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The *N*-oligosaccharyltransferase complex from yeast

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Abstract

N-Oligosaccharyltransferase catalyzes the *N*-glycosylation of asparagine residues of nascent polypeptide chains in the endoplasmic reticulum, a pathway highly conserved in all eukaryotes. An enzymatically active complex was isolated from microsomal membranes from *Saccharomyces cerevisiae*, which is composed of four proteins: Wbp1p and Swp1p (previously found to be encoded by two essential genes necessary for N-glycosylation in vivo and in vitro) and two additional proteins with a molecular mass of 60/62 kDa and 34 kDa. The 60/62 component represents differentially glycosylated forms of a protein that has sequence homology to ribophorin I. Wbp1p and Swp1p reveal homology to mammalian OST 48 and ribophorin II, respectively. Ribophorin I and II and OST 48 were recently shown to be constituents of the mammalian transferase from dog pancreas. The data reveal a high conservation of the organization of this enzyme activity.

Key words: Oligosaccharyltransferase; Protein glycosylation; Glycoprotein; Endoplasmic reticulum; Dolichol; *Saccharomyces cerevisiae*

1. Introduction

N-linked core glycosylation of asparagine residues is an essential protein modification and is highly conserved in all eukaryotic cells [1–4]. The key step of this pathway is the en bloc transfer of the high mannose oligosaccharide $Glc_3Man_9GlcNAc_2$ assembled on the lipid carrier dolichyl phophate to selected Asn-X-Ser/Thr sequences. This so-called core-glycosylation occurs during translocation of nascent polypeptide chains into the endoplasmic reticulum. The reaction is catalyzed by the enzyme *N*-oligosaccharyltransferase. A biochemical characterization of this enzyme has proven to be difficult due to its lability upon solubilization [4–6] and probably also due to the fact that it consists of several subunits forming a membrane-bound complex. In a genetic approach two essential genes have recently been identified in the yeast *Saccharomyces cerivisae*, *WBP1* and *SWP1*, that encode proteins necessary for oligosaccharyltransferase activity in vivo and in vitro [7,8]. Both proteins could be chemically cross-linked suggesting that they are in physical contact [8]. Since overexpression of both proteins does not lead to a significant increase in oligosaccharyltransferase activity, though a Wbp1p–Swp1p complex accumulates, it was assumed that both Wbp1p and Swp1p are non-limiting components of a larger complex. Using an anti-Wbp1p immunoaffinity column an enzymatically active complex could now be isolated, which is composed of Wbp1p, Swp1p and two additional proteins with a molecular mass of 60/62 kDa and 34 kDa, respectively. The data reveal a high conservation of the structural organization of the oligosaccharyltransferase from yeast and components of that enzyme activity recently identified in dog pancrease [9,10].

2. Materials and methods

2.1. Isolation of membranes and solubilized enzyme extract

X2180 wild-type strain was grown to middle of log phase in yeast complete medium, washed in homogenization buffer (50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$ and 1 mM DTT, 0.05% aprotinin, 1 mM PMSF) and broken with glass beads. Membranes were isolated by differential centrifugation. The pellet between $1200 \times g$ and $48{,}000 \times g$ obtained after centrifugation for 45 min was washed once in homogenization buffer and resuspended in 30 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM DTT and 35% glycerol. For solubilization, membranes (12 mg protein/ml) were adjusted to 15 mM Tris/HCl, pH 7.5, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 M KCl, 17% glycerol and 1% Nikkol and incubated for 20 min at 0°C. The solubilized extract was then separated from insoluble material by centrifugation at $150{,}000 \times g$ for 45 min.

2.2. Immunoaffinity chromatography

A polyclonal rabbit anti-Wbp1p-antiserum was coupled to protein A-Sepharose as described [11]. Solubilized extract (120 mg protein) was applied to a column (2 ml vol.) equilibrated with solubilizing buffer. The column was washed with 20 vols. of the solubilizing buffer, containing 0.1% detergent and 1.5 mM phosphatidylcholine. Elution of the complex was carried out with the above buffer containing 1% detergent and 1 mM synthetic antigen peptide or by changing the pH with glycine-HCl buffer to pH 2 followed by immediate retitration to pH 7.5.

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Abbreviations: Swp1p, protein of *SWP1* gene; Wbp1p, protein of *WBP1* gene; Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine.

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2.3. Determination of oligosaccharyltransferase activity and immunoadsorption

Oligosaccharyltransferase activity was determined as described [5]. For immunoadsorption experiments 0.09 ml aliquots of solubilized extract were incubated with anti-Wbp1p antiserum or preimmuneserum as indicated for 2 h to overnight at 4°C. 5 mg proteinA-Sepharose beads equilibrated in solubilizing buffer were added (vol. 0.03 ml) and incubated for 2 h. Beads were centrifuged for 2 min and the supernatant was tested for enzyme activity. In the peptide competition experiments 0.01 ml antiserum was preincubated with 1 mM peptide for 4 h before the solubilized extract was added.

2.4. Other procedures

Polyclonal antibodies were raised in rabbits against two synthetic peptides corresponding to the C-terminal 16 amino acid residues of the Wbp1 and Swp1 protein, respectively, coupled to BSA. Proteins were resolved by SDS-PAGE in the Laemmli system [12] and detected by silver staining. For immunoblots, proteins were transferred to nitrocellulose and visualized using enhanced chemoluminenscence (Amersham). EndoF (Boehringer) digests were carried out following the manufacturer's recommendation.

3. Results and discussion

Two genes have been identified so far in yeast necessary for oligosaccharyltransferase activity: *WBP1* encodes a type I ER membrane protein with a calculated molecular mass of 47 kDa, having a signal sequence, two potential N-glycosylation sites and the majority of the N-terminal domain luminally oriented [7,13]. The other protein, Swp1p, is also of type I with a mass of 29.3 kDa, a potential signal sequence but with no N-glycosylation consensus motif. Originally no sequence homology to other known sequences was detected in several databases [8]. However, direct comparison of *SWP1* with the C-terminal half of the sequence of rat and human ribophorin II [14,15] (≈ 34 kDa, i.e. about the size of Swp1p) reveals 46% similarity and 24% identity. Also three characteristic hydrophobic stretches at the very C-terminus are present in both proteins. Ribophorin II was found to be associated with oligosaccharyltransferase activity from dog pancreas ([9], see also Table 3).

We have obtained a detergent extract from rough microsomal yeast membranes in the presence of 1% Nikkol with about 65% recovery of the enzyme activity. Using an antiserum raised against the 16 cytoplasmically exposed C-terminal residues of Wbp1p, the activity could be immunodepleted from the detergent extract. Depletion was dependent on the amount of antiserum (Fig. 1) and specific for Wbp1p (Table 1), since immunoprecipitation could be prevented by addition of the Wbp1p peptide, but not by other non-Wbp1p related peptides (e.g. a peptide derived from Swp1p or the peptide used as a substrate for the transferase).

This anti-Wbp1p antiserum was used to prepare an immunoaffinity column and to further purify the oligosaccharyltransferase. As can be seen in Fig. 2B an active enzyme could be eluted in the presence of the Wbp1 peptide (used to raise the antibody). The activity cofractionated with four proteins: a 62/60 kDa doublet, Wbp1p, a 34 kDa protein and Swp1p (Fig. 2A). Wbp1p

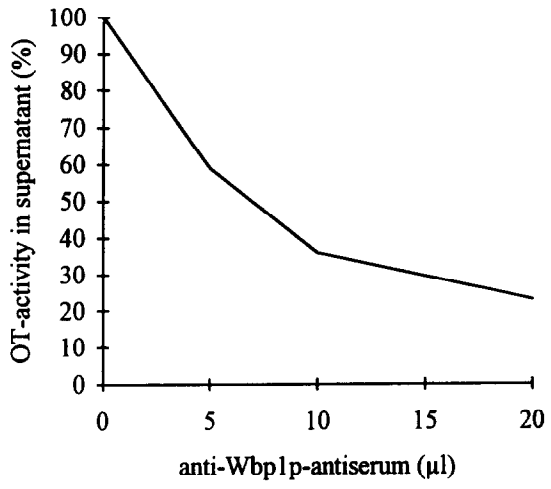

Fig. 1. Immunodepletion of oligosaccharyltransferase activity. Different amounts of anti-Wbp1p antiserum were incubated with solubilized extract. After removing the immune complexes with protein A-Sepharose residual oligosaccharyltransferase activity was determined in the supernatant.

Table 1
Specific inhibition of immunoprecipitation of oligosaccharyltransferase by antigen peptide

	In vitro activity of OT in supernatant
	glycosyl transfer to peptide (%)
Preimmunserum (control)	100
Anti-Wbp1p antiserum	
without peptide	38
plus Wbp1p-peptide	102
plus Swp1p-peptide	42
plus YNLTSV-peptide	39

Solubilized oligosaccharyltransferase was incubated with preimmunserum or anti-Wbp1p antiserum without peptide or 1 mM peptides as indicated. The immuncomplex was precipitated as described in section 2 and the residual enzyme activity of the supernatant was determined. Activity measured with preimmunserum was set to 100%; Wbp1p-peptide is the peptide used to raise the antiserum; Swp1p-peptide corresponds to the 16 C-terminal amino acids of Swp1 protein; YNLTSV peptide is a substrate for oligosaccharyltransferase.

Table 2
Sequence comparison of the N-terminus of 60/62 kDa protein from yeast and human ribophorin I

Yeast 60/62 kDa protein	A	Q	Y	E	-	P	P	A	X	X	E	N	V
Rat Ribophorin I	A	S	S	E	A	P	P	L	I	N	E	D	V
Human Ribophorin I	A	S	P	E	A	P	L	L	V	N	E	D	V

X, no sequence information; –, gap. Immunoaffinity purified complex was subjected to SDS-PAGE (see Fig. 2) and electroblotted onto Immobilon PVDF membrane (Millipore). The 60 and 62 kDa bands were excised and sequenced by automated Edman degradation on a gas-phase protein sequencer. Both bands gave the same sequence.

and Swp1p were identified by their molecular mass and by immunoblot analysis using an anti-Wbp1p and anti-Swp1p specific antiserum, respectively. EndoF treatment revealed that the 62/60 kDa proteins and Wbp1p are glycosylated (data not shown). The 62/60 kDa doublet shifts to a single band of about 56 kDa suggesting the addition of 2–3 core oligosaccharides. Wbp1p shifts from 47 kDa to 43 kDa; this difference is compatible with the N-glycosylation of the two potential glycosylation sites present in *WBP1*. N-terminal sequencing of the 62 and 60 kDa proteins showed that both have an identical N-terminus with sequence homology to ribophorin I (Table 2). Ribophorin I is an N-glycosylated protein with 1–2 core chains [14,16]. Thus we postulate that the 62/60 kDa species are two differentially glycosylated yeast ribophorin I forms. The silver staining of the four co-fractionating bands reveals different intensities. At the moment it cannot be decided, whether this reflects a difference in the stoichiometry or it is due to different intrinsic staining properties of the various bands.

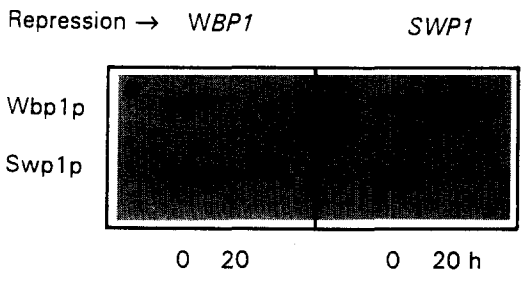

Fig. 3. Depletion of *WBP1* and *SWP1*. Strain 45-C3 (*his*Δ*200*Δ*wbp1::HIS3 URA3:GAL1-WBP1 ade2-101 tyr1*) [7] and strain ATH8 (*ade2-101 his3*Δ*200 ura3-52* Δ*swp1::URA3 pRS313(SWP1- Pst-Bam)*) [8] were shifted from galactose to glucose containing medium. Samples were taken at 0 and 20 h, respectively. Membranes were isolated and aliquots solubilized in SDS sample buffer and analyzed by immunoblotting. In a (lanes 1 and 2, from left to right) *WBP1* gene, in b (lanes 3 and 4) *SWP1* was repressed. Protein expression (Wbp1p and Swp1p) was analyzed with the corresponding antiserum. In lane 4 the two faster moving bands represent underglycosylated forms of Wbp1p.

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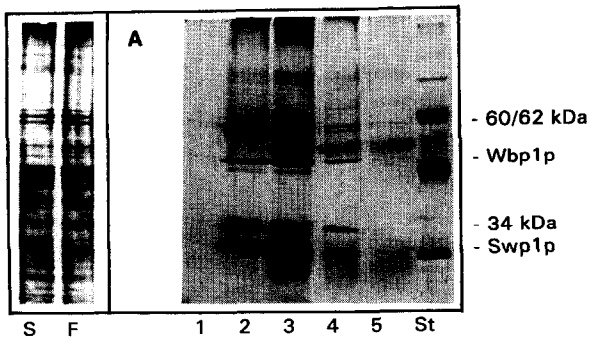

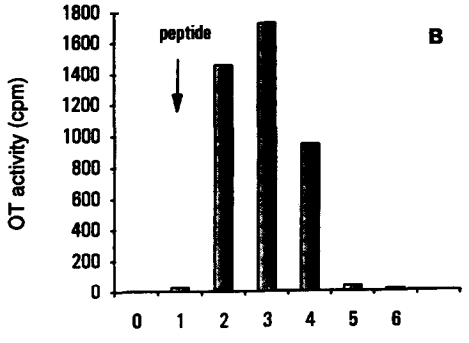

Fig. 2. Immunoaffinity chromatography of the *N*-oligosaccharyltransferase complex. An anti-Wbp1p immunoaffinity column was loaded with solubilized extract (S). After extensive eashing the immunoadsorbed protein was eluted by pH change (A) or antigen peptide (B) and analyzed by silverstaining or by testing OT activity. With both procedures the same protein pattern was obtained. F, first flow through fraction; numbers indicate fractions of eluate; St, protein standard; the asterik indicates non-specific bleeding material from the column.

It has previously been shown that both *WBP1* and *SWP1* genes, when placed under the galactose-inducible and glucose-repressible *GAL1* promotor, lead to a loss of oligosaccharyltransferase activity in vitro and N-glycosylation in vivo under repressible conditions [7,8]. In Fig. 3 an immunoblot is shown following the amount of both proteins in such a repression experiment. Shutting off *WBP1* gene leads, as expected, to a depletion of its encoded protein, but also of Swp1p (Fig. 3a); expression of both proteins also decreases, when *SWP1* gene is repressed (Fig. 3b). In the case of Wbp1p not only a reduction in its amount can be seen but also the appearance of two bands corresponding to underglycosylated Wbp1p forms. This indicates a mutual influence of the two proteins with regard to their formation.

Recently also constituents of the *N*-oligosaccharyltransferase from dog pancreas were identified [9,10]. Table 3 compares the composition of the complexes from yeast and dog pancreas and demonstrates a high conservation not only of the biosynthetic machinery, but also of the structural organization of the transferase. *WBP1* is the functional equivalent to OST48 with 25% overall identity and 50% similarity to *WBP1* [7,9,13]. As discussed above, *SWP1* has homology to the C-terminal half of ribophorin II [14,15,18]. *SWP1* is

Table 3
Homologous components of the oligosaccharyltransferase complex from yeast and dog pancreas

Yeast	Dog pancreas
60/62 gp	ribophorin I (66 kDa)
Wbp1p (47 kDa)	OST 48 (48 kDa)
34 p	?
Swp1p (29 kDa)	ribophorin II (63/64 kDa)

about half the size of ribophorin II and one may speculate that the 34 kDa band found in yeast, but not detected yet in dog pancreas, could serve the function of the N-terminal half of ribophorin II. Inferred from the N-terminal sequence similarity and tryptic peptide sequences the other new identified 62/60 kDa protein seems to be the yeast ribophorin I. Future experiments have to clarify these open questions as well as to identify the specific functions of the individual components in the N-glycosylation process.

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