Applied and Environmental Microbiology, July 2007, p. 4446–4454 Vol. 73, No. 14
0099-2240/07/$08.00+0 doi:10.1128/AEM.02058-06

Engineering of the Yeast *Yarrowia lipolytica* for the Production of Glycoproteins Lacking the Outer-Chain Mannose Residues of N-Glycans[▽]

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Received 31 August 2006/Accepted 8 May 2007

In an attempt to engineer a *Yarrowia lipolytica* strain to produce glycoproteins lacking the outer-chain mannose residues of N-linked oligosaccharides, we investigated the functions of the *OCH1* gene encoding a putative α-1,6-mannosyltransferase in *Y. lipolytica*. The complementation of the *Saccharomyces cerevisiae och1* mutation by the expression of Yl*OCH1* and the lack of in vitro α-1,6-mannosyltransferase activity in the Yl*och1* null mutant indicated that Yl*OCH1* is a functional ortholog of *S. cerevisiae OCH1*. The oligosaccharides assembled on two secretory glycoproteins, the *Trichoderma reesei* endoglucanase I and the endogenous *Y. lipolytica* lipase, from the Yl*och1* null mutant contained a single predominant species, the core oligosaccharide $Man_8GlcNAc_2$, whereas those from the wild-type strain consisted of oligosaccharides with heterogeneous sizes, $Man_8GlcNAc_2$ to $Man_{12}GlcNAc_2$. Digestion with α-1,2- and α-1,6-mannosidase of the oligosaccharides from the wild-type and Yl*och1* mutant strains strongly supported the possibility that the Yl*och1* mutant strain has a defect in adding the first α-1,6-linked mannose to the core oligosaccharide. Taken together, these results indicate that Yl*OCH1* plays a key role in the outer-chain mannosylation of N-linked oligosaccharides in *Y. lipolytica*. Therefore, the Yl*och1* mutant strain can be used as a host to produce glycoproteins lacking the outer-chain mannoses and further developed for the production of therapeutic glycoproteins containing human-compatible oligosaccharides.

Yeast can secrete a variety of proteins in much the same way that mammalian cells do. The presence of yeast-specific outer-chain mannosylation, however, has been a primary hindrance to the exploitation of yeast for therapeutic glycoprotein production, because glycoproteins decorated with yeast-specific glycans are immunogenic and show poor pharmacokinetic properties in humans (1, 24). In the budding yeast *Saccharomyces cerevisiae*, the N-linked oligosaccharides assembled on glycoproteins include hypermannose structures with outer chains that may contain up to 200 mannose units (6). Elongation of the outer chain is initiated by the Och1 protein, which adds the first α-1,6-linked mannose to the core N-linked oligosaccharides upon their arrival in the Golgi apparatus in *S. cerevisiae* (17). Following the addition of the first α-1,6-mannose by Och1p, the core oligosaccharide is elongated by additional α-1,6-mannosyltransferases, Mnn9p and Van1p, which extend the α-1,6-linked polymannose backbone, and the core oligosaccharide is further branched by the addition of α-1,2- and α-1,3-linked mannoses (5, 8). Other yeast species, including *Pichia pastoris*, *Hansenula polymorpha*, and *Schizosaccharomyces pombe*, also use the Och1 protein to extend the mannose outer chain of N-glycans (14, 24, 26, 28). Therefore, the elimination of the Och1 protein was performed to block the yeast-specific outer-chain mannosylation, followed by further engineering of yeast N-glycosylation pathways for the production of glycoproteins with human-compatible oligosaccharides (14, 17, 26).

Yarrowia lipolytica, a heterothallic yeast, is presently considered to be a good potential host for heterologous gene expression due to its ability to secrete large amounts of extracellular proteins (4, 12, 21) and the simplicity with which it is cultivated to a high cell density (13). Despite the potential of *Y. lipolytica* as a valuable host, little information on the structural characteristics of N-linked oligosaccharides of *Y. lipolytica* glycoproteins is available. It was recently reported that *Y. lipolytica OCH1* (Yl*OCH1*), the homolog of *S. cerevisiae OCH1* encoding an α-1,6-mannosyltransferase, may have a minor role in N glycosylation (3). In our study, however, glycoproteins secreted from a Yl*och1* mutant strain showed more homogeneous band patterns upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) than the wild-type strain. Therefore, we have investigated the function of Yl*OCH1* by systematically analyzing the structures of N-linked oligosaccharides assembled on recombinant proteins secreted from the Yl*och1* null mutant strain, and we present several lines of evidence that YlOch1p is a key enzyme responsible for adding the first α-1,6-mannose residue onto the core oligosaccharide, $Man_8GlcNAc_2$, in *Y. lipolytica*. The Yl*och1* mutant strain, which secretes glycoproteins lacking the outer-chain mannoses, can be used as a starting host for further glycoengineering to produce human-compatible oligosaccharides.

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▽ Published ahead of print on 18 May 2007.

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
Y. lipolytica		
SMS397A	*MATA ade1 ura3 xpr2*	11
*och1*Δ (*URA3*)	*MATA ade1 ura3 xpr2 och1*::*tc-URA3-tc*	This study
*och1*Δ (*ura3*)	*MATA ade1 ura3 xpr2 och1*::*tc*	This study
SMS(pAUX-EGI)	SMS397A harboring pAUX-EGI	This study
*och1*Δ(pAUXEGI)	Yl*och1*Δ harboring pAUX-EGI	This study
SMS(pAUX-YlLIP2)	SMS397A harboring pAUX-YlLIP2	This study
*och1*Δ(pAUX-YlLIP2)	Yl*och1*Δ harboring pAUX-YlLIP2	This study
S. cerevisiae		
W303-1A	*MAT***a** *leu2 ura3 trp1 ade2 his3*	Lab collection
*och1*Δ	*MAT***a** *leu2 ura3 trp1 ade2 his3 och1*Δ::*TRP1*	14
TOY137	*MAT***a** *leu2 ura3 trp1 ade2 his3 och1*Δ::*hisG mnn1*Δ::*hisG mnn4*Δ::*hisG*	14
W303-1A(pYEp352GAPII)	W303-1A harboring pYEp352GAPII	This study
*och1*Δ(pYEp352GAPII)	Sc*och1*Δ harboring pYEp352GAPII	This study
TOY137(pYEp352GAPII)	TOY137 harboring pYEp352GAPII	This study
W303-1A(pYEp352GAPII-YlOCH1)	W303-1A harboring pYEp352GAPII-YlOCH1	This study
*och1*Δ(pYEp352GAPII-YlOCH1)	Sc*och1*Δ harboring pYEp352GAPII-YlOCH1	This study
TOY137(pYEp352GAPII-YlOCH1)	TOY137 harboring pYEp352GAPII-YlOCH1	This study

MATERIALS AND METHODS

Strains, plasmids, and media. The *Y. lipolytica* and *S. cerevisiae* strains used in this study are described in Table 1. The yeast strains were routinely grown in YPD (1% yeast extract, 2% Bacto peptone, and 2% glucose) at 28°C (*Y. lipolytica*) or 30°C (*S. cerevisiae*). Synthetic complete medium was composed of 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, and a dropout amino acid mixture including all of the amino acids required. When required, 0.625 mg of 5′-fluoroorotic acid/ml was added to solid medium for the selection of Ura auxotrophic strains. Drug sensitivity was assayed by spotting serially diluted yeast cultures onto YPD solid medium containing 30 μg of hygromycin B (Sigma)/ml, 20 μg of calcofluor white (Sigma)/ml, 100 μg of Congo red (Junsei, Japan)/ml, 0.05% SDS (Sigma), or 7 mM sodium orthovanadate (Sigma).

The plasmid pYEp352GAPII, containing the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene promoter and terminator (18), was used as a backbone vector for the expression of the *Y. lipolytica OCH1* gene in *S. cerevisiae*. The pIMR53-AUX vector (11), containing the *XPR2* promoter and terminator and the *URA3* gene, was used for the secretory expression of *Trichoderma reesei* endoglucanase I (EGI) and *Y. lipolytica* lipase tagged with six histidine residues. To express EGI and lipase, the recombinant *Y. lipolytica* cells were cultivated on YPDm medium (1% yeast extract, 1% proteose peptone, 1% glucose, and 50 mM phosphate-buffered saline, pH 6.8) at 28°C.

Recombinant DNA techniques and gene disruption. Recombinant DNA techniques, Southern blot hybridization, and transformation were carried out essentially as described by Sambrook and Russell (23). PCRs were performed with ExTaq polymerase (Takara, Japan) using the GeneAmp PCR system 2400 (PerkinElmer).

Two pairs of primer sets, YO1F1 (5′-ACTTTTTGCATCTGCGGAC-3′) and YO1R1 (5′-CCATGGATCCGTAGATCTAGGAGTTCGAAGACGTTG-3′) and YO1F2 (5′-AGATCT ACGGATCCATGGGACCGACTCTGTCTTCGA-3′) and YO1R2 (5′-CATCCTCCTGATATACGC-3′), were designed to amplify the 5′ and 3′ flanking regions of the Yl*OCH1* gene. The amplified PCR fragments were fused by performing PCR using the YO1F1 and YO1R2 primers, and the product was subcloned into the pGEMT-Easy vector (Promega). The *tc-URA3-tc* cassette (11) was inserted into the linker site of the fused PCR product. This disruption cassette was made linear by digestion with NotI and used to transform *Y. lipolytica*. Correct disruption was confirmed by PCR and Southern blot analysis. *URA3* of the integrated *tc-URA3-tc* cassette was popped out of the Yl*och1*Δ (*URA3*) strain by growing the strain on synthetic complete medium containing 0.0625% 5′-fluoroorotic acid at 28°C for 5 days. The resulting Yl*och1*Δ (*ura3*) strain was again transformed with the EGI and lipase expression vectors, pAUX-EGI and pAUX-YlLIP2.

Purification and analysis of EGI and lipase. Culture supernatants containing His_6-tagged EGI and lipase were concentrated by ultrafiltration (YM30 membrane, Millipore). The 100-fold-concentrated culture supernatants were dialyzed against 50 mM sodium phosphate (pH 6.0) and 300 mM NaCl, and His_6-tagged EGI and lipase were purified by a His_6-tagged affinity column using the ÁKTA Prime chromatography system (Amersham Pharmacia Biotech AB, Sweden).

Western blot analysis of the purified His_6-tagged EGI was performed with the polyclonal immunoglobulin G Penta-His antibodies (QIAGEN, Germany), and the EGI was detected using the DIG kit (Roche, Germany). Deglycosylation with endoglycosidase H (endo H) was performed in accordance with the instructions of the manufacturer (New England Biolabs). Activity staining of *T. reesei* EGI was carried out as follows. After the cells expressing EGI were grown in 3 ml of YPDm medium for 30 h, the culture supernatant was collected by centrifugation. Each sample (16 μl of supernatant) was subjected to electrophoresis on a 10% PAGE gel. After the completion of electrophoresis, the substrate gel, which contained 50 mM sodium citrate (pH 5.6), 1% carboxyl methyl cellulose, and 1.5% agarose, was overlaid with the PAGE gel. Both gels were wrapped and incubated at 30°C for 3 h. The substrate gel was stained with 1% Congo red solution for 10 min and then washed several times with 1 M sodium chloride. For invertase activity staining, cells were grown in 3 ml of YPD medium to an optical density at 600 nm of 6, transferred into YPsuc medium (2% yeast extract, 2% Bacto peptone, and 1% sucrose) in order to derepress invertase expression, and then incubated at 25°C for 3 h. The cell pellets were homogenized with acid-washed glass beads (425 to 600 μm in diameter) in PAGE sample buffer (125 mM Tris-HCl [pH 6.8], 1% β-mercaptoethanol, 15% glycerol, 3% SDS, 0.1% bromophenol blue, 2 mM phenylmethylsulfonyl fluoride) and were then subjected to 5% PAGE. The gel was incubated in a solution containing 0.1 M sodium acetate (pH 5.1) and 0.1 M sucrose for 30 min at 37°C, washed with H_2O, and then boiled in a 0.5 N NaOH solution containing 2,3,5-triphenyltetrazolium chloride for the visualization of proteins with invertase activity.

Analysis of N-linked oligosaccharides. N-linked oligosaccharides were released from 200 μg of the purified EGI and lipase by PNGase F (New England Biolabs). The oligosaccharides were labeled at their reducing ends with 2-aminopyridine (PA) by using the PALSTATION pyridylamination reagent kit (Takara Shuzo Co., Japan). After pyridylamination, the samples were purified by Sephadex G-15 spin columns (Amersham Pharmacia Biotech AB, Sweden) to remove residual PA. The digestion of PA-labeled oligosaccharides with α-1,2-mannosidase from *Aspergillus saitoi* (Glyko, Japan) or α-1,6-mannosidase from *Xanthomonas manihotis* (New England Biolabs) was carried out according to the instructions of the manufacturer.

Size fractionation high-performance liquid chromatography (HPLC) was per-

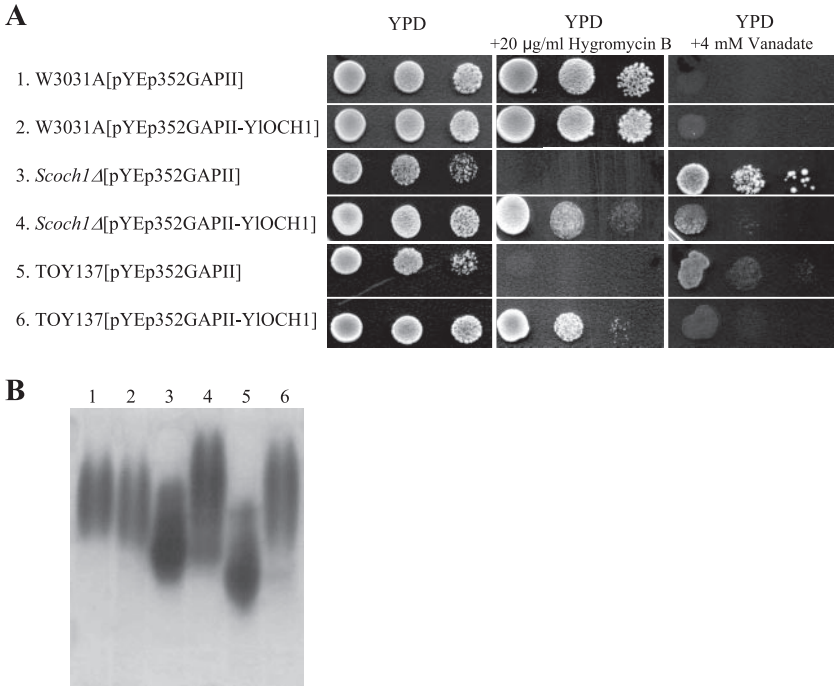


FIG. 1. Functional complementation of *S. cerevisiae och1* mutation by the *Y. lipolytica OCH1* gene. (A) Spotting analysis of growth phenotype. The *S. cerevisiae* wild-type W303-1A, *och1*Δ, and TOY137 (*och1*Δ *mnn1*Δ *mnn4*Δ) strains were transformed with a Yl*OCH1* expression vector, pYEp352GAPII-YlOCH1, and a control vector, pYEp352GAPII. Serial (1/10) dilutions of cells were spotted onto plates containing YPD, YPD with 20 μg of hygromycin B/ml, or YPD with 4 mM vanadate. The plates were incubated for 3 days at 25°C. (B) Activity staining of invertase. Cells grown to an optical density at 600 nm of 6 in 3 ml of YPD medium were transferred onto YPsuc medium (2% yeast extract, 2% Bacto peptone, and 1% sucrose) to derepress invertase expression and were then incubated at 25°C for 3 h. Lanes 1, 3, and 5 contain cell lysates of W303-1A, Sc*och1*Δ, and TOY137 strains transformed with a control vector, respectively. Lanes 2, 4, and 6 contain cell lysates of W303-1A, Sc*och1*Δ, and TOY137 strains transformed with pYEp352GAPII-YlOCH1, respectively.

formed with a Shodex Asahipak NH2P-50 column (0.46 by 25 cm; Showa Denko K. K., Japan) at a rate of 1.0 ml/min. The column was equilibrated with a solution comprising 80% solvent A (200 mM acetic acid-triethylamine [pH 7.3]–acetonitrile, 1:9) and 20% solvent B (200 mM acetic acid-triethylamine [pH 7.3]–acetonitrile, 9:1). After sample injection, the proportion of solvent B was increased linearly up to 95% for 52 min. PA oligosaccharides were detected by fluorescence (excitation λ, 320 nm, and emission λ, 400 nm) with a Waters 2475 fluorescence detector.

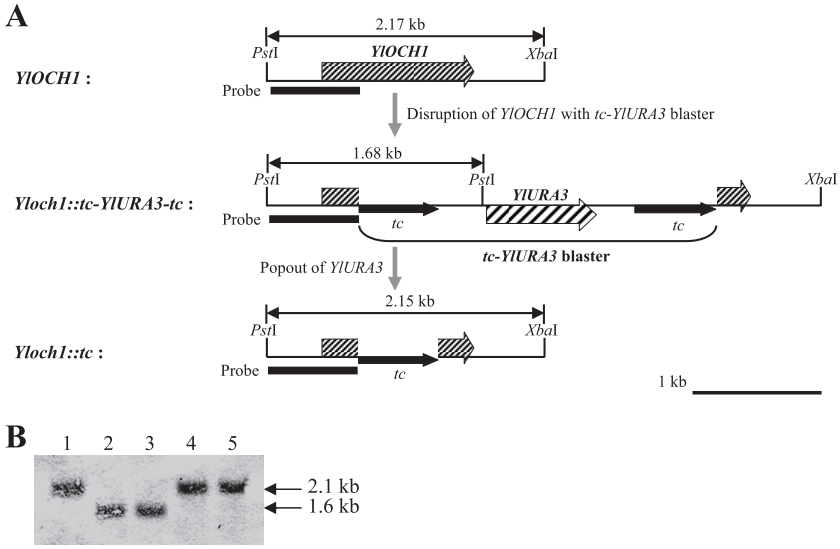


FIG. 2. Disruption of Yl*OCH1*. (A) Schematic representation of various genomic fragments containing Yl*OCH1* or mutated alleles. The 1.2-kb fragment of Yl*OCH1* was replaced with the *tc*-Yl*URA3-tc* cassettes flanked by regions homologous to Yl*OCH1* by in vivo DNA recombination. (B) Southern blot analysis of the Yl*OCH1* disruption. Lane 1, SMS397A (wild type); lanes 2 and 3, Yl*och1*Δ (*URA3*) strain (Yl*och1*::*tc*-Yl*URA3-tc*); lanes 4 and 5, Yl*och1*Δ strain (*ura3*) (Yl*och1*::*tc*). Each genomic DNA strand was digested with PstI and XbaI and hybridized with the digoxigenin-labeled 690-bp DNA fragment.

In vitro α-1,6-mannosyltransferase activity assay. Membrane fractions were obtained as described previously (14). Cells grown in YPD medium were harvested, washed with 1% KCl, and resuspended in 5 ml of PMS buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MnCl_2$, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, and 2 μg of each protease inhibitor [antipain, chymostatin, leupeptin, and pepstatin A]/ml). Glass beads (425 to 600 μm in diameter) were added to half of the cell suspension volume, and the mixture was homogenized four times for 1 min at 4°C. Homogenates were centrifuged at 10,000 × *g* for 20 min, and the supernatant obtained was further centrifuged at 100,000 × *g* for 1 h. High-speed pellets were collected and resuspended in PMS buffer, and protein concentrations were determined using a protein assay agent (Bio-Rad). The α-1,6-mannosyltransferase activity assay was performed as described by Nakajima and Ballou (16). One hundred micrograms of high-speed pellet proteins was incubated in 100 μl of 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM $MnCl_2$, 1 mM GDP-mannose, 0.5 mM 1-deoxymannojirimycin, and 100 pmol of the $Man_8GlcNAc_2$-PA acceptor at 30°C for 2 h. The reaction was terminated by boiling at 99°C for 5 min, and the reaction mixture was filtered through an Ultrafree-MC membrane (10,000-Da cutoff; Millipore) and analyzed by HPLC.

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis. N-linked oligosaccharides released from 100 μg of the purified recombinant EGI were isolated by using a porous graphitized carbon column (Alltech). To elute neutral and acidic glycans, 900 μl of a solution consisting of 25% (vol/vol) acetonitrile (Applied Biosystems) and 0.05% (vol/vol) trifluoroacetic acid (Aldrich) was added onto the column. The effluent was collected, lyophilized on a freeze-dry system (Ilshin, Korea), and dissolved in 10 μl of HPLC-grade water. The glycan sample (0.7 μl) was mixed with 0.7 μl of a mixture of 6-aza-2-thiothymine and 2,5-dihydroxybenzoic acid (vol/vol, 1:1) as a matrix, and the mixture was loaded onto a ground steel MSP 96 target (microScout target; Bruker Daltonics, Germany). The mass spectrum was analyzed with a microflex mass spectrometer (Bruker Daltonics, Germany) in the positive reflector mode (for the detection of neutral sugars) or in the negative linear mode (for the detection of acidic sugars).

RESULTS

Yl*OCH1* is a functional homolog of Sc*OCH1* encoding an initiating α-1,6-mannosyltransferase. Since the role of Yl*OCH1* in outer-chain biosynthesis has not yet been completely defined despite the previous study by Barnay-Verdier et al. (3), we wanted to investigate whether Yl*OCH1* is a functional homolog of Sc*OCH1.* We first examined the effect of Yl*OCH1* expression on the growth phenotypes of *S. cerevisiae och1* mutant strains, such as sensitivity to hygromycin B and resistance to orthovanadate, which are general characteristics observed in yeast mutant strains defective in N glycosylation (2, 7, 18). A complementation vector, pYEp352GAPII-YlOCH1, which expresses Yl*OCH1* under the control of the *S. cerevisiae* GAPDH gene promoter, was constructed and introduced into the *S. cerevisiae* wild-type and *och1*Δ mutant strains. In contrast to the strains transformed with a control plasmid, pYEp352GAPII, the *S. cerevisiae och1* mutant strains transformed with pYEp352GAPII-YlOCH1 recovered the phenotypes of the wild-type strain, including the normal growth rate, resistance to hygromycin B, and sensitivity to sodium orthovanadate (Fig. 1A). Next, we performed an invertase gel electrophoretic mobility assay in order to determine whether Yl*OCH1* can eliminate the glycosylation defect in the Sc*och1*Δ mutant strains. The invertases secreted from the Sc*och1*Δ mutant cells migrated faster than those secreted from the wild-type cells (Fig. 1B), which indicates the lack of heavy glycosylation in the invertases from the Sc*och1*Δ mutants. It is evident, however, that the introduction of the Yl*OCH1* gene restored the ability of the Sc*och1*Δ mutant strains to secrete fully glycosylated invertase. Along with the recovery of the normal growth phenotype, the elimination of the glycosylation defect in the *S. cerevisiae och1* mutant strains by the expression of Yl*OCH1* demonstrates that Yl*OCH1* is a functional homolog of *S. cerevisiae OCH1*.

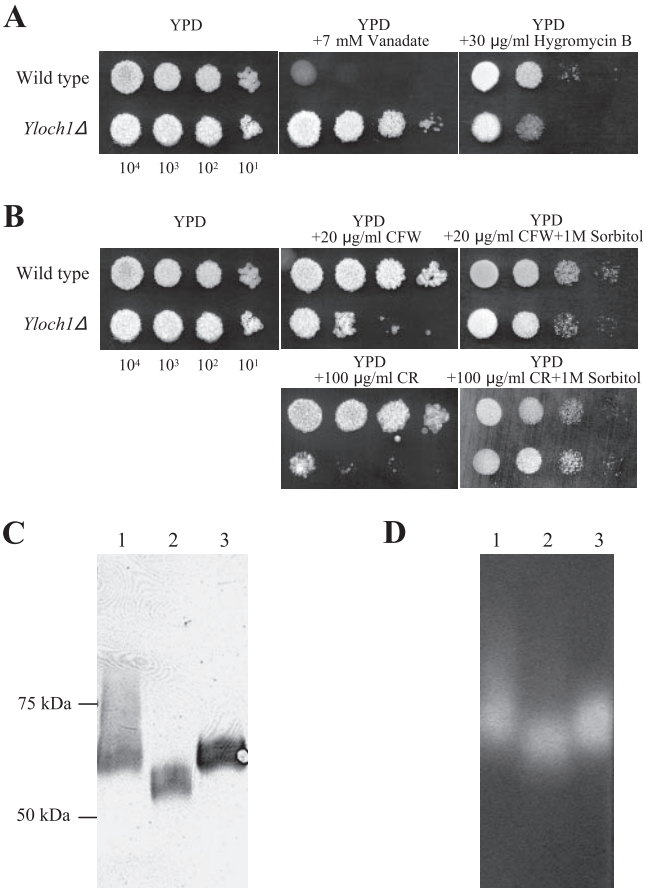


FIG. 3. Phenotypic analysis of the Yl*och1* mutant strain. (A) Vanadate resistance and hygromycin B sensitivity. (B) Calcofluor white and Congo red sensitivity. The wild-type and Yl*och1* mutant cells were grown in YPD, and 10-μl serial (1/10) dilutions of each strain were spotted onto YPD plates containing 7 mM vanadate, 30 μg of hygromycin B/ml, 20 μg of calcofluor white (CFW)/ml, or 100 μg of Congo red (CR)/ml. The plates were incubated for 2 days at 28°C. (C) Western blot analysis of *T. reesei* EGI secreted from the wild-type and Yl*och1*Δ cells. The secreted EGI from the wild-type and Yl*och1*Δ cells was purified, separated by 10% SDS-PAGE, and detected with anti-His antibody. Lane 1, EGI secreted from the wild type; lane 2, EGI treated with endo H; lane 3, EGI secreted from Yl*och1*Δ cells. (D) The supernatants (16 μl) from cell cultures grown in 3 ml of YPD medium for 30 h were subjected to 10% PAGE without SDS. The substrate gel containing 1% carboxyl methyl cellulose was overlaid with the PAGE gel. Both gels were wrapped and incubated at 30°C for 3 h. The substrate gel was stained with 1% Congo red solution for 10 min. Lane 1, EGI secreted from the wild type; lane 2, EGI treated with endo H; lane 3, EGI secreted from Yl*och1*Δ cells.

Effect of Yl*OCH1* deletion on cell growth and N glycosylation. To study the in vivo function of the Yl*OCH1* gene in *Y. lipolytica*, the Yl*och1* null mutant (Yl*och1*Δ) strain was constructed and confirmed by Southern analysis (Fig. 2). Interestingly, unlike the *S. cerevisiae och1* mutant, the Yl*och1*Δ mutant grew as well as the wild-type strain both under normal growth conditions and at a high temperature, 34°C (data not shown). To test whether the Yl*och1*Δ mutant has the phenotypes of defective glycosylation, we grew the Yl*och1*Δ mutant on me-

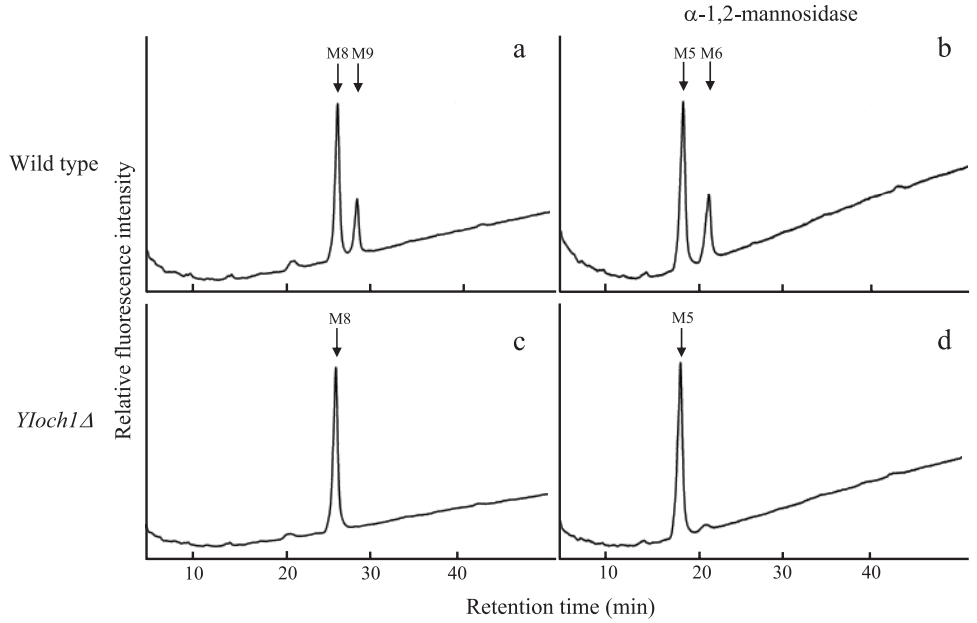


FIG. 4. In vitro activity analysis of *Y. lipolytica OCH1* gene product. Solubilized membrane fractions from the *Y. lipolytica* wild type (a and b) and Yl*och1*Δ (c and d) strains were prepared as enzyme sources, and $Man_8GlcNAc_2$-PA was used as an acceptor. The reaction products were analyzed by HPLC. The reaction products treated with α-1,2-mannosidase were also analyzed by HPLC. M5, $Man_5GlcNAc_2$-PA; M6, $Man_6GlcNAc_2$-PA; M8, $Man_8GlcNAc_2$-PA; M9, $Man_9GlcNAc_2$-PA.

dium containing vanadate or hygromycin B. The Yl*och1*Δ mutant was resistant to vanadate and sensitive to hygromycin B compared to the wild-type strain (Fig. 3A), indicating that the Yl*och1*Δ mutant was defective in terms of glycosylation. The Yl*och1*Δ mutant also showed poor growth on medium containing calcofluor white or Congo red. However, the addition of an osmotic stabilizer, 1 M sorbitol, to the medium restored growth to the level of wild-type growth (Fig. 3B), although supplementation with 0.5 M sorbitol was shown to be insufficient to support the growth of the Yl*och1*Δ mutant (3). This result suggested that the Yl*och1*Δ mutant has alterations in its cell wall composition, probably in mannoproteins (7, 22). The phenotypes of the glycosylation defect and the alteration of cell wall composition and structure in the Yl*och1*Δ mutant imply that the function of Yl*OCH1* may be important for the N-glycosylation process in *Y. lipolytica*.

To further examine protein N glycosylation in the Yl*och1*Δ mutant, *T. reesei* EGI, which has nine potential N-linked glycosylation sites (9), was expressed in *Y. lipolytica*. The Yl*och1*Δ mutant produced relatively homogeneously sized EGI proteins compared to the wild-type strain, which secreted rather heterogenous forms of EGI, as judged by the smeared pattern on Western blots; this smearing was eliminated upon treatment with endo H, which removes oligomannosidic N-glycans (Fig. 3C and D). This result led us to believe that the Yl*och1*Δ mutant certainly lacked hyperglycosylation activity and that YlOch1p might be involved in the outer-chain elongation of N-linked oligosaccharides in *Y. lipolytica*.

Membrane fraction of Yl*och1*Δ mutant lacks an initiating α-1,6-mannosyltransferase activity. To determine whether the Yl*och1*Δ mutant lacks an initiating α-1,6-mannosyltransferase that uses the core oligosaccharide as a substrate, solubilized membrane fractions were prepared from the wild-type and Yl*och1*Δ mutant strains and used as enzyme sources for an in vitro α-1,6-mannosyltransferase assay. Whereas, as expected, the peak corresponding to $Man_9GlcNAc_2$-PA was detected as a reaction product in the membrane fraction of the wild-type strain, the acceptor, $Man_8GlcNAc_2$-PA, was not converted into $Man_9GlcNAc_2$-PA by the membrane fraction of the Yl*och1*Δ mutant (Fig. 4). Furthermore, the $Man_9GlcNAc_2$-PA product was converted into $Man_6GlcNAc_2$-PA and the $Man_8GlcNAc_2$-PA core oligosaccharide was converted into $Man_5GlcNAc_2$-PA when the reaction products were treated with α-1,2-mannosidase from *A. saitoi*, which specifically removes α-1,2-linked mannose residues at the nonreducing ends of manno-oligosaccharides (Fig. 4). These results strongly indicate that Yl*OCH1* encodes an initiation-specific α-1,6-mannosyltransferase acting on the core oligosaccharide.

Structural analysis of N-linked oligosaccharides released from EGI and lipase in the Yl*och1*Δ mutant. To obtain detailed information on the in vivo function of YlOch1p in N glycosylation, we analyzed the structures of N-glycans assembled on EGI secreted from the *Y. lipolytica* wild-type and Yl*och1*Δ strains. In the case of the wild-type strain, two major peaks corresponding to $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ in the chromatogram of the oligosaccharides released from EGI were observed; some other minor peaks also appeared (Fig. 5A). In the case of the Yl*och1*Δ strain, however, we found a single major peak for $Man_8GlcNAc_2$, indicating that the Yl*och1*Δ mutant was defective in its ability to synthesize oligosaccharides larger than $Man_8GlcNAc_2$ (Fig. 5A, panels a and c). To further investigate the structures of N-linked oligosaccharides synthesized from the wild-type and Yl*och1*Δ mutant strains, we analyzed the oligosaccharides by treating them with α-1,2- and α-1,6-mannosidases. The $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ oligosaccharides generated from the wild-type

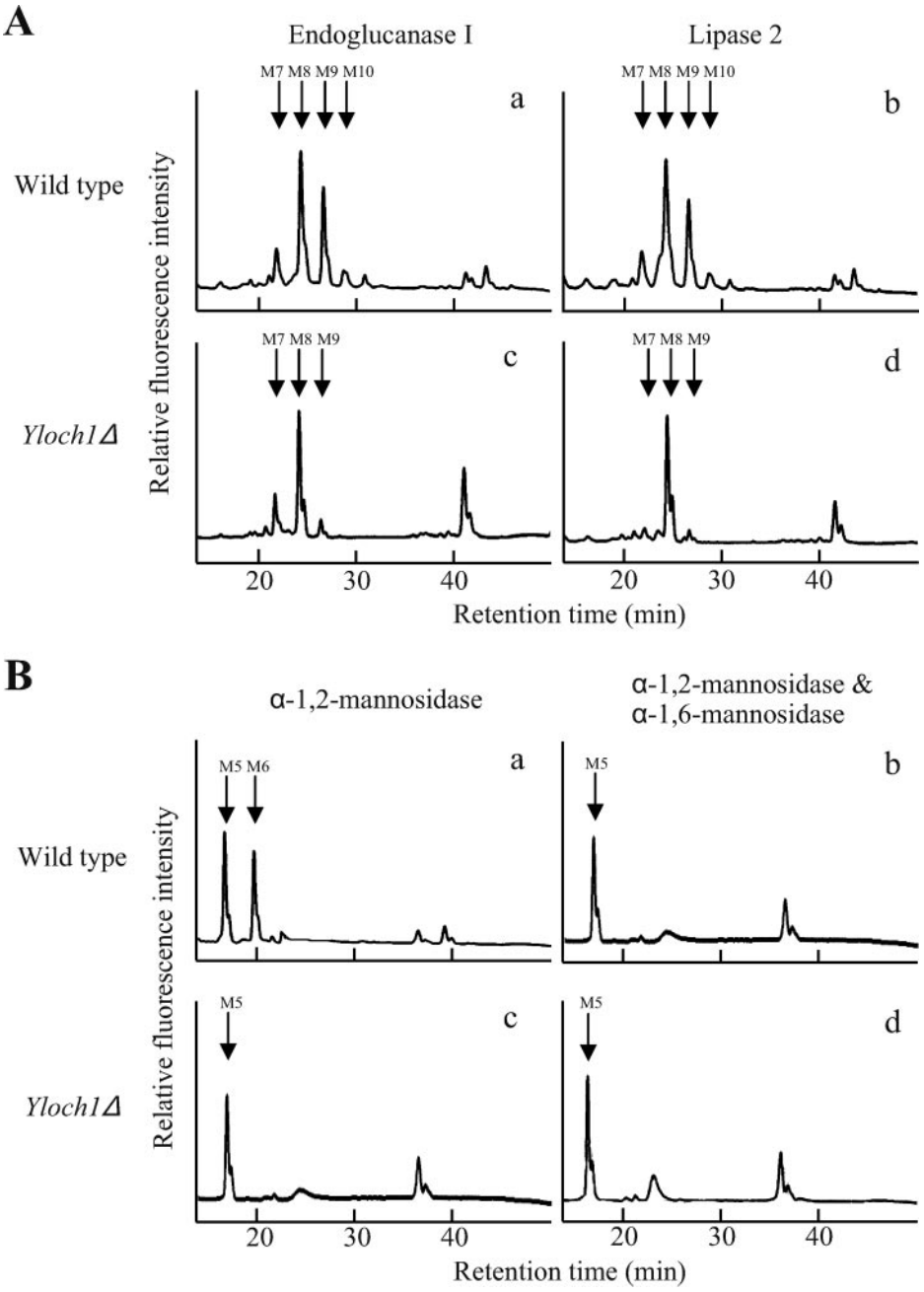
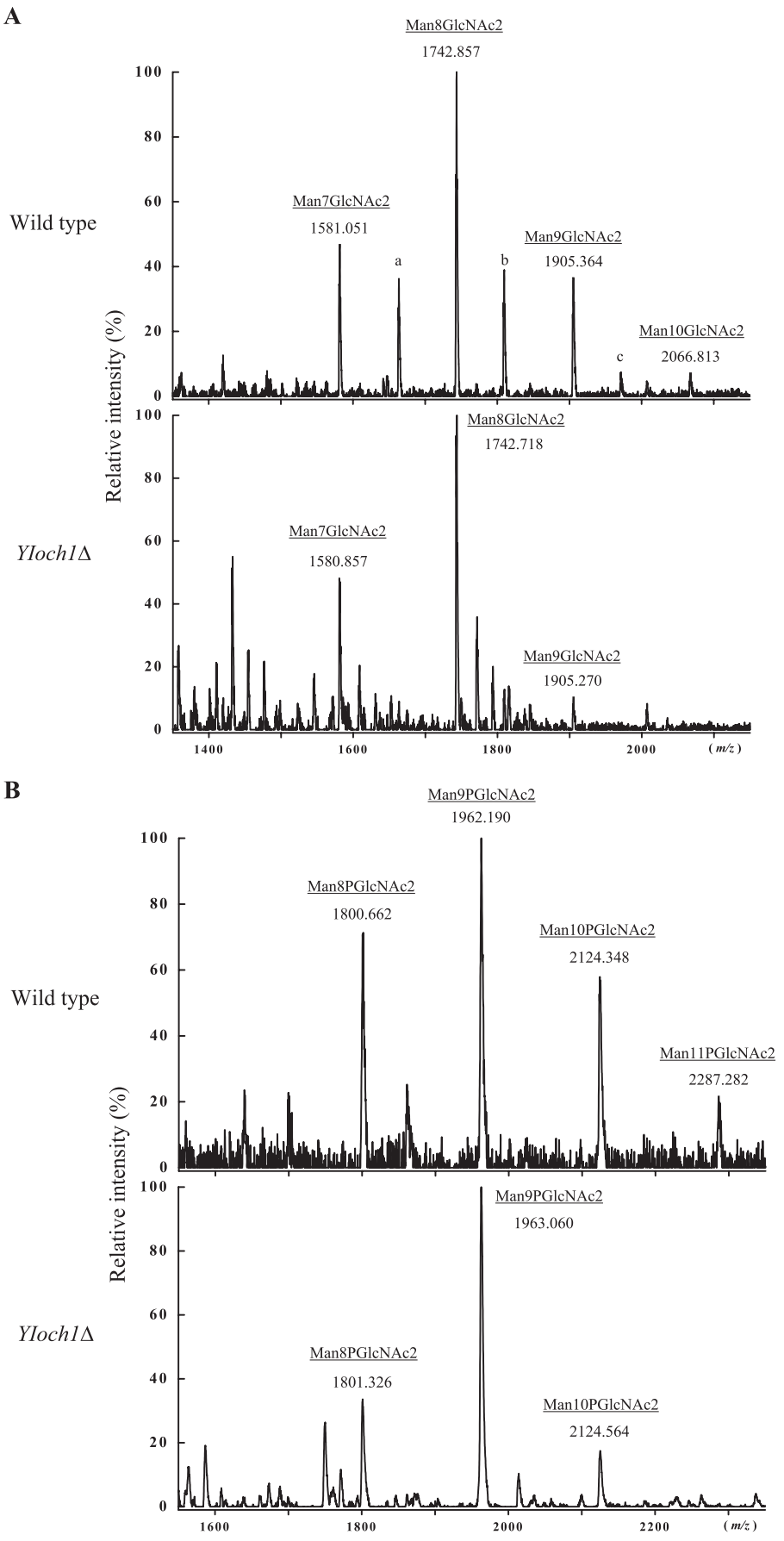


FIG. 5. HPLC analysis of N-linked oligosaccharides assembled on glycoproteins. (A) Chromatograms of the N-linked oligosaccharides released from EGI (a and c) and lipase (b and d) from the wild-type and Yl*och1*Δ strains. (B) Chromatograms of the N-linked oligosaccharides released from EGI secreted by the wild-type (a and b) and Yl*och1*Δ strains (c and d) after treatment with α-1,2-mannosidase and α-1,6-mannosidase. The elution times of authentic PA-sugar chains are indicated by arrows. M5, $Man_5GlcNAc_2$-PA; M6, $Man_6GlcNAc_2$-PA; M7, $Man_7GlcNAc_2$-PA; M8, $Man_8GlcNAc_2$-PA; M9, $Man_9GlcNAc_2$-PA; M10, $Man_{10}GlcNAc_2$-PA.

and Yl*och1*Δ strains were treated with α-1,2-mannosidase from *A. saitoi*. $Man_8GlcNAc_2$ was converted completely into $Man_5GlcNAc_2$, and $Man_9GlcNAc_2$ was converted completely into $Man_6GlcNAc_2$ (Fig. 5B, panels a and c). The $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$ oligosaccharides were subsequently digested with α-1,6-mannosidase from *X. manihotis*, a highly specific exoglycosidase that removes unbranched α-1,6-linked D-mannopyranosyl residues from oligosaccharides. The $Man_6GlcNAc_2$ oligosaccharides were converted into $Man_5GlcNAc_2$ (Fig. 5B, panels b and d), which indicates that the $Man_6GlcNAc_2$ species has a single unbranched α-1,6-linked mannose. These results demonstrate that the Yl*och1*Δ mutant strain has a defect in terms of the addition of the first α-1,6-mannose residue onto the core oligosaccharide, $Man_8GlcNAc_2$.

In order to demonstrate that the function of Yl*OCH1* is generally required for the addition of the first α-1,6-mannose residue onto the core oligosaccharide, $Man_8GlcNAc_2$, in *Y.*



lipolytica, we analyzed the N-linked oligosaccharide structure of an endogenous glycoprotein, lipase (YlLip2p), which is a secreted protein with two potential N-linked glycosylation sites (20). The HPLC profiles of the oligosaccharides assembled on the lipases secreted from the wild-type and Yl*och1*Δ strains were the same as those of the oligosaccharides assembled on EGI (Fig. 5A, panels b and d). Therefore, it is clear that Yl*OCH1* encodes an enzyme that initiates the outer-chain elongation by adding the first α-1,6-linked mannose to the core oligosaccharide in *Y. lipolytica*.

There were two minor peaks appearing much later than the major peaks in the HPLC profiles of the oligosaccharides from the wild-type strain (Fig. 5). Judging from a comparison of the HPLC profiles with the data from the study by Wang et al. (25), in which the mannosylphosphate transferase activity of *S. cerevisiae* Mnn6p was assayed, we suspected that the two minor peaks might represent phosphate-containing oligosaccharides. To determine whether N-linked oligosaccharides in *Y. lipolytica* are phosphorylated, we analyzed N-glycans derived from the recombinant EGI by using MALDI-TOF mass spectrometry. The glycans were analyzed in the positive reflector mode for the detection of neutral sugars (Fig. 6A) and in the negative linear mode for the detection of acidic sugars (Fig. 6B). The major peaks detected in the negative mode corresponded to molecular masses higher than those of the neutral sugars, which nearly equaled the molecular masses of mannosylphosphorylated $Man_7GlcNAc_2$ to $Man_9GlcNAc_2$ ($Man_8PGlcNAc_2$ to $Man_{10}PGlcNAc_2$) species. These results indicate that N-linked oligosaccharides assembled on glycoproteins in *Y. lipolytica* are modified by mannosylphosphate. In addition, the observation that the overall glycan mass spectra are consistent with the HPLC data (Fig. 5A and 6A) confirms that the Yl*och1*Δ mutant is defective in synthesizing oligosaccharides larger than $Man_8PGlcNAc_2$.

DISCUSSION

The Och1 protein has been proven to be an initiating α-1,6-mannosyltransferase that plays a key role in the addition of the first mannose to the core oligosaccharide in several yeast species, including *S. cerevisiae*, *P. pastoris*, *Schizosaccharomyces pombe*, and *H. polymorpha* (14, 15, 17, 19, 28). However, *Y. lipolytica* Och1p was suspected to have a minor role in the outer-chain elongation of N glycosylation or to be important only for specific proteins (3). In this study, we reexamined the *Y. lipolytica OCH1* gene and analyzed the structure of the N-linked oligosaccharide from the Yl*och1*Δ mutant based on our observation that the recombinant glycoprotein EGI secreted from the Yl*och1*Δ mutant appeared to be less heavily glycosylated than the recombinant EGI from the wild-type strain. Here, we present several lines of strong evidence indicating that Yl*OCH1* encodes an α-1,6-mannosyltransferase that plays an important role in the addition of the first mannose to the core oligosaccharide in *Y. lipolytica*, as in other yeasts. First, the ability of the Yl*OCH1* gene to eliminate the defects of the *S. cerevisiae och1* mutant, i.e., retarded growth rate and a reduction in the sizes of N-linked oligosaccharides, demonstrated that Yl*OCH1* is a functional homolog of Sc*OCH1*. Second, the membrane fraction of the Yl*och1*Δ mutant lacked an initiation-specific α-1,6-mannosyltransferase activity. Third, the N-linked oligosaccharides attached to the recombinant EGI and the endogenous lipase secreted from the Yl*och1*Δ mutant cells were composed of a single predominating species, $Man_8GlcNAc_2$, and some minor ones, which is consistent with the rapid mobility of the glycoproteins from the Yl*och1*Δ mutant cells. Last, the structural analysis of N-glycans revealed that the N-linked oligosaccharides from Yl*och1*Δ did not contain additional α-1,6-linked mannoses. Taken together, these data provide clear evidence that the major function of Yl Och1p is to initiate α-1,6-linked mannose elongation of the core oligosaccharide, $Man_8GlcNAc_2$. However, we cannot exclude the possible presence of another α-1,6-mannosyltransferase that may play a minor role in the outer-chain elongation of certain glycoproteins in *Y. lipolytica*.

In *S. cerevisiae*, N-linked oligosaccharides are capped with immunogenic α-1,3-linked mannose residues (16, 27), and α-1,2-linked mannoses of N-linked oligosaccharides are not digested with α-1,2-mannosidase if they are attached to α-1,3-linked mannose. However, in *Y. lipolytica*, $Man_8GlcNAc_2$ was converted completely into $Man_5GlcNAc_2$ upon treatment with α-1,2-mannosidase. This result, along with the finding that no open reading frame that is homologous to *S. cerevisiae MNN1* appears in the *Y. lipolytica* genome database (http://cbi.labri.fr/Genolevures/), strongly suggests that the core oligosaccharides from *Y. lipolytica* may not be terminally capped by α-1,3-linked mannose residues.

Analysis using MALDI-TOF mass spectrometry suggests that N-linked glycans assembled on secreted glycoproteins from *Y. lipolytica* contain phosphates and that the two minor peaks appearing in the HPLC profiles of the oligosaccharides from the wild-type strain (Fig. 5) may represent mannosylphosphorylated forms of $Man_7GlcNAc_2$ to $Man_9GlcNAc_2$ (Fig. 6). The absence of the latter of the two minor peaks in the chromatograms corresponding to the oligosaccharides from the Yl*och1*Δ mutant strain and the α-1,6-mannosidase-treated oligosaccharides from the wild-type strain (Fig. 5B) indicates that the difference between the two peaks comes from an additional α-1,6-linked mannose at the latter peak. Therefore, we think that mannosylphosphates are transferred to the core forms of N-glycans in *Y. lipolytica*. Further studies are presently in progress to identify genes involved in the mannosylphosphorylation of *N*-linked oligosaccharides in *Y. lipolytica*.

Recently, many studies have reported the engineering of

FIG. 6. MALDI-TOF mass spectrometry analysis of N-linked oligosaccharides assembled on EGI. (A) Mass spectra analyzed in the positive reflector mode for the detection of neutral sugars released from the recombinant EGI secreted from the wild-type (top panels) and Yl*och1*Δ (bottom panels) strains. The intermediate peaks for the wild type, designated a, b, and c, are assumed to represent the mannosylphosphorylated forms of $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$, based on their *m*/*z* of 1,662.792, 1,808.617, and 1,970.866, respectively. (B) Mass spectra analyzed in the negative reflector mode for the detection of acidic sugars released from the recombinant EGI secreted from the wild-type (top panel) and Yl*och1*Δ (bottom panel) strains. The analyzed glycan samples are free, nonreduced forms without any labeling.

novel yeast strains secreting therapeutic glycoproteins with human-compatible types of N-linked oligosaccharides (10, 14, 24, 26). *Y. lipolytica* is considered to be one of the most suitable strains for heterologous protein expression (12) and can be developed for the production of glycoproteins. In this study, we found that the Yl*och1*Δ mutant strain, despite the complete block of the outer-chain elongation of N-linked oligosaccharides, could grow as well as the wild-type strain under normal growth conditions. This growth property of the Yl*och1*Δ mutant strain will be very useful for the further development of *Y. lipolytica* as a host for the production of glycoproteins. Along with the possibility of a lack of the immunogenic terminal α-1,3-mannose linkages in *Y. lipolytica*, the Yl*och1*Δ mutant strain can be exploited as a platform strain for developing another potential yeast system that can produce recombinant glycoproteins with human-compatible oligosaccharides.

ACKNOWLEDGMENTS

We thank Y. Jigami for providing *S. cerevisiae och1* mutant strains and Jeong Mi Lee for MALDI-TOF analysis.

This work was supported by grants from the 21C Frontier Microbial Genomics and Application Center Program (MG05-0309-3-0) to J.-Y. Kim and from the MOCIE of the Republic of Korea (Next Generation New Technology Development Program) to H. A. Kang. J.-N. Park is presently a postdoctoral fellow of BK21.

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