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Yeast Wbp1p and Swp1p form a protein complex essential for oligosaccharyl transferase activity

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Asparagine-linked N-glycosylation is an essential protein modification occurring in all eukaryotic cells. The central step is the co-translational transfer of the core oligosaccharide assembled on the lipid carrier dolichol phosphate to selected Asn-X-Ser/Thr residues of nascent polypeptide chains in the endoplasmic reticulum. This reaction is catalyzed by the enzyme *N*-oligosaccharyl transferase. In yeast, Wbp1p is an essential component of this enzyme. Using a high copy number suppression approach, the *SWP1* gene was isolated as an allele specific suppressor of a *wbp1* mutation. Swp1p is a 30 kDa type I transmembrane protein and essential for cell viability. Similar to Wbp1p, depletion of Swp1p results in reduced *N*-oligosaccharyl transferase activity *in vivo* and *in vitro*. Wbp1p and Swp1p can be chemically cross-linked, suggesting that both proteins are essential constituents of the *N*-oligosaccharyl transferase complex.

Key words: endoplasmic reticulum/glycosylation/oligosaccharyl transferase/*Saccharomyces cerevisiae*/transmembrane protein

Introduction

N-linked glycosylation of proteins is an essential modification of secreted proteins in the endoplasmic reticulum and follows a highly conserved pathway in all eukaryotic cells. A core oligosaccharide of the composition [$Glc_3Man_9(GlcNAc)_2$] is first assembled on the lipid carrier dolichol phosphate and then transferred to selected asparagine residues of nascent polypeptide chains by the enzyme *N*-oligosaccharyl transferase (OTase) (Kaplan *et al.*, 1987; Tanner and Lehle, 1987).

A biochemical characterization of the *N*-oligosaccharyl transferase has proven to be difficult: attempts to purify this enzyme have failed due to the lability of the enzyme upon solubilization (Das and Heath, 1980; Sharma *et al.*, 1981; Kaplan *et al.*, 1987). Recently, it has been shown that mammalian *N*-oligosaccharyl transferase activity co-fractionated with a protein complex of ribophorins I and II and a 48 kDa protein (Kelleher *et al.*, 1992). Antibodies directed against ribophorin I were able to precipitate *N*-oligosaccharyl transferase activity (Kelleher *et al.*, 1992). The presence of a potential binding site for dolichol (Albright *et al.*, 1989) in the transmembrane domain of ribophorin I led to the suggestion that this protein might be involved in the binding of the lipid-linked core oligosaccharide substrate (Kelleher *et al.*, 1992). Ribophorins are highly conserved proteins in the rough endoplasmic reticulum and found to be associated with the translocation machinery in the ER membrane (Kreibich *et al.*, 1983; Marcantonio *et al.*, 1984; Rapoport, 1991).

We have recently shown that in the yeast *Saccharomyces cerevisiae* the essential gene *WBP1* encodes a protein necessary for *N*-oligosaccharyl transferase activity *in vivo* and *in vitro*. Depletion of Wbp1p led to a loss of *N*-oligosaccharyl transferase activity (te Heesen *et al.*, 1992). We proposed that Wbp1p is an essential component of the *N*-oligosaccharyl transferase complex. This conclusion is supported by the recent finding that the 48 kDa component of the canine pancreas *N*-oligosaccharyl transferase (Kelleher *et al.*, 1992) has sequence homology to the yeast Wbp1 protein (Silberstein *et al.*, 1992). Therefore, the *N*-oligosaccharyl transferase (or at least one component of it) is structurally conserved from yeast to mammals.

In this report, we describe the identification and characterization of an additional component, Swp1p, of the yeast *N*-oligosaccharyl transferase. We show that this component, like Wbp1p, is essential for *N*-oligosaccharyl transferase activity *in vivo* and *in vitro*. Cross-linking experiments and genetic data indicate that the two proteins form a complex *in vivo*.

Results

Isolation of the SWP1 gene

As noted previously, the temperature-sensitive phenotype of *wbp1* mutants is due to a reduced level of mutant Wbp1p at permissive and non-permissive temperatures (te Heesen *et al.*, 1992). It is possible that a reduced stability of the mutant Wbp1 protein is the cause of this defect. We hypothesized that stability could be (partially) restored by an increased concentration of an appropriate Wbp1p complex partner. Therefore, we applied a high copy number suppressor approach (Rine, 1991) and screened a high copy number plasmid library containing yeast genomic DNA for the presence of a *wbp1* suppressor. To construct a high copy number plasmid library, total genomic DNA of the strain 45-C3 (*Δwbp1::HIS3 URA3:Gal1-WBP1 ade2-101 tyr1*) (te Heesen *et al.*, 1992) was isolated. This strain contains a hybrid *WBP1* gene: the expression of the wild-type *WBP1* protein is directed by the *GAL1* promoter. Since the *GAL1* promoter activity is repressed in the presence of glucose, this modified *WBP1* locus should not be able to complement a *wbp1* mutation when the cells are grown on glucose-containing medium. DNA was partially digested with *Sau*3A, fragments between 4 and 10 kb were isolated and ligated into the *Bam*HI-cleaved *URA3*-containing vector YEp352 (Hill *et al.*, 1986). This plasmid pool, resulting from 120 000 individual transformants, was used to transform the temperature-sensitive strain MA9-D (relevant phenotype:

Fig. 1. (**A**) Restriction map of the *SWP1* locus. The cleavage site for selected restriction enzymes is shown. The location of the Swp1p encoding open reading frame is shown as an arrow. (**B**) Nucleotide sequence of the *Pst*I−*Bam*HI fragment of the *SWP1* locus and the predicted amino acid sequence of Swp1p. Numbering of the nucleotides, starting with the ATG initiation codon, is indicated above the sequence. Numbering of the amino acid residues of Swp1p is given at the right side of the sequence. (**C**) Hydropathy analysis of Swp1p according to Kyte and Doolittle. The N-terminal and the three C-terminal hydrophobic domains are indicated by bars.

wbp1-2 ura3-52) to uracil prototrophy at 23°C on glucose-containing medium. The resulting 60 000 colonies were screened by replica-plating for a temperature-resistant phenotype. Seventeen revertants were isolated, the plasmids conferring the temperature-resistant phenotype were recovered in *Escherichia coli* and analyzed by restriction analysis. All except one of these plasmids contained *WBP1* specific sequences. Restriction analysis of *WBP1* positive plasmids revealed small deletions within the *GAL1-10* promoter region in some of these plasmids, but no detailed analysis was performed. The remaining plasmid was able to confer temperature resistance when transformed into strain MA9-D (*wbp1-2*). Interestingly, no temperature-resistant transformants were recovered when strain MA7-B (*wbp1-1*) was used as recipient, indicating an allele specificity of the suppressor plasmid (see below). Deletion analysis located the suppressing activity on a 1.7 kb *Pst*I−*Bam*HI DNA fragment (Figure 1A). This region was sequenced (Figure 1B) and the corresponding locus was termed *SWP1* (*s*uppressor of *wbp*1).

The SWP1 gene encodes a potential transmembrane protein essential for vegetative growth

The 1.7 kb *Pst*I−*Bam*HI fragment capable of suppressing the temperature-sensitive phenotype of *wbp1-2* strains contains one large open reading frame predicted to encode a protein of 286 amino acid residues (Figure 1B). Hydrophilicity analysis of the corresponding protein revealed a potential N-terminal signal sequence and a long C-terminal hydrophobic region with the capacity to form three transmembrane domains (Figure 1C). The predicted molecular weight of mature Swp1p is 29.3 kDa. No

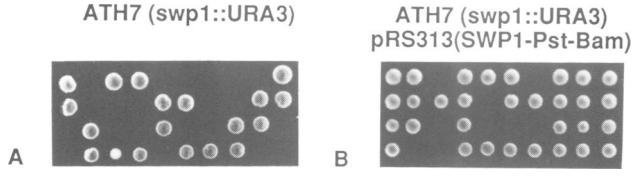

Fig. 2. Swp1p is an essential protein. Tetrad analysis of diploid strains heterozygous for the SWP1 deletion. Tetrads obtained from sporulation of the strain ATH7 (**A**), heterozygous for the deletion in the *SWP1* coding sequence, and the strain ATH7, carrying the *SWP1* gene on the plasmid pRS313(SWP1-Pst-Bam) (**B**), were dissected on YPD medium, the four spores of one tetrad in a column, and grown for 4 days at 30°C. The colonies were replica-plated to determine their phenotype. All growing colonies resulting from strain ATH7 were ura$^-$, indicating the lethal effect of the SWP1 deletion. This lethality could be overcome by the expression of the *SWP1* gene on the plasmid pRS313(SWP1-Pst-Bam). Improper segregation of the complementing plasmid pRS313(SWP1-Pst-Bam) can account for the 1:3 and 2:2 segregation of lethality observed in Figure 2B.

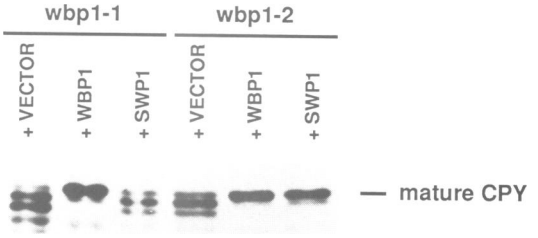

Fig. 3. *SWP1* is an allele specific *wbp1* suppressor. The two strains MA-7B (*wbp1-1*) and MA9-D (*wbp1-2*) were transformed with either YEp352 (+ vector), YEp352(WBP1) (+ WBP1) or YEp352(SWP-Pst-Bam) (+ SWP1) as indicated. Strains were grown under selective conditions at 23°C, labeled for 1 h, lysed and used for CPY specific immunoprecipitation. The position of mature CPY is indicated; the two *wbp1* mutations lead to underglycosylation of CPY.

significant homology to other known sequences was detected in several databases using the FASTA or WORDSEARCH programs. No potential N-linked glycosylation site is present within the Swp1p coding sequence. To test if *SWP1* encodes an essential protein, a deletion version of the *SWP1* locus was constructed in which the *Cla*I−*Bgl*II fragment of *SWP1* (Figure 1A) was replaced by the 1.1 kb *Hin*dIII fragment of the *URA3* locus (Rose *et al.*, 1983). This construct was used to replace one wild-type copy of the *SWP1* locus in the diploid strain SS328×SS330. Correct replacement was monitored by genomic Southern blot analysis of resulting Ura$^+$ transformants. Sporulation of such strains and resulting tetrad analysis showed that haploid segregants containing the *SWP1* deletion were inviable (Figure 2). The deletion-containing spores were able to germinate but only micro-colonies of ~20 cells were formed (data not shown). The lethal phenotype of the *SWP1* deletion could be rescued by a plasmid that contains the intact *SWP1* allele (Figure 2). We conclude that like Wbp1p, the SWP1 protein is essential for the vegetative growth of yeast.

Overexpression of SWP1 restored the glycosylation deficiency in wbp1-2 but not in wbp1-1 strains

As outlined above, overexpression of *SWP1* was capable of suppressing the temperature-sensitive phenotype in *wbp1-2* but not in *wbp1-1* strains. Both *wbp1* alleles result in underglycosylation of proteins in permissive as well as non-permissive conditions (te Heesen *et al.*, 1992). If the growth defect in the *wbp1* strains were due to this underglycosylation, we would expect glycosylation to be restored in a *wbp1-2* but not in a *wbp1-1* strain transformed with a high copy number *SWP1* plasmid. The glycosylation capacity of the cells was measured by the analysis of carboxypeptidase Y (CPY) (Stevens *et al.*, 1982). Mature CPY contains four N-linked oligosaccharides and has a molecular weight of 61 kDa (Hasilik and Tanner, 1978). The *wbp1-1* and *wbp1-2* mutations affect CPY glycosylation at the permissive as well as the non-permissive temperature, resulting in underglycosylated CPY (Figure 3) (te Heesen *et al.*, 1992). Whereas expression of wild-type Wbp1p restored the glycosylation defect in both mutant strains, high copy number expression of *SWP1* had an effect only in the *wbp1-2* strain: fully glycosylated and small amounts of CPY lacking one oligosaccharide chain were detected in this strain. Only a slight influence of *SWP1* expression on the CPY glycosylation pattern was seen in the *wbp1-1* strain. To demonstrate unambiguously that core oligosaccharide transfer was affected, *N*-oligosaccharyl transferase activity was determined in extracts prepared from *wbp1-2* mutant cells transformed with either *SWP1* or *WBP1* (Table I). Extracts from *wbp1-2* mutant cells contained a 10-fold reduced OTase activity compared with wild-type extracts. A high copy number plasmid containing the *SWP1* locus restored OTase activity to 50% of the wild-type level, whereas the *WBP1* gene could fully complement the *wbp1-2* defect. These results establish that *SWP1* is a *wbp1-2* specific high copy number suppressor for both the temperature-sensitive phenotype and the constitutive underglycosylation of proteins.

Table I. *In vitro* activity of OTase in different cell extracts

Strain	Glycosyl transfer to peptide (%)
SS328 (wild-type)	100
MA9-D (*wbp1-2*)	10
MA9-D (YEp352/SWP1-Pst-Bam)	50
MA9-D (YEp352/WBP1)	90

The different strains were grown to mid-log phase, extracts were prepared and oligosaccharyl transferase activity was measured as given in Materials and methods. The value obtained from wild-type extracts was taken as 100%.

Depletion of the SWP1 protein results in underglycosylation of CPY in vivo and a reduced N-oligosaccharyl transferase activity in vitro

The depletion of the *WBP1* protein leads to underglycosylation of CPY due to a reduced *N*-oligosaccharyl transferase activity. We asked if depletion of the *SWP1* protein results in a similar phenotype. For that purpose, the diploid strain heterozygous for the *SWP1* deletion was transformed with a low copy number plasmid carrying a *GAL1*−*SWP1* hybrid gene. In this gene, the expression of the *SWP1* coding region is controlled by the *GAL1* promoter. After sporulation of such a strain and tetrad dissection, haploid strains were recovered carrying a deletion of the chromosomal *SWP1* gene but containing the complementing plasmid. Such strains grow on galactose- but not on glucose-containing medium. We analyzed the time-dependent effect of Swp1p depletion on CPY glycosylation (Figure 4). We observed the first reduced glycosylation of

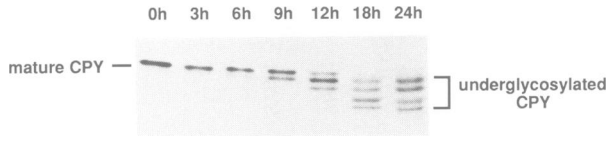

Fig. 4. Depletion of Swp1p alters CPY glycosylation. Strain ATH8 was shifted from galactose- to glucose-containing selective minimal medium, resulting in the repression of Swp1p synthesis. Samples were taken at the time indicated, labeled for 1 h, lysed and used for CPY specific immunoprecipitation as given in Materials and methods. The position of fully glycosylated, mature CPY is indicated. Depletion of Swp1p results in the accumulation of underglycosylated CPY.

CPY after 6 h in glucose-containing medium, and the amount of underglycosylated CPY increased with time. Underglycosylated CPY gave rise to a multiplet of bands with a higher mobility than mature CPY, representing underglycosylated forms lacking one, two, three or all four N-linked oligosaccharide chains. The same CPY pattern was observed in strains depleted for the *WBP1* protein (te Heesen *et al.*, 1992) or carrying the *wbp1-1* (or *wbp1-2*) mutation. A similar underglycosylation due to Swp1p depletion was observed for proteinase A (PrA) (data not shown). The hypothesis that this underglycosylation is due to a reduced *N*-oligosaccharyl transferase activity was verified by determining the *in vitro* activity of this enzyme in Swp1p-depleted cells (Table II). It is evident that growth in glucose medium of a *GAL1−SWP1* strain leads to a time dependent decrease in OTase activity. Therefore, the SWP1 protein is as essential for *N*-oligosaccharyl transferase activity *in vivo* and *in vitro* as Wbp1p.

Wbp1p and Swp1p form a protein complex

The observation that (i) high copy number expression of the *SWP1* gene could suppress the *wbp1-2* mutation and (ii) that Wbp1p and Swp1p depletion lead to a similar phenotype suggests that these two proteins form a complex *in vivo* essential for *N*-oligosaccharyl transferase activity. To test this hypothesis, cross-linking experiments were performed. Membrane fractions of metabolically labeled wild-type cells were prepared, solubilized with Triton X-100 and treated with the cross-linking reagent dithiobis(succinimidylpropionate) (DSP) (Deshaies *et al.*, 1991). Cross-linked products were denatured and immunoprecipitation was performed under non-reducing conditions using affinity-purified anti-Wbp1p antibodies. After cleavage of the cross-linker with DTT, the products were analyzed on SDS−PAGE. Beside Wbp1p, additional proteins were precipitated from cross-linked extracts: proteins migrating with a mobility of 80, 70, 68 and 30 kDa were detected (Figure 5, lane 3). Among these proteins, the 30 kDa protein is cross-linked most efficiently to Wbp1p. Immunoprecipitation without cross-linking resulted primarily in the detection of Wbp1p (Figure 5, lanes 1 and 2). Since 30 kDa is the expected molecular weight of Swp1p, we tested if the 30 kDa protein is Swp1p. We constructed a yeast strain with a modified SWP1 locus. A tag sequence encoding 12 amino acids was inserted at the 3′ end of the *SWP1* open reading frame. A yeast strain, ATH1, containing only the tagged Swp1p was constructed. Phenotypically, this strain could not be distinguished from a wild-type strain, indicating that the C-terminal extension did not impair Swp1p function. We have used this strain to perform cross-linking experiments as described above. If the 30 kDa protein cross-linked to Wbp1p is Swp1p, we expected that the 30 kDa protein from the *SWP1* modified cells would be replaced by a protein of ~31.5 kDa. Indeed, the expected mobility-shift of the small cross-linked protein was detected in extracts derived from *SWP1* modified cells as compared with wild-type cells (Figure 5, lanes 3 and 4). Our results prove that Swp1p can be specifically cross-linked to Wbp1p in solubilized membrane fractions, arguing for a direct interaction between these two proteins.

Table II. *In vitro* activity of OTase in Swp1p-depleted cell extracts

Strain	Time after shift to glucose (h)	Glycosyl transfer to peptide (%)
AT-H8	0	100
	6	70
	12	23
	18	9
	24	8

Strain ATH8 was grown in selective minimal medium containing galactose, washed and used to innoculate minimal medium with glucose as carbon source. Cultures were grown at 30°C for the time indicated, extracts were prepared and oligosaccharyl transferase activity was measured as given in Materials and methods. The value obtained at the shift from galactose to glucose medium was taken as 100%.

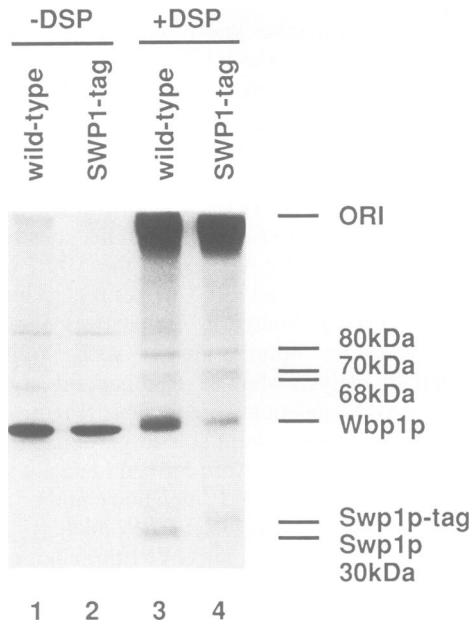

Fig. 5. Cross-linking of Wbp1p to Swp1p. Radiolabeled, solubilized membrane extracts were treated with dithiobis(succinimidylpropionate) (+DSP) at 23°C (lanes 3 and 4). Products were immunoprecipitated with affinity-purified anti-Wbp1p antibodies, the cross-linker was reduced and the samples were analyzed by SDS−PAGE. In control experiments, the cross-linking step was omitted and SDS was added before immunoprecipitation (−DSP) (lanes 1 and 2). Extracts were prepared from strain SS328 (wild-type) (lanes 1 and 3) and ATH1 (*SWP1*−tag) (lanes 2 and 4). The positions of Wbp1p, Swp1p (30 kDa), the tagged Swp1p (31.5 kDa) and additional cross-linked proteins are indicated.

Discussion

We have isolated the essential *SWP1* gene of *S.cerevisiae* based on its capacity to suppress the temperature-sensitive phenotype of the *wbp1-2* mutation. Several experimental findings suggest that Swp1p and Wbp1p form a complex essential for *N*-oligosaccharyl transferase: (i) Depletion of Wbp1p and Swp1p lead to underglycosylation of proteins and to a concomitant reduction of oligosaccharyl transferase activity in extracts from depleted cells. (ii) Swp1p could be cross-linked to Wbp1p in solubilized membranes, suggesting a close contact between the two proteins. (iii) The presence of *SWP1* on a high copy number plasmid suppresses only a *wbp1-2* but not a *wbp1-1* mutation. This allele specificity is best explained by a direct interaction between the two proteins (Huffaker *et al.*, 1987).

Based on the primary amino acid sequence we propose that Swp1p (like Wbp1p) is a type I transmembrane protein. The first 22 amino acids represent a potential signal sequence to direct import into the endoplasmic reticulum (von Heijne, 1986). The C-terminal part of the protein is very hydrophobic and three potential transmembrane domains can be predicted. We therefore propose that the N-terminal portion of Swp1p is located in the lumen of the endoplasmic reticulum, separated by three membrane-spanning domains from the short, cytoplasmic tail. In support of this hypothesis, we could recently show that fusing the histidinol dehydrogenase protein domain to the C-terminus of Swp1p allowed growth of a *his4* strain on histidinol (M.Zecca, S.te Heesen and M.Aebi, unpublished). This result indicates that the C-terminus of Swp1p is located in the cytoplasm, since histidinol can only be converted to histidine by a cytoplasmic histidinol dehydrogenase (Sengstag *et al.*, 1990). Since Wbp1p is also a type I transmembrane protein, the Wbp1p−Swp1p complex is exposed to the lumen of the ER.

At present, we have isolated two essential components of the yeast *N*-oligosaccharyl transferase, but we have no direct indication for the individual functional role of these components in the transfer reaction of the core oligosaccharide to asparagine residues of nascent polypeptides. *wbp1-1* and *wbp1-2* mutants are characterized by a reduced level of mutant Wbp1p, resulting in a decreased *N*-oligosaccharyl transferase activity (te Heesen *et al.*, 1992). This reduction of N-linked glycosylation results in a temperature-sensitive phenotype. The observation that the presence of *SWP1* on a high copy number plasmid can rescue a temperature-sensitive *wbp1-2* mutation suggests that Wbp1p is stabilized in a complex with Swp1p. In support of this notion we observe that depletion of Swp1p results in a concomitant reduction of Wbp1p (data not shown), but we do not know at present whether depletion of Wbp1p also results in a similar reduction of Swp1p.

One substrate of *N*-oligosaccharyl transferase is the dolichol-bound core oligosaccharide. It has been noted that several dolichol binding enzymes contain an 11 residue consensus sequence in the membrane-spanning domains. This sequence was implicated in the binding of dolichol, but no experimental evidence exists to support this hypothesis (Albright *et al.*, 1989; Lehrman, 1991). Interestingly, this consensus sequence was also observed in the transmembrane domain of ribophorin I (Kelleher *et al.*, 1992) (see below). We note that two of the three potential membrane-spanning domains of Swp1p also contain sequences similar to the dolichol consensus sequence: within amino acid positions 196−206 and 234−244 (see Figure 1) we observe a four out of seven and three out of seven match to the consensus sequence. However, we have no direct proof that these regions interact with dolichol.

Recently, Kelleher *et al.* (Kelleher *et al.*, 1992) have found that *N*-oligosaccharyl transferase activity from dog pancreas is present in a complex consisting of ribophorins I and II and a 48 kDa protein. This 48 kDa protein, called OST48p, is homologous to the yeast Wbp1p (Silberstein, 1992) suggesting that *N*-oligosaccharyl transferase is structurally conserved during evolution. However, the 48 kDa Wbp1p homolog OST48p was found in a complex with ribophorins I and II (Kelleher *et al.*, 1992). two proteins which have not been identified so far in yeast (Sanderson *et al.*, 1990). The purification of dog pancreas *N*-oligosaccharyl transferase did not yield a protein similar in size to Swp1p (30 kDa) (Kelleher *et al.*, 1992) and Swp1p has no sequence similarity to either ribophorin I or II. It is possible that only one component of *N*-oligosaccharyl transferase (Wbp1p/OST48p) is conserved in evolution. Alternatively, the multi-component complex is conserved between different species, but the yeast ribophorin homologs and the Swp1p homolog of higher eukaryotes have not been isolated so far. Our results that additional proteins beside Swp1p can be cross-linked to Wbp1p (Figure 5) suggest that additional component(s) of yeast *N*-oligosaccharyl transferase complex may exist. Indeed, we observe that simultaneous overexpression of both Wbp1p and Swp1p does not lead to a significant increase of *N*-oligosaccharyl transferase activity in extracts from such cells, though a Wbp1p−Swp1p complex accumulates in these cells (data not shown). Both Wbp1p and Swp1p are therefore non-limiting components of the yeast *N*-oligosaccharyl transferase. Combined genetic and biochemical approaches will allow us to identify such additional components. With these components identified we should be able to define the functional role of the different subunits in the transfer of the core oligosaccharide to nascent polypeptide chains.

Materials and methods

Yeast strains and media

The following yeast strains were used in this study: X2180-1A (Yeast Genetic Stock Center, Berkley, CA); SS328 (MATa *ade2-101 his3Δ200 ura3-52 lys2-801)* (Vijayraghavan *et al.*, 1989); SS330 (MATα *ade2-101 his3Δ200 ura3-52 tyr1*) (Vijayraghavan *et al.*, 1989); MA7-B (MATa *wbp1-1 ade2-101 his3Δ200 ura3-52 lys2-801*) (te Heesen *et al.*, 1992); MA9-D (MATa *wbp1-2 ade2-101 his3Δ200 ura3-52 lys2-801*) (te Heesen *et al.*, 1992); 45-C3 (MATα *his3Δ200 Δwbp1::HIS3 URA3:GAL1-WBP1 ade2-101 tyr1*) (te Heesen *et al.*, 1992); ATH1 (MATa *ade2-101 his3Δ200 ura3-52 lys2-801 SWP1−tag:URA3*) (this study); ATH7 (MATa/α *ade2-101/ade2-101 his3Δ200/his3Δ200 ura3-52/ura3-52 lys2/+ tyr1/+ Δswp1::URA3/+*) (this study); ATH8 [MATa *ade2-101 his3Δ200 ura3-52 Δswp1::URA3* pRS313(SWP1-*Pst-Bam*)] (this study). Standard yeast media (Sherman *et al.*, 1983) were used.

Isolation of the SWP1 locus

DNA techniques have been described (Sambrook *et al.*, 1989). Chromosomal DNA was isolated from strain 45-C3 and partially digested with *Sau*3A. DNA fragments between 4 and 10 kb in size were fractionated by agarose-gel electrophoresis and ligated into the vector YEP352 (Hill *et al.*, 1986). After transformation and amplification in the *E.coli* strain DH5α (Hanahan, 1983), the plasmid pool was transformed into the yeast strain MA9-D (Ito *et al.*, 1983) and uracil prototrophic transformants were selected at permissive

temperature (23°C). Temperature-resistant colonies were screened by replica-plating on selective minimal medium and YEPD medium and incubation of the plates at 37°C. Resistant strains were colony-purified, total DNA was isolated and plasmid DNA was recovered by transformation into *E.coli* strain DH5α, analyzed by restriction digest analysis and tested if they were able to transform yeast strain MA9-D to temperature resistance. Plasmid YEP352/SWP1 was analyzed further and the *wbp1-2* complementing *Pst*I−*Bam*HI fragment was sequenced. The DNA sequence was submitted to the EMBL Data Bank, accession number X67705. For further experiments, the *Pst*I−*Bam*HI fragment of the *SWP1* locus was ligated into the 2μm-derived vector YEp352 [YEp352(SWP1-*Pst-Bam*)] and the CEN-ARS plasmid pRS313 (Sikorski and Hieter, 1989) [pRS313(SWP1-*Pst-Bam*)].

Disruption of the SWP1 locus

The 1.1 kb *Hin*dIII fragment of the *URA3* locus (Rose *et al.*, 1983) was inserted between the *Cla*I and *Bgl*II sites of the *SWP1* locus (see Figure 1), resulting in the deletion of codons 51−280 of the *SWP1* ORF. This version of the *SWP1* locus was used to replace one wild-type copy of the *SWP1* gene of the diploid strain SS328×SS330 by homologous recombination, selecting for uracil prototrophy.

Construction of pRS313(GAL1−SWP1)

A *Hin*dIII site was introduced at position −29 of the *SWP1* gene by standard PCR methods, resulting in the sequence 5′-**AAGCTT**AGACCATAAAA-3′. The resulting *SWP1* gene was cleaved with *Hin*dIII and *Bam*HI, located downstream of the *SWP1* coding sequence, mixed with the *Eco*RI−*Hin*dIII *GAL1-10* promoter fragment of YIpGAL(WBP1) (te Heesen *et al.*, 1992) and ligated into the *Eco*RI−*Bam*HI cleaved vector pRS303 (Sikorski and Hieter, 1989). The *GAL1−SWP1* unit was then transferred as a 2 kb *Sal*I−*Bam*HI fragment to the CEN-ARS vector pRS313 (Sikorski and Hieter, 1989) to yield pRS313(GAL1−SWP1). The coding sequence of this modified *SWP1* gene was sequenced. The diploid strain ATH7 was transformed with this plasmid to histidine prototrophy, sporulated and tetrads were dissected on plates containing galactose as sole carbon source.

Construction of the SWP1−tag strain ATH1

First, a *Nar*I site was introduced at position 63 of the *SWP1* gene by standard PCR methods. The resulting sequence was 5′-**GGCGCC**AGATGTTCA-TG-3′. The *Nar*I−*Kpn*I fragment containing a 5′-truncated *SWP1* locus was ligated into the *Cla*I−*Kpn*I cleaved vector pBS/URA3. This vector contains the 1.1 kb *Hin*dIII fragment of the *URA3* locus (Rose *et al.*, 1983) within the polylinker sequence of pBluescript KS(+). Transcription of the *URA3* gene is toward the *Sac*I site of the polylinker. The resulting plasmid was called pBS(SWPΔNarI)URA3. In this plasmid, the 3′ non-translated region of the *SWP1* gene was replaced by a synthetic, tag encoding sequence and 0.7 kb of the 3′ non-translated region of the *WBP1* gene. The new C-terminus encoding sequence is as follows: 5′-**GATCTTTTGGTCAA-ACAATC**ctcgagcaaaagttgatctctgaagaagacttgaactag*TATATGTTTT*-3′. The bold letters represent *SWP1* sequence from position 839−858, the lower case letters an artificial sequence encoding the c-*myc* tag-containing sequence LEQKLISEEDLN and the italic letters *WBP1* sequence from position 1294−1303 of the *WBP1* locus (te Heesen *et al.*, 1991). This plasmid, pBS(SWP1ΔNarI/tag)URA3, was linearized with *Cla*I, located at position 145 in the *SWP1* coding sequence, and used to transform strain SS328 to uracil prototrophy. Correct integration of the plasmid into the *SWP1* locus was monitored by PCR analysis of genomic DNA of the transformants. Integration of the plasmid at the *Cla*I site results in an inactive copy due to the *Nar*I deletion and an active copy expressing the tagged Swp1p. The resulting strain was called ATH1.

Immunological methods

Recombinant Wbp1p (codons 14−396) was produced in *E.coli* BL21 cells using the pET system (Studier and Moffat, 1986) and purified from inclusion bodies by preparative SDS−PAGE. The material was used to immunize a New Zealand White rabbit. The serum obtained was purified by affinity columns containing the pure recombinant rWbp1p coupled in 100 mM MOPS (pH 7.5) to AffiGel 15 matrix using standard techniques (Harlow and Lane, 1988).

For cross-linking experiments, yeast strains were grown in minimal medium containing the appropriate amino acid and nucleoside supplements to a density of ~10^7 cells/ml. 10^9 cells were labeled with 1.5 mCi of [^{35}S]methionine (>1000 Ci/mmol, Amersham) for 1 h at 30°C. Preparation of labeled yeast membranes, cross-linking with DSP (Fluka, Switzerland) and immunoprecipitation were as described (Deshaies *et al.*, 1991) except that 20 μl of affinity-purified anti-Wbp1p antiserum was used and the treatment of extracts with IgSorb prior to incubation with antibody was omitted. Immunoprecipitation of CPY was as described (te Heesen *et al.*, 1992). Labeled proteins were analyzed by 9% (Wbp1p/Swp1p) or 8% (CPY) SDS−PAGE, followed by fluorography (Chamberlain, 1979) and exposure to Kodak XAR-5 films at −80°C.

Determination of N-oligosaccharyl transferase activity

Preparation of membrane fractions and determination of OTase activity have been described (te Heesen *et al.*, 1992).

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