The Oligosaccharyltransferase Complex from Saccharomyces cerevisiae

ISOLATION OF THE OST6 GENE, ITS SYNTHETIC INTERACTION WITH OST3, AND ANALYSIS OF THE NATIVE COMPLEX

(Received for publication, March 8, 1999)

Roland Knauer and Ludwig Lehle‡
From the Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, Universitätsstrasse 31, 93053 Regensburg, Germany

The key step of N-glycosylation of proteins, an essential and highly conserved protein modification, is catalyzed by the hetero-oligomeric protein complex oligosaccharyltransferase (OST). So far, eight genes have been identified in Saccharomyces cerevisiae that are involved in this process. Enzymatically active OST preparations from yeast were shown to be composed of four (Ost1p, Wbp1p, Ost3p, Swp1p) or six subunits (Ost2p and Ost5p in addition to the four listed). Genetic studies have disclosed Stt3p and Ost4p as additional proteins needed for N-glycosylation. In this study we report the identification and functional characterization of a new OST gene, designated OST6, that has homology to OST3 and in particular a strikingly similar membrane topology. Neither gene is essential for growth of yeast. Disruption of OST6 or OST3 causes only a minor defect in N-glycosylation, but an ∆ost3Δost6 double mutant displays a synthetic phenotype, leading to a severe underglycosylation of soluble and membrane-bound glycoproteins in vivo and to a reduced OST activity in vitro. Moreover, each of the two genes has also a specific function, since agents affecting cell wall biogenesis reveal different growth phenotypes in the respective null mutants. By blue native electrophoresis and immunodetection, a ~240-kDa complex was identified consisting of Ost1p, Stt3p, Wbp1p, Ost3p, Ost6p, Swp1p, Ost2p, and Ost5p, indicating that probably all so far identified OST proteins are constituents of the OST complex. It is also shown that disruption of OST3 and OST6 leads to a defect in the assembly of the complex. Hence, the function of these genes seems to be essential for recruiting a fully active complex necessary for efficient N-glycosylation.

Asparagine-linked glycosylation is one of the most common types of eukaryotic protein modifications (1–3). N-Glycans are essential for cell viability (4–6) and have a profound role in the biological function and physicochemical properties of many secreted and integral membrane proteins (7). The key step of this pathway is the en bloc transfer of the core oligosaccharide Glc3Man9GlcNAc2 from dolichyl pyrophosphate to selected asparagine residues in an Asn-X-Ser/Thr consensus sequon of nascent polypeptides, where X can be any amino acid except proline (8–13). The reaction is catalyzed by the ER1-resident enzyme N-oligosaccharyltransferase (OST) (for review see Refs. 14 and 15).

Previous attempts to purify and characterize this enzyme have met with limited success due to its lability upon solubilization (16–18) and probably also due to the fact that it is a multimeric membrane protein complex. Meanwhile, however, OST complexes have been purified from different sources, such as dog pancreas (19, 20), yeast (21–23), hen oviduct (24), and human (25) and pig (26) liver. The subunit composition of the various isolated complexes and the protein sequences, so far obtained, reveal in part a high conservation of the structural organization of this enzyme throughout evolution. Independent OST purifications from Saccharomyces cerevisiae have yielded active complexes consisting of four polypeptides (Ost1p (64/62 kDa), Wbp1p (47 kDa), Ost3p (34 kDa), and Swp1p (30 kDa)) (21, 23) or of six subunits (Ost1p, Wbp1p, Ost3p, Swp1p, Ost2p (16 kDa), and Ost5p (9.5 kDa)) (22). Cloning and functional analysis of OST1 (23, 27), WBP1 (28), SWP1 (29), and OST2 (30) have indicated that these genes are essential for the vegetative growth of the yeast cell and reveal significant homology to components of the canine complex: to ribophorin I (27), OST48 (31), to the C-terminal half of ribophorin II (22), and DAD1 (defender against apoptotic cell death) (20), respectively. In contrast, OST3 (32) and OST5 (33) coding for the 34- and 9.5-kDa subunits, respectively, are not essential, but their deletion yields glycosylation defects and reduces OST activity in vitro. In addition to these six proteins, genetic screens have identified two other loci, OST4 (34) and STT3 (35), that are required for optimal OST function in vivo and in vitro. Recent evidence indicates now that the derived proteins are indeed part of the complex (36, 37). OST4 encodes an unusually small, hydrophobic polypeptide of 3.6 kDa; its deletion leads to underglycosylation of N-glycoproteins and a temperature-sensitive growth phenotype. STT3 encodes a 78-kDa transmembrane protein with the highest conservation among the proteins associated with OST function. The essential Stt3p was found to be necessary for stability or assembly of the complex, and its lack affects the substrate specificity for the lipid-linked oligosaccharide donor (35).

So far, the specific function of the various enzyme subunits is obscure and remains to be defined. In addition, the question must be answered which subunit is a bona fide constituent of

* This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 521) and Fonds der Chemie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, Universitätstrasse 31, 93053 Regensburg, Germany. Fax: 49-941-943-3352; E-mail: lehle.ludwig@biologie.uni-regensburg.de.

1 The abbreviations used are: ER, endoplasmic reticulum; OST, oligosaccharyltransferase; ORF, open reading frame; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Dol, dolichol; BN, blue native; Bistris, bis[2-hydroxyethyl]iminotris(hydroxy-methyl)methane; Tricine, N-tris(hydroxymethyl)methylglycine; HPLC, high pressure liquid chromatography; CPY, carboxypeptidase Y; DPAP, dipeptidyl aminopeptidase; CFW, Calcofluor White.
The complex or serves only an auxiliary function. In this report, we describe the isolation and functional characterization of a new, not essential gene, designated OST6, that has sequence homology and in particular a very similar membrane topology to OST3. Disruption of OST6 causes only a minor defect in N-glycosylation, both in vivo and in vitro. However, a Δost6Δost3 double mutant exhibits a synthetic phenotype with a strong underglycosylation of soluble and membrane-bound glycoproteins as well as a defect in complex formation. By blue native electrophoresis, we demonstrate that the OST complex has a molecular mass of about 240 kDa and consists of all hitherto defined OST subunits (except for the small 3.6-kDa Ost4p, for which no antibody is available for detection).

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Genetic Methods**—Yeast strains were SS330 (MATa ade2–101 ura3–52 his3A200 tyr1), MATB (MATa ade2–101 ura3–52 his3A200 tyr1 st3–3), RKY325 (MATa ade2–101 ura3–52 his3A200 Δost6::HIS3), RKY326 (MATa ade2–101 ura3–52 his3A200 Δost3::HIS3 Δost6::URA3), RKY327 (MATa ade2–101 ura3–52 his3A200 Δost3::HIS3 Δost6), RKY346 (MATa ade2–101 ura3–52 his3A200 Δost3::HIS3 YEp352-OST6), and RKY348 (MATa ade2–101 ura3–52 his3A200 Δost3::HIS3 YEp352-OST6). Strain SS330 is the isogenic wild-type. Plasmids encoding recombinant CPY forms (38) were transformed into yeast using standard techniques. Cells were grown in standard yeast media (39).

**Cloning and Disruption of OST6**—The OST6 coding sequence was originally reported as an ORF of unknown function on chromosome XIII by the yeast genome sequencing project (ORF YML019W). A genomic clone of OST6 was isolated from a yeast gene bank with 4-kilobase pair Sau3A-DNA fragments in the multicopy vector YEp352 (40). As a probe for colony hybridization, a PCR-amplified, digoxigenin-labeled genomic OST6 fragment was used. A genomic clone containing the OST6 open reading frame together with 5′- and 3′-flanking sequences was isolated from ~20,000 Escherichia coli colonies.

For disruption of the OST6 locus by homologous recombination, DNA fragments of 0.9 and 1.2 kilobase pairs from the 5′- and 3′- untranslated regions of OST6 were ampiclonic from genomic wild-type DNA by PCR and ligated into an URA3 cassette. The 5′ fragment covered ~840 to +58 base pairs (relative to the start ATG of the OST6 locus); the 3′ fragment 41 base pairs upstream of the stop codon and 115 base pairs of the 3′-untranslated region. Successful replacement of the OST6 locus was confirmed by PCR using genomic DNA from uracil prototrophic transformants.

**Isolation of Microsomal Membranes and Preparation of Solubilized Enzyme Extract**—Rough microsomal membranes were isolated as described (12). Yeast cells were grown to mid-log phase in yeast complete medium. Of [35S]methionine was added and cells were labeled for 30–45 min. In case of invertase, cells were derepressed for 20 min in yeast minimal medium without glucose, and labeled with 100 μCi of [35S]methionine. For Western blotting, proteins were transferred to nitrocellulose or Immobilon (in case of BN-PAGE) membranes using the semidry blotting technique (46).

Immunodetection was performed according to standard procedures and was visualized by the ECL method (Amersham Pharmacia Biotech). Polyclonal antisera against Wbp1p and Swp1p were raised in rabbits using synthetic peptides corresponding to the 8-20 C-terminal amino acids coupled to bovine serum albumin (47). Western blotting, proteins were transferred to nitrocellulose or Immobilon (in case of BN-PAGE) membranes using the semidry blotting technique (46).

**RESULTS**

**Isolation and Sequence Analysis of the OST6 Gene**—In the process of cloning and characterizing the OST3 gene encoding the 34-kDa subunit (our accession number X79596, GeneEMBL data base) a report appeared dealing with the same gene (32). Our nucleotide sequence analysis is in agree-
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Ost6p, Solid lines represent identical amino acids; single and double points indicate conservative amino acid replacements. Ost3p, the sequence of the N terminus of the mature protein (starting with Asn) and of an internal tryptic peptide fragment, determined by direct amino acid sequencing, are underlined. Ost6p, the arrow indicates the potential signal sequence cleavage site. B, Hydropathy analysis of Ost3p and Ost6p according to the algorithm of Kyte and Doolittle (50) using a window of 17 amino acids. Note the nearly identical arrangement of Ost3p according to the algorithm of Kyte and Doolittle (50) using a window of 17 amino acids. Note the nearly identical arrangement of Ost3p and Ost6p with the published data. However, the N terminus of the mature Ost3 protein, isolated by us and determined by gas phase sequencing, was found to start three amino acids further (Fig. 1A). However, the two proteins have a strikingly similar membrane topology (Fig. 1B) with a typical N-terminal cleavable signal sequence (49) and a C-terminal arrangement of four transmembrane domains using the algorithm of Kyte and Doolittle (50). Searches of protein sequence data bases have revealed that OST3 and OST6, respectively, have homology to three other ORFs: to an ORF of Caenorhabditis elegans on chromosome 3 (identity 21%, similarity 46%) encoding a 37.7-kDa protein, to a candidate human tumor suppressor gene on chromosome band 8p22, named N33 (51), and to the mammalian protein αTRAP, a component involved in protein translocation into the ER (identity 23%, similarity 48%; in both cases) (52). Whereas the first two proteins have a similar hydropathy profile, and thus may be structural homologues to Ost3p/Ost6p, αTRAP is a type I membrane protein with only one transmembrane domain and probably different function. OST6 encodes a protein of 332 amino acid residues with a calculated molecular mass of 37.9 kDa, but runs on SDS gels as 32 kDa, somewhat faster than Ost3p (34 kDa) and hardly to resolve from Swp1p (see below). The presumed signal sequence cleavage site (Fig. 1A) is in agreement with the predictive method of von Heijne (53). A consensus site for N-glycosylation is located at Asn-175, but not used, since endo H treatment or the N-glycosylation inhibitor tunicamycin does not alter the mobility of Ost6p (data not shown). Thus OST6, like OST3, is not a glycoprotein. A genomic clone of OST6 was obtained by PCR techniques amplifying a fragment that comprises the sequence from 233449 through 234455 from chromosome XIII (YML019W).

OST3 and OST6 Are Required for N-Glycosylation in Vivo, and Their Disruption Leads to a Synthetic Phenotype—To analyze the function of OST3 and OST6 and, in particular, to disclose a possible distinction between the two genes, we examined the glycosylation pattern of well characterized soluble and membrane glycoproteins in single and double mutant strains. Either cell extracts from metabolically labeled cells followed by immunoprecipitation and gel electrophoresis (in case of carboxypeptidase, invertase, dipetidyl aminopeptidase B), or protein immunoblots (in case of Wbp1p) were analyzed. As depicted in Fig. 2 and Table I, soluble vacuolar carboxypeptidase Y (CPY) shows in single mutants only a slight reduction in glycosylation indicated by the appearance of a minor band of CPY lacking one N-linked chain; the defect in αost6 is consistently more pronounced than in αost3. Mature CPY contains four N-linked core type oligosaccharide chains and has a molecular mass of 61 kDa (54). In striking contrast, the double mutant αost3αost6 severely underglycosylates CPY and lacks one, two, and, to some extent, also three and four chains. Such a reduction in glycosylation can also be observed in OST mutants defective in the essential genes WPB1 (28) or OST1 (27). All underglycosylated bands were endo H-sensitive and shifted to a 53-kDa band, the predicted size of unglycosylated mature CPY, indicating that the bands differ in oligosaccharide content rather than in polypeptide mass (data not shown). A similar underglycosylation pattern in the order Δost6 < Δost3 < Δost3Δost6 was demonstrated also for the soluble, extracellular glycoprotein invertase (only the core glycosylated ER form has been considered, since the high molecular weight cell wall form does not allow a reliable quantification of the glycosylation defect), for the vacuolar membrane protein dipetidyl aminopeptidase B (DPAP B) and for the OST subunit Wbp1p (Fig. 2; Table I). The glycoproteins vary to some extent in the degree of underglycosylation. Our results, however, do not allow to postulate a bias for underglycosylation of membrane glycoproteins, as has been previously discussed for the case of ost3 (32).

Since OST3 and OST6 show a synthetic phenotype, we asked whether an overexpression of OST6 in a multicopy vector can
analyzed the dolichol-linked oligosaccharides from isogenic or to a reduced OST activity (27, 28, 30). To clarify this, we
The phenotype of Formation of Lipid-linked Oligosaccharide—
is rather mild.

D and DPAP B was performed by metabolic labeling of yeast cells with mWbp1p
2
mWbp1p
membrane protein were loaded per lane. Mature Wbp1p (not shown). We did not examine overexpression of

Table I

Reduced in vivo glycosylation activity in Δost3, Δost6, and Δost3Δost6 disruptants. Analysis of glycosylation of CPY, invertase, and DPAP B was performed by metabolic labeling of yeast cells with

D
D
D



TABLE I

Number of N-glycosylation sites of each protein.

Glycoprotein     Type   Number of N-glycosylation sites   Percent of Wild-type glycosylation

| Glycoprotein | Type            | Number of N-glycosylation sites | Percent of Wild-type glycosylation |
|--------------|-----------------|---------------------------------|-----------------------------------|
| CPY          | Soluble         | 4 (4)                           | 90 97 45                          |
| Invertase    | Soluble         | 13 (9–10)                       | 86 81 23                          |
| DPAP B       | Type I membrane | 8 (6–7)                         | 70 90 10                          |
| Wbp1p        | Type I membrane | 2 (2)                           | 53 72 20                          |

functionally replace OST3 and thus cure underglycosylation of CPY in an Δost3 single and an Δost3Δost6 double mutant, respectively. Indeed, this is the case as shown in Fig. 3, lanes 1 and 4. No suppression of CPY underglycosylation was observed, when OST1, WBP1, or SWP1 were overexpressed (data not shown). We did not examine overexpression of OST3 in an Δost6 mutant, since the underglycosylation defect in this strain is rather mild.

Ost3p and Ost6p Influence OST Activity in Vitro, but Not Formation of Lipid-linked Oligosaccharide—The phenotype of underglycosylation of proteins could be attributed to a defect in the assembly of lipid-linked oligosaccharide (alg mutants) (55–57) or to a reduced OST activity (27, 28, 30). To clarify this, we analyzed the dolichol-linked oligosaccharides from isogenic

wild-type, Δost3, Δost6, and Δost3Δost6 cells labeled with [3H]mannose. In all strains synthesis of fully assembled core oligosaccharide was observed (Fig. 4). The oligosaccharide pattern in the double mutant was slightly shifted toward Glc3Man9GlcNAc2, which is in accord with the observed underglycosylation in this strain. We conclude that the glycosylation defect is not due to a specific defect in the biosynthesis of the lipid-oligosaccharide precursor, as is the case in different alg mutants.

Next, we tested whether OST activity was affected in vitro in these strains. Since we had previously observed that the stt3Δ3 mutation results in a sensitivity of OST toward only a suboptimal, non-full-length substrate (35), two different glycosyl donor substrates (lipid-linked chitobiose and lipid-linked Glc3Man9GlcNAc2) were used; the synthetic hexapeptide Tyr-Asn-Leu-Thr-Ser-Val served as the oligosaccharide acceptor (16). As shown in Table II, the measured OST activity reflects the underglycosylation observed in vivo. Whereas the single mutations display only a small decrease in activity, the Δost3Δost6 double mutant is severely affected, in a similar fashion as, e.g., a wbp1 mutant (28). OST activity in Δost6 using the chitobiose donor was consistently somewhat lower than in Δost3, opposite to the situation in vivo; however, glycosyl transfer using the full-length substrate is in accord with the in vivo data. The data also indicate that OST6 may influence the glycosyl donor recognition, but compared with stt3Δ3 only to a very minor extent.

We have also determined various enzyme activities involved in biosynthesis of lipid-linked oligosaccharide such as DolP-Man, DolP-Glc, and DolPP-GlcNAC2, respectively, and found no differences between the mutants and wild-type (data not shown). Similarly, biosynthesis of O-linked glycans, which is initiated in yeast in the ER (3, 58), is not affected. Chitinase, an exclusively O-mannosylated glycoprotein, is not altered in its mobility when analyzed by Western technique and, furthermore, no defect in O-mannosylation activity is detectable in vitro (data not shown).

Ost3p and Ost6p Are Not Involved in Peptide Substrate Recognition but Are Needed for Optimal Oligosaccharyltransfer—In search of a specific function for OST3 and OST6, we considered among other possibilities that these genes could be involved in peptide substrate recognition. To address this point, we expressed simultaneously with wild-type CPY a recombinant form (in single copy as is the case for the wild-type form) containing only one of the four carbohydrate chains, either A, B, C, or D (Fig. 5A). The corresponding carboxypeptidase species are called CPY-A, CPY-B, CPY-C, and CPY-D, respectively. As shown in Fig. 5B, both in the Δost3 single and in the Δost3Δost6 double null mutant, all four recombinant CPY forms are glycosylated. This indicates that Ost3p and

FIG. 2. In vivo N-glycosylation defects in Δost3, Δost6, and Δost3Δost6 disruptants. Analysis of glycosylation of CPY, invertase, and DPAP B was performed by metabolic labeling of yeast cells with [35S]methionine, followed by immunoprecipitation, PAGE, and autoradiography. The position of the mature form of CPY (mCPY) and of the underglycosylated forms lacking one to four oligosaccharide chains (CPY−1 to −4) are indicated; mDPAP B, mature DPAP B. Glycosylation of Wbp1p was analyzed by Western blot technique. 50 μg of membrane protein were loaded per lane. Mature Wbp1p (mWbp1p) and the two underglycosylated forms (−1, −2) are indicated. A presumably proteolytic fragment of Wbp1p running somewhat faster than the unglycosylated form (−2) is marked by an asterisk (*)

FIG. 3. High copy number suppression of the Δost3 glycosylation defect by OST6. OST6 was expressed in the multicopy vector YEp352 (40) in the Δost3 single mutant (lane 1) and the Δost3Δost6 double mutant (lane 4). CPY was analyzed in the transformants by metabolic labeling with [35S]methionine, followed by immunoprecipitation, PAGE, and autoradiography. The vector control is shown in lanes 2 and 3. The positions of mature CPY (mCPY) and of glycoforms lacking one to four oligosaccharide chains are designated accordingly (−1 to −4).
Lipid-linked oligosaccharides were labeled by incorporation of [3H]-cells (radiolabeled oligosaccharide from DolPP-GlcNAc2 or DolPP-GlcNAc2Man8; D type and recombinant CPY forms in the GlcNAc2Man9Glc3 as glycosyl donor to the synthetic hexapeptide YN-
The slight increase in mobility of CPY-C mutant (Fig. 5) particular glycosylation site of the nascent polypeptide chain. Ost6p, respectively, are not involved in the recognition of a LTSV as glycosylacceptor (cf. 16).

In vitro OST activity of wild-type and mutant strains with different lipid-linked oligosaccharides as substrates

In vitro OST activity was determined by measuring the transfer of a radiolabeled oligosaccharide from DolPP-GlcNAc2 or DolPP-GlcNAc2Man9Glc3 as glycosyl donor to the synthetic hexapeptide YN-LTSV as glycosylacceptor (cf. 16).

| Strain | Glycosyl transfer to peptide from DolPP-GlcNAc2 | Glycosyl transfer to peptide from DolPP-GlcNAc2Man9Glc3 |
|--------|-----------------------------------------------|--------------------------------------------------------|
| Wild-type | 100 | 100 |
| Δost3 | 84 | 83 |
| Δost6 | 73 | 95 |
| Δost3Δost6 | 29 | 39 |
| stt3–3 | 8 | 72 |
| wbp1–1 | 21 | 24 |

Ost6p, respectively, are not involved in the recognition of a particular glycosylation site of the nascent polypeptide chain. The slight increase in mobility of CPY-C mutant (Fig. 5B, lane 3) is an inherent feature of the recombinant form and not due to altered glycosylation (38); it is also observed when expressed in a wild-type strain (Fig. 5B, upper panel).

In order to estimate the degree of glycosylation of the wild-type and recombinant CPY forms in the Δost3Δost6 double mutant, the following calculation was made. Since the recombinant glycosylated and unglycosylated CPY forms comigrate with the −3 and −4 wild-type forms, respectively, the amount of radioactivity of the −3′−4 bands of the control (lane 5) was subtracted from the corresponding bands in lanes 1–4 to give the amount of radioactivity in the recombinant forms (assuming that underglycosylation of wild-type CPY is the same, when both species are expressed). We calculate that in Δost3Δost6 cells wild-type CPY contains on average 1.8 instead of four N-linked saccharide chains, corresponding to 45% glycosylation compared with wild-type cells (Table I). In the recombinant forms, having only one glycosylation site, 0.58 chains of CPY-A, 0.75 of CPY-B, 0.71 of CPY-C, and 0.65 of CPY-D are glycosylated, corresponding on average to 67% glycosylation. Thus, a nascent polypeptide with multiple glycosylation sites is somewhat what more affected, indicating that OST activity is limiting in the double mutant.

In order to exclude a defect in the protein translocation machinery that could also account for the underglycosylation, we investigated by Western analysis whether the amount of components of the translocon, such as Sec61p, Sec62p, and Sec63p, was altered in the Δost3Δost6 double mutant. However, no difference was observed as compared with wild-type cells (data not shown).

**Growth Phenotypes of OST3 and OST6 Disruptants—**Both OST3 and OST6 are not essential for yeast growth, and also the double knock-out mutant does not exhibit an altered growth rate or morphology as compared with wild-type when tested up to 37 °C in complete medium (data not shown). Mutations in other OST subunits, like WBP1, OST1, or OST2, cause temperature-sensitive growth defects (27, 28, 30). However, we noticed that ost3 and ost6 null mutants display at 30 °C differential phenotypes, when stressed by agents that interfere with cell wall biogenesis, such as Calcofluor White, caffeine, or SDS. Cells with weakened cell walls are sensitive...
against Calcofluor White (CFW) (59) at concentrations that do not impair growth of wild-type cells. As shown in Fig. 6A, growth of the Δost6 disruptant is completely abolished in the presence of 5 μg/ml CFW, whereas in Δost3 no inhibition occurs. A similar effect is also exhibited by the phosphodiesterase inhibitor caffeine (Fig. 6B). Evidence has been obtained (60–62) that a protein kinase C is a central element in the regulation of cell integrity via regulating cell wall formation. Likewise, Δost6 but not Δost3 cells are sensitive to caffeine and SDS. In the case of Δost3, it was found that the mutant is even somewhat more resistant against SDS, compared with the isogenic wild-type strain (Fig. 6C). Various mutants with a defective cell wall biosynthesis or N-glycosylation were reported to reveal an SDS sensitivity (63, 64). The different behavior of Δost6 and Δost3 null mutants toward these compounds could mean that both genes may in part be involved in different functions important for cell wall biogenesis and/or stability.

Subunit Composition of the OST Complex by Blue Native and Denaturing Electrophoresis—Our affinity purification procedure using an anti-Wbp1p affinity column (21) led to the isolation of a tetrameric, enzymatically active OST complex consisting of Ost1p, Wbp1p, Ost3p, and Swp1p, whereas the complex purified by Kelleher and Gilmore (22) contained in addition Ost2p and Ost5p. In none of the two procedures the essential and genetically identified subunits Stt3p and Ost4p, or the newly discovered Ost6p were present in detectable amounts by silver or Coomassie staining. Using the more sensitive method of Western analysis, we show now that the tetrameric OST preparation from wild-type cells contains also Stt3p, Ost2p, and Ost5p, as well as Ost6p (no antibodies are available for Ost4p; Fig. 7B). We find that Stt3p, which has a predicted molecular mass of 78 kDa, migrates on SDS gel as a diffuse band with an anomalous mobility of ~60 kDa that is not resolved from Ost1p (lanes 1 and 3). Proof for the correct identification of the Stt3p signal comes from the observation that it disappears, when Stt3p depleted membranes were analyzed (data not shown). Ost6p turns out to have a mobility almost identical to that of Swp1p and, therefore, may have escaped detection so far.

Affinity purification and silver staining (Fig. 7A) of OST from Δost3Δost6 cells reveals a reduction of Ost1p and, as expected, a lack of Ost3p (and Ost6p by Western blot, see Fig. 7B). Ost1p and Wbp1p, respectively, the two glycosylated subunits of the complex are partly underglycosylated (Fig. 7, A, lane 2; B, lanes 4 and 6). By Western analysis, the presence of Stt3p, Ost2p, and Ost5p could also be demonstrated. Furthermore, a comparison of the composition of the complexes from wild-type strain and Δost3Δost6 double mutant (Fig. 7B) indicates that the amount of Stt3p, Ost1p, and Ost5p is reduced. Thus, the structural organization of OST is severely affected and leads, as shown above (Table I), to a defect in enzyme activity, observed so far for cells lacking an essential OST protein.

In order to estimate the size of the native complex and identify possible subcomplexes (see below), we applied the method of blue native-polycrylamide electrophoresis that was reported to be a powerful tool for the separation and size determination of native membrane bound complexes (41, 42, 65). By this technique, in combination with Western analysis, we can identify in wild-type membranes a complex of ~240 kDa (Fig. 8), in which all OST subunits are assembled. In the case of Wbp1p, we also observe in low amounts an additional band (larger than the expected mass of Wbp1p) that is recognized by the antibody; its origin remains obscure, however. The differences in signal intensities do not reflect different amounts or the stoichiometry of the subunits in the complex, but are rather due to differences in the antibody titer.

**Discussion**

We have described the identification and functional characterization of OST6, a novel gene encoding a subunit of the OST from yeast. Ost6p is a 32-kDa transmembrane protein not disclosed in any of the previously purified OST complexes. It is homolog to Ost3p, the 34-kDa γ-subunit of yeast OST (32). The identity between both proteins with 21% is rather low, but they display a strikingly similar membrane topology containing four predicted C-terminal membrane-spanning domains. Such a hydrophathy profile and the same degree of identity (23%) is also found for a Caenorhabditis elegans ORF of unknown function and a human candidate tumor suppressor. The relationship of OST6 and OST3 to a neoplastic phenotype is obscure, but altered glycosylation of cell surface proteins is a well known feature of tumor cell lines (66, 67).

Disruption of OST3 was reported (32), and is confirmed in the present study, to cause an only moderate underglycosylation of proteins in vivo and a small decrease in OST activity measured in vitro. In the case of disruption of OST6 the underglycosylation defect is even less. However, an Δost3Δost6 double mutation leads to a synthetic phenotype with a severe underglycosylation, both in vivo and in vitro (Fig. 2; Table I), as
is the case for ts mutations of essential OST genes (27, 28, 30). Surprisingly, however, growth is not impaired at temperatures up to 37 °C. Overexpression of Ost6p not only complements the Δost6 knockout, but also rescues the Δost3 underglycosylation effect. Although we have not performed the reverse experiment (due to the very mild Δost6 defect), it seems that both genes have in part a redundant function and are able to partially replace each other. On the other hand, they also reveal specific effects (cf. Fig. 6). We observed that upon stressing the cells by compounds interfering with cell wall biogenesis, like Calcofluor White, caffeine, or SDS, an Δost6 mutant behaves differently compared with an Δost3. Even though no distinct target reaction can be given for these findings, they may eventually help to unravel the complexity of formation and function of N-linked saccharide chains.

Previous biochemical investigations of the yeast OST suggested that the enzyme consists of four (Ost1p, Wbp1p, Ost3p, Swp1p) (21, 23), five (68), or six subunits (Ost1p, Wbp1p, Ost3p, Swp1p, Ost2p, Ost5p) (22). Genetic screens have identified in addition STT3 (35) and OST4 (34). In particular the lack of the essential and highly conserved Stt3p in all OST preparations was puzzling, and it was hypothesized that Stt3p could be a substoichiometric assembly factor (35). We have shown now by more sensitive probing with antibodies that the “tetrameric,” enzymatically active complex isolated by us (21) also contains Stt3p, Ost2p, Ost5p, and the newly discovered Ost6p. The present study, and also recent experiments employing affinity purification of tagged Stt3 protein, or analysis of co-immunoprecipitates of in vivo radiolabeled subunits clearly identify Stt3p (36, 37) and also Ost4p (37) as components of the complex. Moreover, an estimation of the amount of radioactivity incorporated into the subunits of the co-immunoprecipitates (37) is compatible with the notion that the eight subunits identified therein (Stt3p, Ost1p, Wbp1p, Ost3p, Swp1p, Ost2p, Ost5p, Ost4p) may be present in equimolar amounts. Thus, the underrepresentation of some subunits in the various complex preparations seems to be due to their depletion during isolation rather than to a real substoichiometric participation in the in vivo complex. This interpretation is also in agreement with our analysis of the native complex composition using the method of blue native electrophoresis. A complex in the range of ~240 kDa was found, which agrees with a calculated molecular mass assuming all 9 subunits are present in equimolar amounts. Nevertheless, the isolation of an active “tetrameric” complex may indicate that for the actual catalysis of the glycosylation reaction less subunits are sufficient, and the additional components may be essential or important only for in vivo function.

OST3 and OST6 are not essential for OST activity, but their simultaneous lack drastically decreases glycosylation in vitro and in vivo. Therefore, one could envisage that the products of these genes are needed for optimal OST activity either directly by interacting with and regulating the catalytic subunit, or indirectly by affecting the assembly of an optimal complex, or being involved in proper positioning the OST to the polypeptide at the translocation site or to the site of formation of the lipid-linked oligosaccharide precursor. Analysis of the subunit composition of the affinity-purified complex in the Dost3Dost6 double mutant by Western analysis clearly indicates a severe defect in the structural organization of the complex (Fig. 7).

Studies demonstrating genetic interactions among different OST genes, either by using a high copy number suppression approach or by constructing double mutants with a synthetic phenotype, have led to the suggestion that OST subunits can be sorted into three groups: I, Ost1p-Ost5p; II, Wbp1p-Swp1p-Ost2p; and III, Stt3p-Ost4p-Ost3p (15, 36, 37). The structural similarity of Ost3p and Ost6p, their synthetic interaction, as well as the suppression of Dost3 by OST6 overexpression suggests a grouping of Ost6p to (III). Complementary biochemical evidence indicates a direct physical interaction between Wbp1p and Swp1p (29) and Ost2p,2 as well as between Stt3p, Ost3p, and Ost4p (37), supporting the idea that these groups may represent discrete subcomplexes. In this context, it is interesting to note that upon simultaneous disruption of the OST3 and OST6 genes, Ost5p and Ost1p (subcomplex I) and Stt3p (subcomplex III) are decreased, whereas Wbp1p, Swp1p, and Ost2p (subcomplex II) are not affected in their amount (Fig. 7). Since glycosylation still occurs in the Dost3Dost6 strain, albeit reduced, one could speculate that Wbp1p, Swp1p, and Ost2p represent the catalytic core unit of the complex.

At present it is not clear, however, whether the proposed subcomplexes comprise intermediates in the assembly of a fully functional complex, or whether they are autonomous in vivo pools that may assemble into OST complexes with slightly different composition and function. Recent findings indicate the occurrence of multiple pathways of protein translocation into the ER (69–72), one dependent on the signal-recognition particle and the other independent. The identified translocation complexes share in part subunit components. The fact that no distinct OST subcomplexes can be detected in wild-type cells by blue native polyacrylamide gel electrophoresis makes it less likely that OSTs of different composition are associated with the respective translocation machineries. In agreement with this idea is also our observation that in the Dost3Dost6 mutant proteins are underglycosylated irrespective whether they use the SRP-dependent (e.g. invertase, DPAP B) or the SRP-independent (e.g. CPY) sorting pathway. Nevertheless, this view does not exclude specific interactions of particular OST subunits and/or not yet identified auxiliary proteins with the different targeting pathways.

The combination of biochemistry and powerful yeast genetic methods has advanced tremendously our knowledge of the N-glycosylation of proteins. Now that the composition of one of the most complex enzymes in nature as well as some of the interactions between its nine subunits has been defined, further work needs to concentrate on a number of intriguing issues. These include the specific functions of the various subunits of the complex, the regulation of the enzyme and the coupling of OST to protein translocation and protein folding.

2 R. Knauer and L. Lehle, unpublished results.
Finally, the results obtained in yeast may also lead to the isolation of the homologous mammalian proteins, not yet identified in respective complexes.

Acknowledgments—We are very grateful to Markus Aebi, Reid Gilmore, and Satoshi Yoshida for antibodies, Jacob Winther for generously providing CPY plasmids, and Eckhard Loos for critical reading of the manuscript.

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