoglu *et al.*, 1995), and Ost5p (Reiss *et al.*, 1997).

In addition to these six proteins, genetic screens have identified two other loci, *STT3* and *OST4* that are required for full OTase activity *in vivo* (Zufferey *et al.*, 1995; Chi *et al.*, 1996). *OST4* encodes 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 (Chi *et al.*, 1996). Additional evidence suggests that Ost4p is a component of the OTase complex (Karaoglu *et al.*, 1997; Spirig *et al.*, 1997; Kim *et al.*, 2003). 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* (Zufferey *et al.*, 1995). Recent experiments show that Stt3p is also a structural component of the yeast OTase complex (Karaoglu *et al.*, 1997; Spirig *et al.*, 1997). Ost3p and Ost4p have been proposed to act together in a subcomplex with the OTase subunit Stt3p (Karaoglu *et al.*, 1997; Spirig *et al.*, 1997; Knauer and Lehle, 1999; Kim *et al.*, 2003). Furthermore, the overexpression of either Ost3p or Ost4p in defined *stt3* mutant strains restores the growth of the strains at 37°C and improves glycosylation of carboxypeptidase Y (CPY) (Spirig *et al.*, 1997).

A search in the available databases for homologues of the nonessential Ost3p revealed a hypothetical 37-kDa protein, now termed Ost6p, with 46% amino acid sequence homology and 21% sequence identity to Ost3p (Knauer and Lehle, 1999). 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. It was shown that this protein is indeed part of the OTase complex (Knauer and Lehle, 1999a). Functional OTase complexes from *Saccharomyces cerevisiae* have been isolated that do not contain all the components described above but retain transferase activity *in vitro* (Knauer and Lehle, 1994; Pathak and Imperiali, 1997). Based on sequence similarity, two mammalian homologs of Ost3/Ost6 have been identified (Kelleher *et al.*, 2003).

We analyzed the OTase complex by blue native polyacrylamide gel electrophoresis that allows the separation of proteins and protein complexes under native conditions (Schägger and von Jagow, 1991; Schägger *et al.*, 1994,

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1995). We were able to confirm 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 was needed for the incorporation of either Ost3p or Ost6p into the complex. Furthermore, using a combination of genetic techniques and blue native electrophoresis, we showed that there are two different OTase complexes in yeast only differing by the presence of Ost3p and Ost6p. These two complexes have different functions *in vivo*.

Results

The yeast OTase complex visualized by blue native gel electrophoresis

Blue native gel electrophoresis is a powerful technique for the isolation and characterization of native-protein complexes from biological membranes (Schägger and von Jagow, 1991). To analyze the native form of the OTase in wild-type (wt) 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 wt yeast cells were supplemented with Serva blue G and subsequently separated on polyacrylamide gels using a 5–12% acrylamide gradient. After transfer to nitrocellulose membrane, 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 (Figure 1) by comparing the mobility of the complex with those of thyroglobulin and apoferritin. This complex was termed wt OTase complex I. Antibodies against the largest OTase subunit Stt3p detected a protein complex running at the same position (Figure 1, lane 1), confirming that Stt3p is a component of the yeast OTase (Karaoglu *et al.*, 1997; Spirig *et al.*, 1997). In addition, antibodies against Ost6p (Knauer and Lehle, 1999a) revealed a complex running at the same position as well (Figure 1, lane 6), indicating that Ost6p is indeed a component of the OTase complex. Blots were also incubated with antibodies directed against a component of the protein-translocation machinery, Sec61p (Figure 1, lane 9), as well as antibodies against Gas1p, a glycopospholipid-anchored surface glycoprotein (data not shown). Both antibodies revealed proteins running at a position different from the complexes detected by antibodies against OTase subunits, indicating that complex I was not an unspecific aggregate 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 OTase complex (Spirig *et al.*, 1997). In addition, this complex was destroyed upon the addition of sodium dodecyl sulfate (SDS) (Figure 1, lanes 7 and 8), demonstrating the detergent-sensitivity of the complex. These data suggested that complex I represented the native OTase complex.

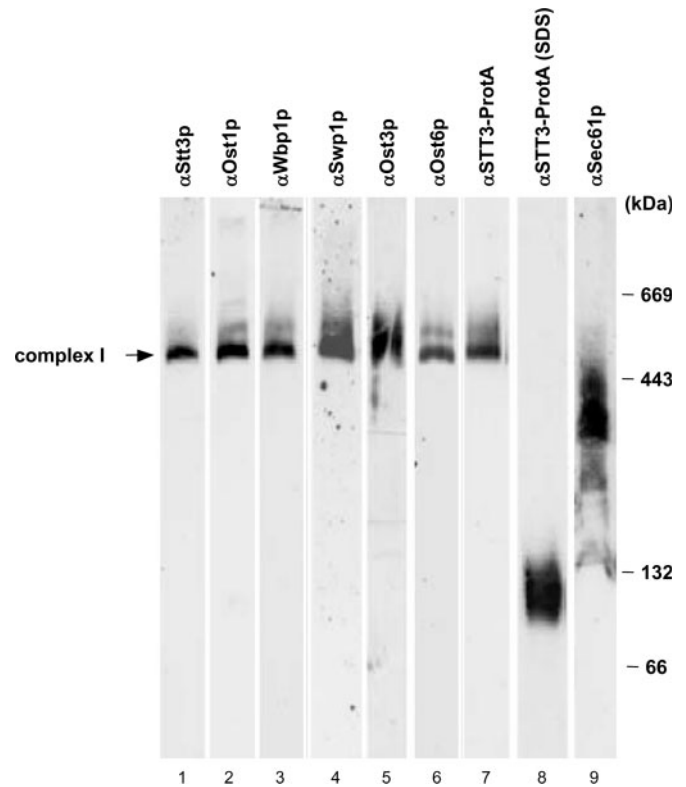


Fig. 1. The wild-type (wt) yeast OTase complex analyzed by blue native gel electrophoresis. SS328 wt (lanes 1–6 and 9) and YG469 cells (*STT3-ProtA*, lanes 7 and 8) were grown at 30°C. Membranes were prepared, and proteins were solubilized with 1.5% digitonin or 2% SDS (lane 8). About 40 µg of solubilized membrane proteins were analyzed by blue native gel electrophoresis by 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 (α Stt3p, α Ost1p, α Wbp1p, α Swp1p, α Ost3p, and α Ost6p) (lanes 1–6), or against PAP (a soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase) (lanes 7 and 8), or against the translocon component α Sec61p (lane 9). The positions of marker proteins (albumin, monomeric, 66 kDa, and dimeric form, 132 kDa; apoferritin, 443 kDa; and thyroglobulin, 669 kDa) are indicated. The position of wt OTase complex I is indicated by arrow.

Ost4p was required for recruiting either Ost3p or Ost6p into OTase complex

To demonstrate that this protein complex I visualized by blue native gel electrophoresis was functionally linked with OTase activity, we analyzed complex composition in mutant cells carrying a deletion of the *OST4* locus or the *OST3* locus. 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 (Chi *et al.*, 1996).

In a screen for high-copy suppression of the Δ *ost4* temperature-sensitive phenotype, we recovered plasmids encoding both *OST3* (Spirig *et al.*, 1997) and *OST6*. pOST3 gave a strong suppression of the temperature-sensitivity, whereas pOST6 suppressed to a lesser extent the ts phenotype (Figure 2A). Correspondingly, the overexpression of

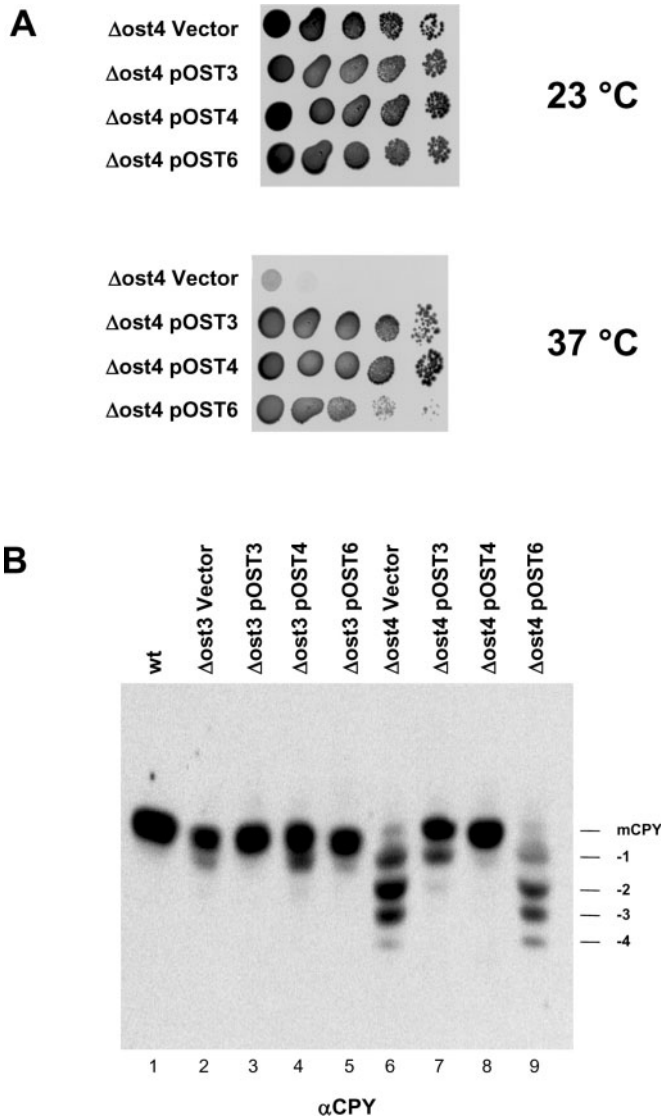


Fig. 2. *OST3* and *OST6* are high-copy number suppressors of an *OST4* deletion. **(A)** YG493 ($\Delta ost4$) cells harboring either the vector YEp352 or the high-copy number plasmids containing the loci *OST3* (pOST3), *OST4* (pOST4), or *OST6* (pOST6) were grown at 23°C to mid-log phase in liquid minimal medium lacking uracil. Serial 1:10 dilutions starting at 5×10^5 cells were spotted onto plates containing minimal medium lacking uracil. The plates were incubated for 4 days at 23°C or 37°C and photographed. **(B)** YG191 ($\Delta ost3$) and YG493 ($\Delta ost4$) 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. Wild-type (wt) SS328 cells were grown at 23°C to mid-log phase in complete medium. Protein extracts were prepared, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), 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.

OST3 in the $\Delta ost4$ strain yielded nearly normal glycosylation of CPY, whereas the overexpression of *OST6* did not improve the hypoglycosylation in the $\Delta ost4$ strain (Figure 2B, lanes 6–9). Cells containing a deletion of *OST3* are fully viable and show only modest changes in glycosylation

compared with wt cells (Karaoglu *et al.*, 1995) (Figure 2B, lane 2). Although 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, was further the evidence of a functional interaction between Ost3p and Ost4p.

We therefore examined the OTase complex in $\Delta ost4$ strains and $\Delta ost4$ strains overexpressing Ost3p or Ost6p. Owing to the observed interaction between Ost4p and Ost3p, a parallel set of $\Delta ost3$ strains containing the vector, pOST3, pOST4, or pOST6, was also examined. Membranes from each of these strains were solubilized, and proteins were subjected to blue native gel electrophoresis. Using α Ost1p antibody, very low levels of complex I were detected in $\Delta ost4$ cells, but instead, a complex that migrated faster was revealed (Figure 3A, lane 1). Complex I was restored in the $\Delta ost4$ cells that were complemented with pOST4 (Figure 3A, 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 the overexpression of Ost3p in $\Delta ost4$ cells (Figure 3A, lane 2) and, to a lesser extent, by the overexpression of Ost6p (Figure 3A, lane 4). These results were consistent with the strong suppression of the temperature-sensitivity (Figure 2A) and hypoglycosylation phenotypes (Figure 2B) observed when Ost3p was overexpressed and with the weaker suppression seen by overexpression of Ost6p in a $\Delta ost4$ strain. These data provided a functional link between OTase activity and complex I and suggested that complex I represented the active OTase complex. Complex II was also detected in $\Delta ost4$ cells and $\Delta ost4$ cells overexpressing either Ost3p or Ost6p by 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 $\Delta ost3$ strains, using the α Ost1p antibody. Interestingly, the $\Delta ost3$ strain contained both complexes, and the overexpression of *OST4* did not change the composition of the complexes (Figure 3B, 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 (Figure 3B, lanes 3 and 5). In contrast, the $\Delta ost6$ strain produced only complex I (data not shown).

When α Ost6p antibody was used to analyze the OTase complexes in the set of $\Delta ost3$ and $\Delta ost4$ 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 wt cells yielded a signal with α Ost6p antibodies, indicating that at least a fraction of wt complex I did contain Ost6p (Figure 3C, lane 1). The complementation of $\Delta ost4$ with pOST4 led to a complex I that contained Ost6p (Figure 3C, lane 8), whereas the complex I that was detected in $\Delta ost4$ strains transformed with pOST3 (Figure 3A, lane 2) did not produce a signal with α Ost6p antibody and thus lacked Ost6p (Figure 3C, lane 7). The small amount of complex I seen after overexpression of Ost6p in the $\Delta ost4$ strain also contained Ost6p (data not shown). These results suggested that there were two forms of complex I, one containing and one

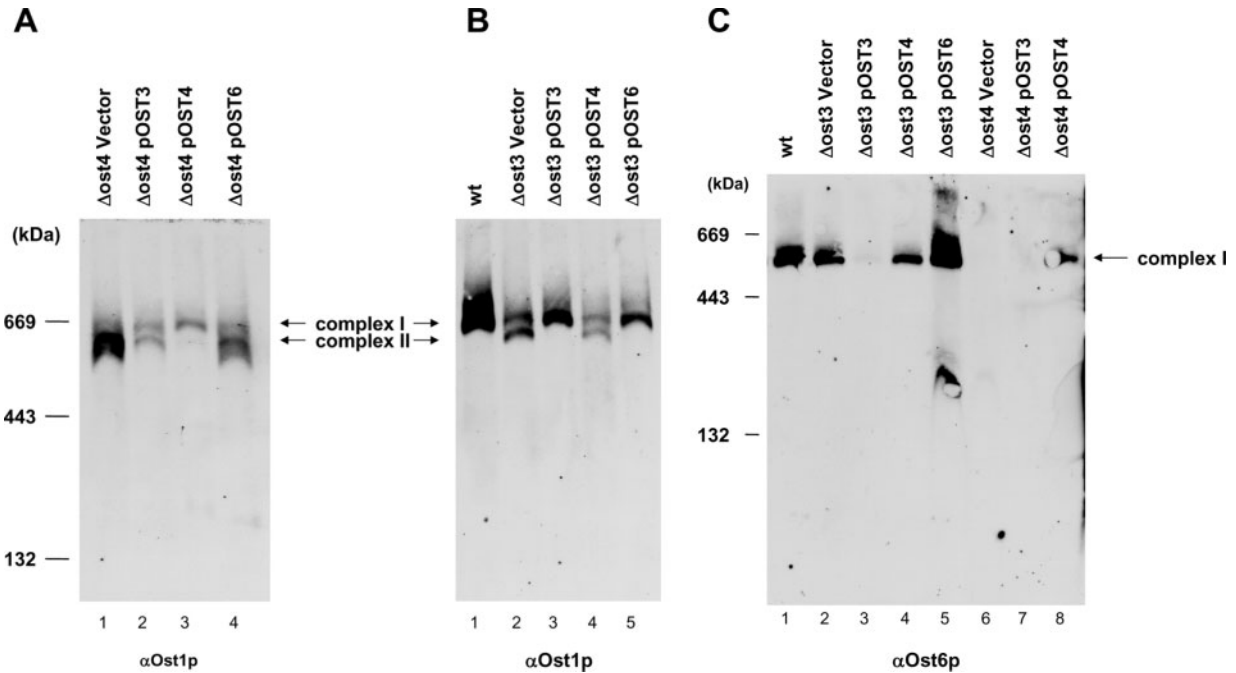


Fig. 3. Analysis of OTase complexes in $\Delta ost3$ and $\Delta ost4$ cells. SS328 wild-type (wt) cells were cultured in complete medium at 30°C to mid-log phase. YG191 ($\Delta ost3$) and YG493 ($\Delta ost4$) cells harboring either the vector or the high-copy number plasmid pOST3, pOST4, or pOST6 were grown in liquid minimal medium lacking uracil at 30°C ($\Delta ost3$) or at 23°C ($\Delta ost4$) 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, and proteins were blotted onto nitrocellulose and detected with α Ost1p antibodies (A and B) or with α Ost6p antibodies (C). The relevant genotypes of the different strains and the high-copy number plasmids present are indicated above each lane. The positions of OTase complexes I and II are indicated by arrows. Marker proteins are as in the legend to Figure 1.

lacking Ost6p. This heterogeneity was also seen in the $\Delta ost3$ strains. The complex I seen in $\Delta ost3$ cells and $\Delta ost3$ cells overexpressing *OST4* contained Ost6p (Figure 3C, lanes 2 and 4). In contrast, the complex I formed in $\Delta ost3$ cells complemented by pOST3 did not contain Ost6p (Figure 3C, lane 3). Because the $\Delta ost3$ /vector and $\Delta ost3$ /pOST3 strains differed only by the overexpression of Ost3p and yet showed different forms of complex I, this result argued that the overexpression of Ost3p precluded Ost6p from taking part in complex I. We therefore conclude that there are two different complexes I in yeast. One form contains Ost3p and lacks Ost6p (termed complex Ia), and a second form contains Ost6p and lacks Ost3p (complex Ib). Ost4p is required for the stable integration of either of these proteins into complex I, because complex I was hardly detected in $\Delta ost4$ cells. Because overexpression of either Ost3p or Ost6p in a $\Delta ost4$ strain resulted in complex I, we concluded that there are parallel pathways that led either to the formation of complex Ia or complex Ib.

Two OTase complexes with different functions *in vivo*

To test the hypothesis of two different OTase complexes more directly, $\Delta ost3\Delta ost6$ double mutant strains were generated and transformed with a multicopy plasmid carrying either the *OST3* or the *OST6* locus. Solubilized membrane proteins of these cells were separated by blue native gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membrane, and the OTase complexes were analyzed

using α Ost1p antibody (Figure 4). The $\Delta ost3\Delta ost6$ double mutant strains did not produce complex I but only low levels of complex II (Figure 4, lane 2), whereas in both strains overexpressing either Ost3p or Ost6p wt levels of complex I were detected (Figure 4, lanes 3 and 4, respectively). Comparable levels of either complex Ia or complex Ib were present in these two strains. Therefore, these strains represented ideal tools to test if complexes Ia and Ib fulfill different functions *in vivo*. First, strains were tested for a temperature-sensitive phenotype. $\Delta ost3\Delta ost6$ strains did show a severe temperature-sensitive phenotype at 39°C, a temperature both wt and complex Ib-expressing strains were viable but not strains expressing only complex Ia (Ost3p) (Figure 5). OTase complex Ib was required for growth at elevated temperatures. Next, we analyzed the glycosylation of glycoproteins in these strains (Figure 6). A strain lacking both complex Ia and complex Ib expressed strongly hypoglycosylated proteins, as shown by immunodetection of the glycoproteins CPY, Ost1p, and Wbp1p (Figure 6, lane 2). Therefore, as shown above, complex II showed reduced OTase activity *in vivo*. The strain expressing only OTase complex Ia did not show any alterations of glycosylation with respects to these three proteins examined (Figure 6, lane 3), whereas complex Ib showed a mild hypoglycosylation of Ost1p and Wbp1p (Figure 6, lane 4). These results suggested that glycosylation of proteins by complex Ia OTase was more efficient *in vivo*, at least toward the two membrane proteins tested, as compared with complex Ib. Therefore, we determined the affinity of the different

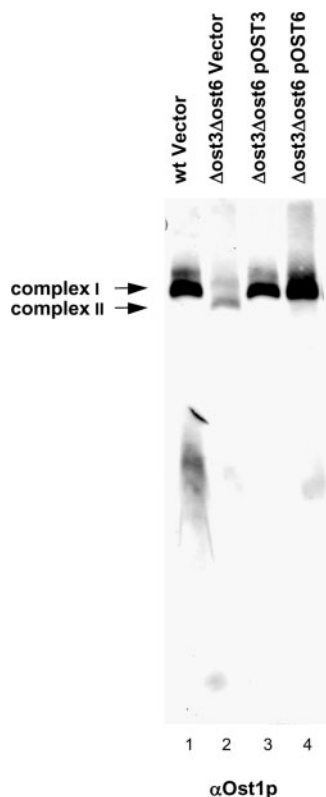


Fig. 4. Normal levels of OTase complex in engineered cells expressing only complex Ia or complex Ib. SS328 wild-type (wt) (lane 1) and YG889 ($\Delta ost3\Delta ost6$) cells harboring either the vector (lane 2) or the plasmid pOST3 (lane 3) or pOST6 (lane 4) were grown in liquid minimal medium lacking uracil at 23°C to mid-log phase. Cells were harvested, membranes prepared and, proteins solubilized in 1.5% digitonin. Protein complexes were separated by blue native gel electrophoresis of 10 μ g protein in a 5–12% gradient gel and blotted onto PVDF membrane. OTase complexes (indicated by arrows) were detected by α Ost1p antibodies.

OTase complexes toward the acceptor peptide *in vitro*. When we tested OTase activity in extracts derived from cells expressing only complex Ia, complex Ib, or complex II, we observed a difference in the K_m values. A slight increase of affinity toward the peptide was observed for complex Ia when compared with complex Ib, confirming the results of the glycosylation studies *in vivo*. For the mixture of the two complexes present in wt-derived extracts, an intermediate value for the apparent K_m was determined. Complex II had a higher K_m than the fully assembled OTase (Table I). In $\Delta ost3\Delta ost6$ cells, a much lower conversion rate was observed, but this low *in vitro* OTase activity correlated with the low level of complex II OTase detected in these extracts (Figure 4, lane 2). We concluded that the hypoglycosylation observed in strains expressing only complex Ib was because of the reduced affinity of this OTase toward the polypeptide substrate.

We tested whether the two OTase complexes differed in their sensitivity toward alterations in the structure of the lipid-linked oligosaccharide (LLO) substrate, as reported for mutations in the Stt3p subunit (Zufferey *et al.*, 1995). At the same time, we examined whether the two complexes act during a different period in glycoprotein maturation in the

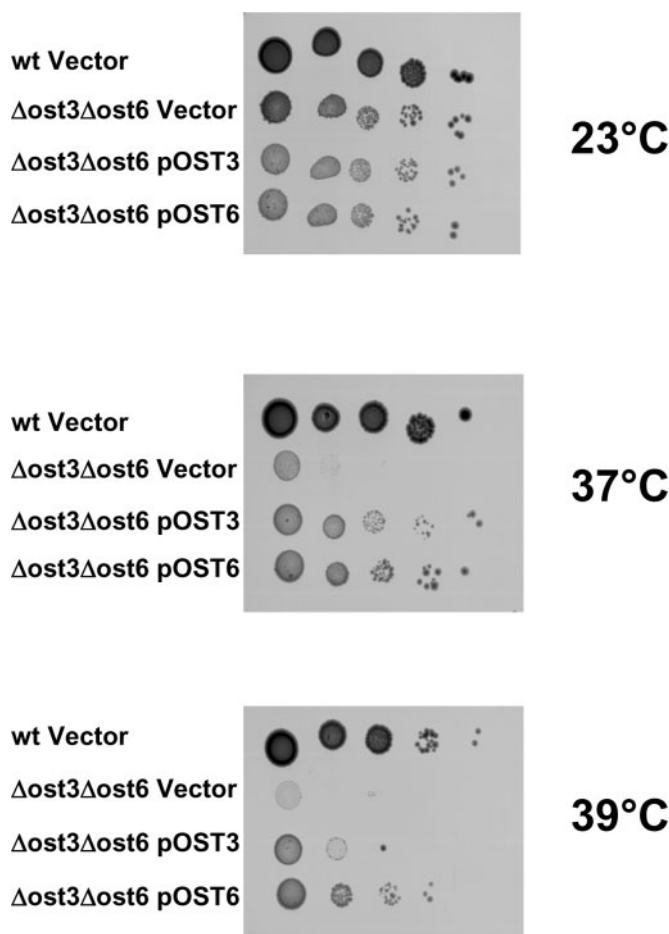


Fig. 5. Cell expressing only OTase complex Ia are temperature sensitive for growth at 39°C. YG889 ($\Delta ost3\Delta ost6$) cells harboring either the vector or the plasmid pOST3 or pOST6 were grown in liquid minimal medium lacking uracil at 30°C to mid-log phase. Serial 1:10 dilutions starting at 5×10^5 cells were spotted onto plates containing minimal medium lacking uracil. The plates were incubated for 4 days at 23°C or 39°C as indicated.

ER. N-linked glycosylation of proteins can occur on the translocating protein (co-translocational glycosylation) but also on a protein that is fully translocated into the lumen of the ER (post-translocational glycosylation). We had reported previously that *alg* mutant cells, unable to synthesize the complete LLO $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, hypoglycosylated glycoproteins because of a reduced affinity of the OTase complex toward the incomplete oligosaccharide substrate (Stagljar *et al.*, 1994). However, when the glycosylation of CPY*, a mutant glycoprotein trapped in the ER because of incomplete folding (Hiller *et al.*, 1996), had been followed in such *alg* mutant cells, and complete glycosylation had been observed, because of post-translocational glycosylation of this malformed protein (Jakob *et al.*, 1998). Therefore, strains deficient in the assembly of complete LLO ($\Delta alg9$) expressing either complex Ia or complex Ib (in this set of experiments, $\Delta ost6$ strains overexpressing OST3 and $\Delta ost3$ strains overexpressing OST6 were used) and mutant CPY* were generated. In cells with normal biosynthesis of the LLO, low steady-state levels of CPY* were observed, indicating efficient degradation of this malformed

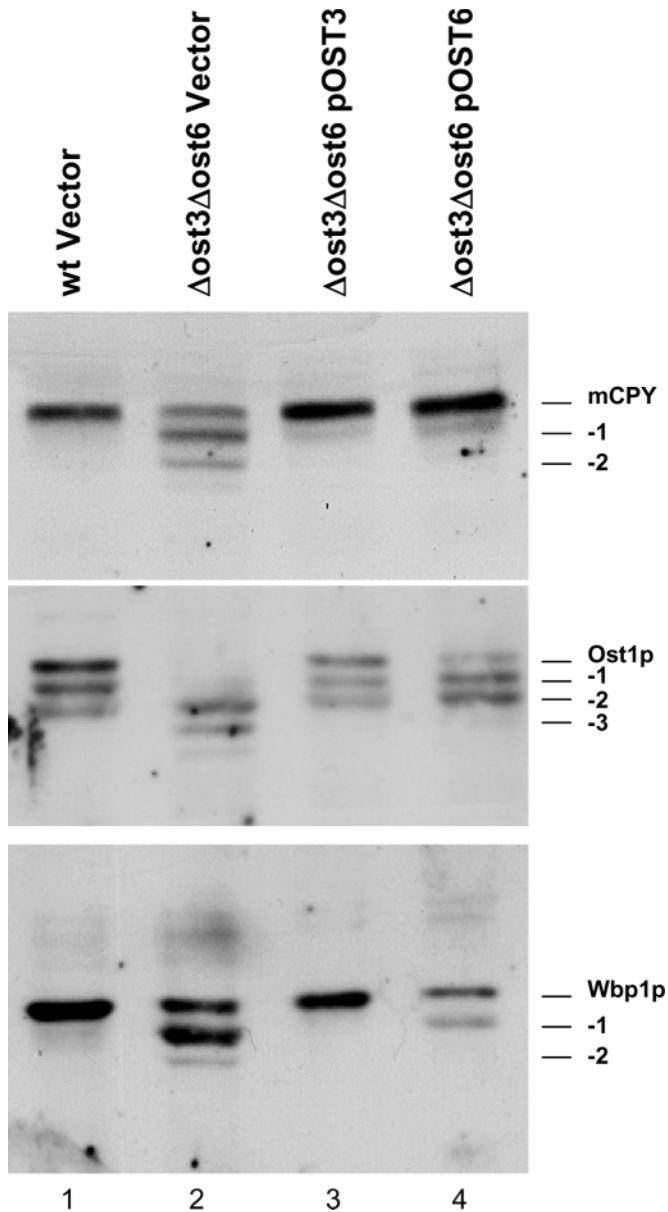


Fig. 6. Reduced glycosylation efficiency in cells expressing OTase complex Ib. SS328 wild-type (wt, lane 1) and YG889 ($\Delta ost3\Delta ost6$) cells harboring either the vector YEp352 (lane 2) or the plasmid pOST3 (lane 3) or pOST6 (lane 4) grown in liquid minimal medium lacking uracil at 30°C to mid-log phase were analyzed. Protein extracts were prepared, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and western blot analysis was performed using CPY- (CPY-) (top), Ost1p- (middle), or Wbp1p- (bottom) specific antibodies. The positions of mature proteins (mCPY, Ost1p, and Wbp1p) and the glycoforms lacking one (–1), two (–2), or three (–3) oligosaccharides are indicated.

protein in the ER (Figure 7, lane 2). In $\Delta alg9$ cells, CPY* was stabilized due to the incomplete oligosaccharide structure required for efficient degradation in the ER (Jakob *et al.*, 1998). As reported previously, this protein is completely glycosylated, and we observed the identical mobility of CPY* in cells expressing only complex Ia or complex Ib (Figure 7, lanes 4 and 5, respectively), indicating that both OTase complexes are competent for posttranslocational

glycosylation. In the same cells, hypoglycosylation of Wbp1p owing to the $\Delta alg9$ mutation was observed. This hypoglycosylation was more pronounced in cells expressing only complex Ib when compared with wt or complex Ia-expressing cells, confirming a reduced glycosylation efficiency of complex Ib *in vivo* when compared with complex Ia.

Discussion

Blue native gel electrophoresis as a tool to analyze native OTase complexes

In the recent years, the OTase was shown to be composed of several different subunits, not only in yeast but also in higher eukaryotes (for review, see Silberstein and Gilmore, 1996; Knauer and Lehle, 1999b). To analyze the OTase complexes accumulating in different OTase mutants, we took advantage of blue native gel electrophoresis that allows the visualization of proteins and protein complexes in their native form. The phenotype of the different mutant strains and the analysis of the corresponding OTase complexes by blue native gel electrophoresis led us to conclude that “native” OTase complexes were indeed resolved by this technique. Thus, blue native gel electrophoresis represented an additional tool to explore the composition, function, and assembly of the OTase. In wt extracts, one band was detected by antibodies against different OTase subunits. This band was termed complex I. We assumed that complex I represented native OTase. Complex I was detected by six different antisera, namely $\alpha Stt3p$, $\alpha Ost1p$, $\alpha Wbp1p$, $\alpha Ost3p$, $\alpha Swp1p$, and $\alpha Ost6p$ antiserum (Figure 1). All of these components had been shown to be part of the OTase (Kelleher and Gilmore, 1994; Karaoglu *et al.*, 1997; Spirig *et al.*, 1997). We did not directly assay for the presence of Ost2p (Silberstein *et al.*, 1995a) and the two small proteins Ost5p (Reiss *et al.*, 1997) and Ost4p (Chi *et al.*, 1996); however, biochemical characterization of the OTase complex had shown that these proteins were an integral part of the complex (Karaoglu *et al.*, 1997; Knauer and Lehle, 1999). A direct correlation of the presence of complex I to OTase function was provided by the analysis of different mutant strains with altered OTase activity: low levels of complex I were found in strains with strong hypoglycosylation of proteins, and the reversion of such hypoglycosylation phenotypes by high copy number suppressors also resulted in an increased amount of complex I (Figure 3). In addition, mutations affecting Wbp1p, Ost1p, or Stt3p also altered OTase-complex mobility upon blue native gel electrophoresis (data not shown). Therefore, native gel electrophoresis represented a valuable tool to analyze the OTase complex.

We 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 complexes of yeast mitochondria (Dekker *et al.*, 1996). Based on these markers, the mobility of complex I suggested a mass of around 500 kDa. However, the expected molecular mass of OTase consisting of Stt3p, Ost1p, Wbp1p, Ost3p, Swp1p, Ost2p, Ost5p, and Ost4p (Kelleher and Gilmore, 1994; Karaoglu *et al.*, 1997; Spirig *et al.*, 1997) is 280 kDa. Knauer and Lehle (1999a) showed the existence of an OTase complex running at

Table I. K_m values of different OTase complexes for the acceptor peptide

Strain (genotype)	SS328 + YEp352 (wild type)	YG889 + pOST3 ($\Delta ost3 \Delta ost6$ pOST3)	YG889 + pOST6 ($\Delta ost3 \Delta ost6$ pOST6)	YG889 + YEp352 ($\Delta ost3 \Delta ost6$)
Complex formed	Ia and Ib	Ia	Ib	II
K_m (μ M)	39 ± 12	40 ± 6	75 ± 17	106 ± 17

The mean K_m values of OTase toward the acceptor peptide in extracts derived from the strains indicated are given. Three independent measurements using two different extract preparations were performed.

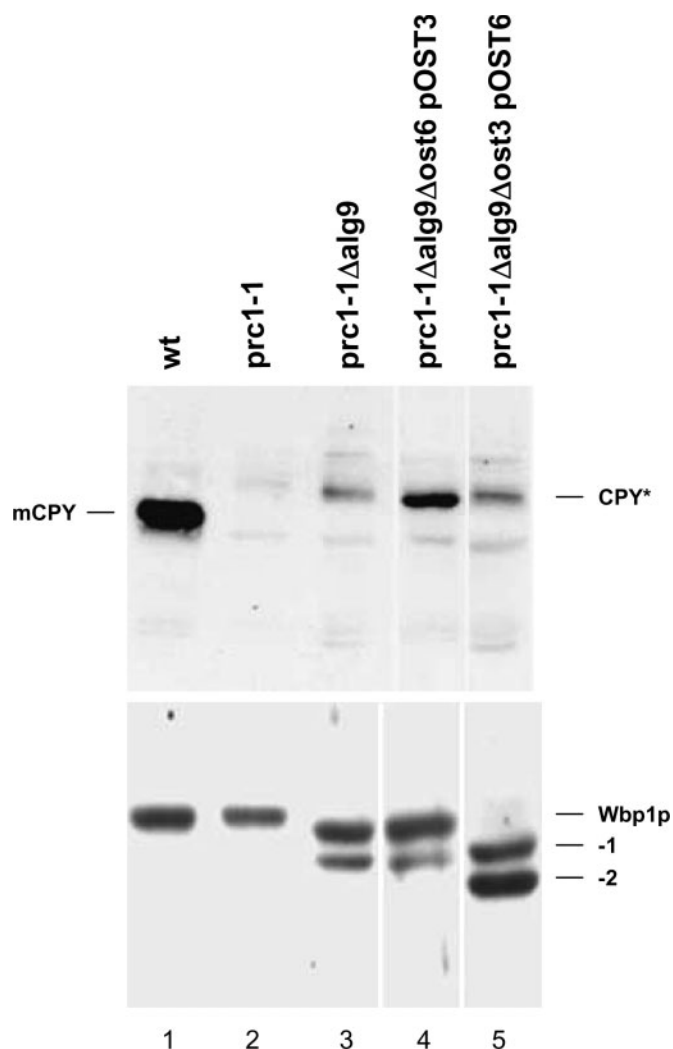


Fig. 7. Efficient glycosylation of CPY* by OTase complex Ia and complex Ib. YG618 (*prc1-1*, lane 2), YG796 (*prc1-1Δalg9*, lane 3), YG1033 (*prc1-1Δalg9Δost6*) harboring the plasmid pOST3 (lane 4), or YG1033 (*prc1-1Δalg9Δost3*) cells harboring the plasmid pOST6 (lane 5) were grown in liquid minimal medium lacking uracil at 23°C to mid-log phase. SS328 (wild-type [wt], lane 1) cells grown in complete medium served as a control. Protein extracts were prepared and western blot analysis was performed using CPY*- (top) or Wbp1p- (bottom) specific antibodies. The positions of mature CPY (mCPY), CPY*, and completely glycosylated Wbp1p, and the corresponding glycoforms lacking one (–1) or two (–2) oligosaccharides are indicated.

around 240 kDa by using blue native gel electrophoresis. However, they used a different detergent to solubilize the enzyme complex. Therefore, it is possible that active OTase is represented as a dimer using digitonin for solubilization of membrane proteins. Furthermore, because we do not know all the parameters that influence mobility of protein complexes in blue native gel electrophoresis, it is premature to estimate the molecular mass of the OTase complex based on its relative mobility in this gel system. In a recent publication, Shibatani *et al.* (2005) identified the mammalian OTase as a complex of 500 kDa in blue native gel electrophoresis, and even larger complexes resulted from the association of the OTase with the translocation machinery.

Two different OTase complexes are present in yeast

This article presents solid evidence that yeast expresses two different OTase complexes. We used both biochemical and genetic methods to show that there were two distinct OTase complexes present *in vivo*. These two complexes consisted of eight subunits, only differing in the highly conserved Ost3p (complex Ia) and Ost6p (complex Ib), respectively. Our results confirmed the previous observation that Ost6p was part of the OTase complex (Knauer and Lehle, 1999a). In addition, it became evident that Ost3p and Ost6p were not assembled in the same complex but did form two different complexes. In the accompanying study, Yan and Lennarz reached the same conclusion using a membrane-based yeast two hybrid system (Yan and Lennarz, 2005b).

Cells lacking Ost3p contained complex Ib (with Ost6p) and complex II (lacking both Ost3p and Ost6p) (Figure 3). Complex II in the $\Delta ost3$ strain was chased into complex Ib by overexpression of *OST6*. Interestingly, overexpression of *OST3* yielded only complex Ia and not complex Ib, suggesting that Ost3p and Ost6p competed for presence in the fully assembled OTase complex (Figure 3C, lane 3). Ost3p and Ost6p share sequence similarity, and the similar hydrophathy profiles suggest a similar topology of the two proteins in the membrane (Knauer and Lehle, 1999a). The existence of two distinct OTase is consistent with the observation that most but not all of the cellular pool of Wbp1p, Swp1p, or Ost1p copurified with epitope-tagged Ost3p (Karaoglu *et al.*, 1997). Also, the presence of a 31-kDa-band speculated to represent YML019W (Ost6p) in purified OTase preparations from strains expressing haemagglutinine-tagged Stt3p but not from strains expressing tagged Ost3p (Karaoglu *et al.*, 1997) supports our suggestion that these distinct OTase complexes exist in yeast. Based on published

results (Karaoglu *et al.*, 1997) and our analysis of native OTase complexes, we assume a four to one ratio of complex Ia versus complex Ib *in vivo*.

We were able to generate yeast strains expressing only complex Ia or complex Ib by overexpression of either Ost3p or Ost6p in a $\Delta ost3\Delta ost6$ strain. Analysis of native complexes in these strains confirmed that wt levels of fully assembled OTase were present. Our analysis of these strains clearly showed that the two complexes are functionally different. In the absence of complex Ib, no hypoglycosylation of glycoproteins was detected; however, a temperature-sensitive phenotype at 39°C of complex Ia-only strains was discovered. In contrast, a mild hypoglycosylation was observed in strains expressing only complex Ib, but this complex ensured growth at 39°C. In conjunction with the finding that complex Ib had a lower affinity toward the peptide substrate *in vitro*, we speculate that both Ost3p and Ost6p are involved in the recognition of the different polypeptide substrates. They might modulate the affinity of the OTase toward different N-linked glycosylation sites. We speculate that complex Ib is specifically required for the glycosylation of (a subset of) proteins essential for growth at elevated temperature; however, it is also possible that complex Ia is not stable at this elevated temperature. Additional experiments will be required to address the specific functions of these two complexes *in vivo*. Strains lacking both Ost3p and Ost6p were viable, hypoglycosylated proteins and expressed reduced levels of complex II OTase. These results suggested that both Ost3p and Ost6p were dispensable for the catalytic activity of OTase.

A role of Ost4p in the assembly of the OTase complexes

Our results suggested that both Ost3p and Ost6p are assembled at a late point in the assembly pathway of the OTase, because we observed only fully assembled OTase complex (Ia or Ib) containing either of these subunits (Figure 3 and data not shown). Compatible with this observation is the hypothesis that Ost3p and Ost6p are peripherally associated components of the OTase complex (Karaoglu *et al.*, 1997). For the stable association of both Ost3p and Ost6p, the small Ost4p subunit was required. We therefore propose that the Ost4p subunit acts as an assembly factor for the integration of the two membrane proteins Ost3p and Ost6p into the complex. However, complex I was isolated from $\Delta ost4$ cells overexpressing Ost3p or Ost6p (Figure 3A, lanes 2 and 4), suggesting that incorporation of Ost3p or Ost6p was possible in the absence of Ost4p. The suppression of the $\Delta ost4$ phenotype by overexpression of either *OST3* or *OST6* can now be explained by the elevated levels of complex I in such strains: increased levels of Ost3p (or Ost6p) shift the equilibrium of the assembly reaction toward complex I, resulting in an improved glycosylation. Our results also explain recent reports that immunoprecipitation of Ost3p did not coprecipitate other OTase subunits in a $\Delta ost4$ strain, whereas precipitation of Stt3p coprecipitated other OTase subunits except Ost3p (Karaoglu *et al.*, 1997). In addition, cross-linking of Stt3p with Ost3p was dependent on the presence of this small protein (Kim *et al.*, 2003). Whether the role in assembly of the OTase is the single function of Ost4p remains to be determined, but the

lethal phenotype of $\Delta ost3\Delta ost4$ double mutant strains suggests that there are other roles of this protein besides the assembly of either Ost3p or Ost6p.

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 (Kim *et al.*, 2003, Li *et al.*, 2003). Such interactions seem to be crucial for the assembly or the stability of a functional OTase complex. Small, hydrophobic proteins are found in other membrane-protein complexes, such as the protein translocase in the outer mitochondrial membrane, where the small, 6-kDa Tom6 protein is required for the assembly of Tom22p into the complex (Dekker *et al.*, 1998).

The analysis of the eukaryotic OTase has advanced rapidly. The best studied enzyme, the yeast OTase, is composed of eight subunits. Work presented in this and the accompanying report (.) showed the presence of two, functionally distinct OTase complexes that differ in one subunit. Kelleher *et al.* (2003) have shown that in mammalian cells, at least two different OTase complexes exist that differ in the central STT3 subunit. Based on the individual properties of the different OTase complexes, specific functions within the reaction mechanism of the enzyme can be assigned: the STT3 subunit is the catalytically active component (Wacker *et al.*, 2002; Yan and Lennarz, 2002; Kelleher *et al.*, 2003; Nilsson *et al.*, 2003), the Ost1 (ribophorin I) subunit associates with the polypeptide emerging from the translocation channel (Wilson *et al.*, 2005), whereas the Ost3/6 subunit seem to modulate peptide-substrate specificity. One of the small subunits (Ost4) is essential for OTase assembly. In view of the multiple functions of the N-glycan in the processing of glycoproteins (Helenius and Aebi, 2004), the presence of multiple OTase complexes with different specificities and function allows a fine tuning of the N-glycosylation process in the ER.

Materials and methods

Materials

Strains. SS328 (*MAT α ade2-101 ura3-52 His3 Δ 200 lys2-801*) (Vijayraghavan *et al.*, 1989), YG191 (*MAT α ade2-101 ura3-52 His3 Δ 200 Δ ost3::HIS3*), YG469 (*MAT α ade2-101 ura3-52 His3 Δ 200 lys2-801 Δ stt3::STT3-ProtA*) (Spirig, *et al.*, 1997), YG493 (*MAT α ade2-101 ura3-52 His3 Δ 200 lys2-801 Δ ost4::kanMX4*) (Spirig, *et al.*, 1997); YG618 (*MAT α ura3-52 His3 Δ 200 lys2-801 prc1-1*) (Jakob *et al.*, 1998), YG796 (*MAT α ade2-101 ura3-52 lys2-801 His3 Δ 200 prc1-1 Δ alg9::kanMX4*) (Jakob *et al.*, 1998), YG889 (*MAT α ade2-101 ura3-52 His3 Δ 200 tyr1 Δ ost3::HIS3 Δ ost6::kanMX4*), YG1033 (*MAT α ade2-101 ura3-52 His3 Δ 200 lys2-801 prc1-1 Δ alg9::kanMX4 Δ ost3::HIS3*), YG1034 (*MAT α ade2-101 ura3-52 His3 Δ 200 lys2-801 prc1-1 Δ alg9::kanMX4 Δ ost6::kanMX4*).

Yeast manipulations

Standard yeast media and genetic techniques (Guthrie and Fink, 1991) were used for growth of yeast cultures.

Preparation of microsomal membranes and solubilization of membrane proteins

Microsomal membranes were prepared as described (Reiss *et al.*, 1997) 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 MgCl₂, 1 mM MnCl₂, 35% glycerol), containing 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (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 MgCl₂, 1 mM CaCl₂, 1 mM MnCl₂), 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, Buchs, Switzerland) for 45 min at 25°C on a thermoshaker. Glycerol was added to a final concentration of 10%. Membrane proteins were solubilized by the addition of digitonin (1.5% final concentration) (Sigma, St. Louis, MO) and 6-aminocaproic acid (750 mM final concentration) (Fluka). Incubation was for 45 min at 4°C with shaking. Insoluble material was removed by centrifugation for 30 min at 40,000 rpm in a Kontron TFT 80.4 rotor (Kontron, Zurich, Switzerland) at 4°C. Protein concentration of the supernatant was determined by the Bio-Rad protein assay or by the method of Sailer and Weissmann (1991) using bovine serum albumin as a standard. Samples were 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 × 15 × 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 (Schägger and von Jagow, 1991) except that Tris-Base was used in all buffers instead of Bis-Tris and that the pH was adjusted to 7.5 (4°C) instead of 7.0 (4°C). Protein concentration of the solubilized membrane protein samples was adjusted to 1 µg/µL with TM buffer containing 10% glycerol 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 h, the cathode buffer (50 mM Tricine, 15 mM Tris-HCl, 0.02% Serva blue G, pH 7.5, 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, pH 7.5, (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) or PVDF membrane (Millipore, Bedford, MA) using a semi dry blotter from Kem-En-Tec (Copenhagen, Denmark) with a constant current of 1 mA/cm² for 135 min. Removal of Coomassie dye from the nitrocellulose was achieved by soaking the blots for 2 × 7 min in 50% methanol, 10% acetic acid and subsequent washing in phosphate buffered saline (136 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.1% Tween 20 [v/v]). PVDF membranes were destained in 90% methanol. Blots were then air-dried and incubated with appropriate antisera. Antibody binding was visualized with peroxidase-labeled protein A using enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, UK).

OTase assay

Protein extracts were adjusted to 5 mg/mL with membrane buffer. LLOs were extracted from 180 g of bovine pancreas as described (Spiro *et al.*, 1976), dissolved in 40 mL chloroform/methanol/water (10:10:3, v/v), separated from insoluble material by centrifugation in a Sorvall HB-6 rotor (Kendro, Zurich, Switzerland) at 10,000 rpm at room temperature and stored at -20°C. The diethylaminoethyl dextran purification step was omitted. The terminally acetylated and amidated hexapeptide N α -Ac-YNLTSV-NH₂ (Wieland *et al.*, 1987) was purchased from Tana Laboratories (Houston, TX) and iodinated as described (Kelleher *et al.*, 1992) except that the amount of chloramine T was doubled. The iodinated hexapeptide was dissolved in dimethylsulfoxide and stored as a 100 µM solution at 4°C.

OTase activity was assayed as follows (Das and Heath, 1980; Kelleher *et al.*, 1992): 15 µL of LLO (0.45 µM final concentration) were dried in a Savant Speedvac (Kendro) and suspended in 50 µL 66 mM Tris-HCl (pH 7.5), 33 mM NaCl, 4 mM MnCl₂, 185 mM sucrose, 0.3% NP-40, and 1 mM DTT. Insoluble material was separated by centrifugation and 30-µL hexapeptide (50–7000 cpm/pmol, 2–300 µM final concentration, diluted with cold hexapeptide). The reaction was started adding 20 µL of membrane solution (100 µg protein) in membrane buffer. After incubation at 23°C for 30 min, the reaction was stopped on ice by the addition of 100 µL NP-40 (2%). Heat-inactivated membranes served as a control. Glycosylated peptide was recovered by adding 1 mL ice-cold wash buffer (50 mM Tris-HCl, pH 7.4, 1 M NaCl, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM CaCl₂, 0.01% NP-40) to the assay mixture followed by 100 µL of concanavalin A-Sepharose beads (Pharmacia, Pfizer, New York, NY, freshly suspended in wash buffer). The tubes were incubated for 20 min on a rotating wheel at 4°C, and the beads were washed three times with 1 mL of wash buffer. The radioactivity retained on the beads was quantified using a COBRA II (Canberra Packard, Zurich, Switzerland) auto-gamma counter.

To determine the K_m value for the hexapeptide substrate, the final substrate concentration was varied between 2 and 300 µM. For each substrate concentration, triplicate reactions were performed.

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 (Spirig *et al.*, 1997), and the *OST6* locus was isolated as a high-copy number suppressor of the temperature-sensitive phenotype of the Δ *ost4* strain YG493. The YEp352-bound library described by Fleischmann *et al.* (1991) was used.

Western blot analysis

Western blot analysis of different glycoproteins has been described (Burda *et al.*, 1996).

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Abbreviations

CPY, carboxypeptidase Y; DTT, dithiothreitol; ER, endoplasmic reticulum; LLO, lipid-linked oligosaccharide; OTase, oligosaccharyltransferase; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate.

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