Functional Characterization of the *Hansenula polymorpha* HOC1, OCH1, and OCR1 Genes as Members of the Yeast OCH1 Mannosyltransferase Family Involved in Protein Glycosylation*

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The α-1,6-mannosyltransferase encoded by *Saccharomyces cerevisiae* OCH1 (ScOCH1) is responsible for the outer chain initiation of N-linked oligosaccharides. To identify the genes involved in the first step of outer chain biosynthesis in the methylotrophic yeast *Hansenula polymorpha*, we undertook the functional analysis of three *H. polymorpha* genes, HpHOC1, HpOCH1, and HpOCR1, that belong to the OCH1 family containing seven members with significant sequence identities to ScOCH1. The deletions of these *H. polymorpha* genes individually resulted in several phenotypes suggestive of cell wall defects. Whereas the deletion of *HpHOC1* (*Hphoc1Δ*) did not generate any detectable changes in N-glycosylation, the null mutant strains of *HpOCH1* (*Hpoch1Δ*) and *HpOCR1* (*Hpocr1Δ*) displayed a remarkable reduction in hypermannosylation. Although the apparent phenotypes of *Hpocr1Δ* were most similar to those of *S. cerevisiae och1* mutants, the detailed structural analysis of N-glycans revealed that the major defect of *Hpocr1Δ* is not in the initiation step but rather in the subsequent step of outer chain elongation by α-1,2-mannose addition. Most interestingly, *Hpocr1Δ* showed a severe defect in the O-linked glycosylation of extracellular chitinase, representing *HpOCR1* as a novel member of the OCH1 family implicated in both N- and O-linked glycosylation. In contrast, addition of the first α-1,6-mannose residue onto the core oligosaccharide ManαGlcNAc2 was completely blocked in *och1* mutants. As expected, the core oligosaccharide ManαGlcNAc2 was extended with 50 or more α-1,2-linked mannoses in the *och1* mutant strains with the targeted expression of *Aspergillus saitoi* α-1,2-mannosidase in the endoplasmic reticulum, shown to produce human-compatible high mannose-glycans.

Glycosylation is one of the most ubiquitous forms of post-translational modification, and the early stages of N-linked glycosylation are highly conserved among eukaryotes. The formation of N-linked oligosaccharides assembled on glycoproteins begins in the endoplasmic reticulum (ER), where an identical Glc3Man9GlcNAc2 oligosaccharide is transferred to the Asn residues on nascent proteins by oligosaccharyltransferase complex (1). Subsequent trimming by glucosidases I and II and a specific ER-residing α-1,2-mannosidase led to the formation of a core oligosaccharide (ManαGlcNAc2). Glycoproteins containing ManαGlcNAc2 are then collected into transport vesicles and delivered to the Golgi apparatus. It is in the Golgi where the diversity of N-glycans structures is generated by a series of glycidosidases and glycosyltransferases acting in a manner that varies enormously between species, and even between individual proteins within a species (2).

In the traditional yeast *Saccharomyces cerevisiae*, where the N-linked glycosylation has been most studied, the maturation of N-linked oligosaccharides in the Golgi often leads to hypermannosylated glycoproteins possessing a large structure called the outer chain. This outer chain biosynthesis is initiated by the addition of the first α-1,6-mannose onto the ManαGlcNAc2 core structure in the early Golgi, a process mediated by the OCH1 gene product in *S. cerevisiae* (3). After the action of the *S. cerevisiae OCH1* gene product (ScOch1p), the extension of an α-1,6-linked polymannose backbone occurs by the sequential action of two enzyme complexes, mannan polymerase (M-Pol) I and II (2). The linear backbone of the outer chain is often extended with 50 or more α-1,6-linked mannosides, highly branched by the addition of α-1,2-linked mannosides and terminally capped by α-1,3-linked mannosides (4). The mannosyltransferase activity and the substrate specificity of the *S. cerevisiae* Och1p are well characterized and shown to require the intact structure of ManαGlcNAc2 for efficient mannoside outer chain initiation (5, 6). The functional homologues of the *S. cerevisiae OCH1* gene in other yeast species, including *Schizosaccharomyces pombe* (7) and *Pichia pastoris* (8), have been reported and shown to be involved in the initiation of α-1,6-linked mannoside outer chain biosynthesis. The *S. cerevisiae HOC1* gene (Homologous to OCH1), isolated as a high copy suppressor of a protein kinase C mutant, encodes a putative α-1,6-mannosyltransferase that strongly resembles ScOch1p. However, no obvious defects in N-linked or O-linked glycosylation were detected in the null mutation of *HOC1*, and the overexpression of *HOC1* cannot...
H. polymorpha OCH1 Mannosyltransferase Family

suppress an och1 mutation (9). The S. cerevisiae HOC1 gene product (SchHoc1p) was found to reside in the M-Pol II complex, but whether SchHoc1p directly contributes to the α-1,6-mannosyltransferase activity is not yet proven (2). At present, the function of SchHoc1p is unclear, although the S. cerevisiae och1 mutants show phenotypes associated with glycosylation defects, including sensitivity to Calcofluor White and hygromycin B. SchHoc1p might be a protein-specific mannosyltransferase or have a function overlapping with another glycosyltransferase or function only under certain conditions.

The thermotolerant methylotrophic yeast, Hansenula polymorpha, has emerged as a promising host for the high level expression of heterologous genes, because of its well established expression toolboxes along with the feasibility of its high cell density culture in methanol-containing media (10, 11). Furthermore, the less extensive hyperglycosylation of glycoproteins from H. polymorpha than from S. cerevisiae has been suggested to be another factor that favors the production of mammalian cell-originated proteins in this yeast (12, 13). Our recent study on the structural characterization of the oligosaccharides assembled on glycoproteins secreted by H. polymorpha showed that most N-linked glycans synthesized in H. polymorpha have core-type structures (Man₅GlcNAc₂) and that they are mainly branched by α-1,2-mannose addition, whereas the functions of SchHoc1p are closely associated with the subsequent elongation step by α-1,2-mannose addition. Furthermore, we evaluated the potential of HpOCH1 deletion as a first step toward the N-glycan engineering of H. polymorpha to produce human-compatible N-linked oligosaccharides.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Media—H. polymorpha DL1-L (leu2) and DL-LdU (leu2 ura3 Δ: lacZ) strains were derivatives of DL-1 (ATCC26012) (15). S. cerevisiae och1Δ (MATa leu2 ura3 trp1 ade2 his3 och1Δ:TRP1) and S. cerevisiae TOY137 (MATa leu2 ura3 trp1 ade2 his3 och1Δ:hisG mnn1Δ:hisG mnn4Δ:hisG) were derived from S. cerevisiae W303-1A.3 The vector pDLMOX-GOD(H) (14) was used for the secretory expression of Aspergillus niger glucose oxidase tagged with 6 residues of histidine (GOD-His) in H. polymorpha. pMOX-YPs1p-His vector was constructed for the secretion of C-terminally truncated H. polymorpha Yps1p tagged with histidine residues.4 The plasmid YEp352GAPII, containing the S. cerevisiae GAPDH promoter and terminator,5 was used as a backbone vector for H. polymorpha gene expression in S. cerevisiae. To construct the HA-HDEL-tagged Aspergillus sativus α-1,2-mannosidase, the HA-HDEL tag sequence was introduced by PCR using the sense oligonucleotide primer, Msd-N (containing an EcoRI restriction site), and the antisense oligonucleotide primer, Msd-C (containing the coding sequences of the HA tag and the HDEL signal, followed by a stop codon and an Ncol restriction site), using pGAMH1 as a template (16). The resulting PCR product was digested with EcoRI and Ncol and used to substitute the 1.8-kb EcoRI/Ncol GOD-His fragment in pDLMOX-GOD(H) to generate the plasmid pDLMOX-MsdS(HA-HDEL). The auxotrophic marker HpLEU2 in pDLMOX-MsdS(HA-HDEL) was exchanged with HpURA3 to generate pDUMOX-MsdS(HA-HDEL). Drug sensitivity was assayed by spotting serially diluted yeast culture onto YPD (1% yeast extract, 2% peptone, and 2% glucose) solid media containing 40 μg/ml hygromycin B, 7 mg/ml Calcofluor White, or 0.4% sodium deoxycholate. Secretory expression and immunoblot analysis of A. niger GOD-His in H. polymorpha were performed as described previously (14). For deglycosylation, glycoproteins were digested with peptideN-glycosidase F (PNGase F) according to the supplier’s instructions (New England Biolab, Beverly, MA).

Cloning of the H. polymorpha OCH1 Family Genes—Two pairs of primers, R1for/R1rev and R2for/R2rev, were designed using information on two random sequenced tags of H. polymorpha (GenBank™ accession numbers AL435940 and AL433499), which shares significant homology with S. cerevisiae HOC1 and OCH1 genes. Two PCR products of 1.0 kb were amplified from H. polymorpha DL1-L chromosomal DNA using the primers R1for/R1rev and R2for/R2rev and used as probes for Southern blot analysis. Strong hybridization signals at the 7.5-kb HindIII and 5.0-kb BglII fragments of H. polymorpha DL1-L genomic DNA were detected using these probes, respectively. HindIII fragments of 6.7–7.7-kb and BglII fragments of 4.5–6.0-kb of H. polymorpha DL1-L genomic DNA were gel-eluted and ligated into the HindIII and BamHI sites of pBluescript (Stratagene, La Jolla, CA), respectively. To screen H. polymorpha HindIII and BglII genomic DNA libraries, colony PCR was carried out using the same primers used for synthesizing the two probes mentioned above. Plasmids from each positive clone, designated pH305 and pB52, were partially sequenced. Plasmid pH305 contained a 7.5-kb HindIII insert with a full-length open reading frame (ORF) highly similar to that of the S. cerevisiae HOC1 gene product, and thus the ORF was designated as H. polymorpha HOC1 (HpHOC1). However, plasmid pB52 containing a 5.0-kb BglII insert was found to contain only the 3’ portion of a putative ORF, which showed relatively low sequence similarity with the S. cerevisiae HOC1 and OCH1 gene products. To clone the full length of the ORF, the 3’ region of ORF obtained from pB52 was amplified using the primers R3for and R3rev from H. polymorpha DL1-L genomic DNA, and this was then used as a probe in Southern blot analysis. A strong hybridization signal was detected at an ~2.3-kb BamHI fragment, and the BamHI fragments of 1.8–3.0 kb of H. polymorpha DL1-L genomic DNA were gel-eluted and subcloned into the BamHI site of pBluescript. After screening the partial H. polymorpha genomic DNA library by colony PCR, positive clones were isolated and sequenced. Plasmid pBA302 from a positive clone was found to contain a 2.3-kb BamHI insert carrying the 5’-upstream region and a portion of the ORF, which partially overlapped with the ORF from pB52. The full length of an intact ORF, showing relatively low sequence identity (22%) with S. cerevisiae OCH1, was generated by combining two ORFs from pBA302 and pB52 and was designated as H. polymorpha OCH1-related gene 1 (HpOCR1). The nucleotide sequences of HpHOC1 and HpOCR1 have been submitted to GenBank™ under accession numbers AF540063 and AF490971, respectively. H. polymorpha OCH1 (HpOCR1) was isolated by PCR from H. polymorpha DL1-L genomic DNA using two primers, 168Not-N and 168Not-C, that were designed based on information obtained from the whole genome sequence of H. polymorpha (17). The nucleotide sequence of the resulting PCR product was determined and submitted to GenBank™ under accession number AF540063.
H. polymorpha OCH1 Mannosyltransferase Family

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Preparation of Membrane Proteins and α-l,6-Mannosyltransferase Activity Assay—Yeast membrane fractions were obtained as described previously (5). Yeast cells were cultured in YPD medium and harvested at mid-logarithmic phase (A600 = 3–4). Cells were collected by centrifugation at 3,000 × g for 5 min, washed with 1% KCl, and resuspended in 5 ml of PMS buffer (50 mM Tris-HCl (pH 7.5), 10 mM MnCl2, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, and 2 μg/ml each protease inhibitor (antipain, chymostatin, leupeptin, and pepstatin A)). Glass beads (425–600 μm) were added to half of the cell suspension volume and 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 Bio-Rad protein assay agent (Bio-Rad). α-1,6-Mannosyltransferase activity was assayed as described by Nakajima and Ballou (20). 100 μg of high speed pellet proteins was incubated in 100 μl of 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM MnCl2, 1 mM GDP-mannose, 0.5 mM 1-deoxymannojirimycin, and 100 pmol of ManαGlcNAc2-Pα 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 cutoff; Millipore, Bedford, MA), and the filtrate was submitted for HPLC.

O-Glycosylation Analysis of Chitinase—Native chitinases from supernatants of cultured cells were isolated, and their degrees of O-glycosylation were analyzed by SDS-PAGE as described previously (21). Thirty ml of culture supernatants of H. polymorpha cells grown in YPD for 24 h were collected and transferred into centrifuge tubes. After adding 40 mg of chitin, the tubes were rotated end-over-end at 4 °C for 4 h. The chitin was then pelleted by centrifugation and washed three times with cold phosphate-buffered saline buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4 (pH 7.4)). Chitinase was eluted from the chitin in 100 μl of SDS-PAGE sample buffer by boiling for 5 min and then separated in 6% SDS-PAGE, which was visualized by silver staining.

α-1,2-Mannosidase Assay—For in vitro A. saitoi α-1,2-mannosidase activity assay, yeast cell extracts were prepared and analyzed as described by Chiba et al. (16) with slight modification. Soluble cell extracts were incubated with 30 pmol of ManαGlcNAc2-Pα at 37 °C for 30 min and analyzed by reversed-phase HPLC using a TSK-GEL ODS-80T’s column (Tohos, Tokyo, Japan, 0.46 × 25 cm3) equilibrated with a mixture of solvents A (100 mM acetic acid/triethylamine (pH 4.0)) and B (solvent A + 0.5% 1-butanol) (98:2, v/v). The ratio of solvent B was increased linearly up to 20% over 60 min at a flow rate of 1.0 ml/min.

RESULTS

Isolation of HplHOC1, HplOCR1, and HplOCH1 in H. polymorpha—In an effort to identify the gene(s) coding for α-1,6-mannosyltransferase contributing to the initiation of outer chain elongation on ManαGlcNAc2 in H. polymorpha, we had initially isolated two H. polymorpha genes, designated HplHOC1 and HplOCR1 (OCH1-related gene 1), using sequence information from random sequenced tags of H. polymorpha (22). Sequence analysis revealed that the HplHOC1 gene product (HplHoc1p) showed 40 and 24% overall identities with the S. cerevisiae HOCl and OCH1 gene products, respectively. On the other hand, the HplOCR1 gene product (HplOcr1p) showed relatively low sequence identity with both the ScHOC1 and ScOCH1 gene products (21 and 22% identity, respectively). A hydrophathy analysis performed using PSORT II (www.psort.org; see Ref. 23) predicted that HplHoc1p and HplOcr1p

6 J. H. Bae, unpublished observations.
FIGURE 1. Overall sequence comparison of Och1p homologues in yeast. The amino acid sequences of gene products of *S. cerevisiae* HOC1 (*ScHoc1p*, NCBI protein accession number NP_012609), *S. cerevisiae* OCH1 (*ScOch1p*, NCBI protein accession number NP_011477), *H. polymorpha* HOC1, OCR1, and OCH1 (*HpHoc1p*, NCBI protein accession number AAQ1191; *HpOcr1p*, NCBI protein accession number AAQ06498; and *HpOch1p*, NCBI protein accession number AAS77488), *Candida albicans* OCH1 (*CaOch1p*, NCBI protein accession number XP_716632), *P. pastoris* OCH1 (*PpOch1p*, NCBI nucleotide accession number E12456), and *S. pombe* OCH1 (*SpOch1p*, NCBI protein accession number CAD24818) are shown. The alignment was performed using the program ClustalW 1.8 (searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) and shaded using the program Boxshade 3.21 (www.ch.embnet.org/software/BOX_form.html). Identical residues and conservative amino acid substitutions in proteins are shaded with black or gray. Putative membrane-spanning regions, which act as signal-anchor domains, are indicated by boxes, and the DXD motif is shown above a sequence as hhhDXD.
possess a single potential transmembrane-spanning region, which acts as a signal-anchor domain (24) near the \( \text{N} \) terminus of the proteins, suggesting that they are type II membrane proteins. In addition, they possess a DXD motif well conserved in many glycosyltransferase families (25). An alignment of amino acid sequences of \( \text{HpHoc1p} \) and \( \text{HpOcr1p} \) with \( \text{Och1p} \) homologues from other yeast species reveals that several conserved regions are shared by \( \text{Och1p} \) homologues, although \( \text{N} \)-terminal portion is largely unique to each protein (Fig. 1). Most interestingly, \( \text{HpOcr1p} \) has a long C-terminal region that is absent in other \( \text{Och1p} \) homologues.

While we were analyzing the functions of \( \text{HpHOC1} \) and \( \text{HpOCR1} \), the entire genome of \( H. \text{polymorpha} \) was completely sequenced (17). Thus we searched this \( H. \text{polymorpha} \) genome data base to identify other possible \( H. \text{polymorpha} \) ORFs showing significant similarity to the \( S. \text{cerevisiae} \text{OCH1} \) and \( \text{HOC1} \) genes. In addition to the ORFs identified as \( \text{HpHOC1} \) and \( \text{HpOCR1} \), at least five other ORFs were found to encode putative mannosyltransferases with significant sequence identities to \( \text{ScOch1p} \), ranging from 15 to 37% (Table 1). These additional ORFs were also predicted to have a potential transmembrane domain at the \( \text{N} \)-terminal region and a conserved region encompassing a DXD motif. Of these, an ORF with the highest homology to \( \text{ScOch1p} \), designated \( \text{HpOCH1} \), and its functions in cell growth and \( \text{N} \)-linked glycosylation were investigated.

Effect of \( \text{HpHOC1}, \text{HpOCR1}, \) and \( \text{HpOCH1} \) Deletion on Cell Growth and \( \text{N} \)-Glycosylation—To investigate the effect of \( \text{HpHOC1}, \text{HpOCR1}, \) or \( \text{HpOCH1} \) gene deletion on cell growth and \( \text{N} \)-linked glycosylation, the single deletion (\( \text{Hphoc1}\Delta, \text{Hpopcr1}\Delta, \) or \( \text{Hpoch1}\Delta \)) or the double deletion (\( \text{Hphoc1}\Delta\text{Hpocr1}\Delta \) or \( \text{Hpoch1}\Delta\text{Hpoch1}\Delta \)) mutant strains were constructed and analyzed. Disruptions of either of these genes in \( H. \text{polymorpha} \) resulted in hypersensitivity to hygromycin B or sodium deoxycholate (Fig. 2, D and E), which are characteristic phenotypes of cell wall and \( \text{N} \)-linked glycosylation defects (26). In particular, the \( \text{Hporc1} \) null mutant strain showed slow-growing and temperature-sensitive phenotypes (Fig. 2, A and B), as was reported previously in \( \text{Scoch1} \) mutant strain. In addition, the growth of \( \text{Hporc1}\Delta \) was completely inhibited by Calcofluor White, which binds to chitin and disrupts cell wall assembly (27) (Fig. 2F). The growth defect and temperature sensitivity of the \( \text{Hporc1}\Delta \) strain were partially recovered by supplementing an osmotic stabilizer, 1 M sorbitol (Fig. 2C). Although \( \text{Hphoc1}\Delta \) cells displayed no obvious growth defects besides hygromycin B sensitivity, the introduction of the \( \text{Hphoc1}\Delta \) deletion into the background of the \( \text{Hporc1}\Delta \) strain (\( \text{Hporc1}\Delta\text{Hpocr1}\Delta \)) resulted in more significant growth retardation but restored hygromycin B sensitivity (Fig. 2, A and D). Most interestingly, the \( \text{Hporc1}\Delta \) deletion mutant strain (\( \text{Hporc1}\Delta \)) also exhibited a temperature-sensitive growth phenotype, which was complemented by the presence of 1 M sorbitol, but did not show a slow-growing phenotype under normal growth conditions (Fig. 2, A–C). The \( \text{Hporc1}\Delta \) strain was less sensitive to Calcofluor White but was more sensitive to hygromycin B than the \( \text{Hporc1}\Delta \) strain (Fig. 2, D and F). Moreover, unlike the \( \text{Hporc1}\Delta \) strain, additional inactivation of \( \text{Hphoc1} \) in the \( \text{Hporc1}\Delta \) strain background did not cause any further changes in the growth characteristics of the \( \text{Hporc1}\Delta \) strain.

### Table 1

| ORF   | ScHoc1p (396 aa) | ScOch1p (480 aa) | HpHoc1p (402 aa) | HpOcr1p (428 aa) | HpOcr2p (436 aa) | HpOcr3p (414 aa) | HpOcr4p (362 aa) | HpOcr5p (369 aa) |
|-------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ScHoc1p | 20              | 40              | 21              | 23              | 18              | 19              | 18              | 17              |
| ScOch1p | 36              | 40              | 24              | 22              | 37              | 18              | 21              | 17              |
| HpHoc1p | 63              | 40              | 19              | 28              | 22              | 22              | 21              | 17              |
| HpOcr1p | 36              | 36              | 34              | 32              | 32              | 32              | 32              | 32              |
| HpOcr5p | 30              | 31              | 30              | 33              | 32              | 50              | 33              | 47              |

### Figure 2

**Phenotypic analysis of the Hphoc1Δ, Hpopcr1Δ, Hporc1Δ, Hphoc1ΔHpopcr1Δ, and Hphoc1ΔHporc1Δ mutant strains.** Yeast cells were grown on YPD plates incubated at 37 °C (A) or 45 °C (B) on YPD supplemented with 1 M sorbitol at 45 °C (C), on YPD supplemented with 0.4% sodium deoxycholate (D), or 7 mg/ml Calcofluor White (E) at 37 °C for 2 days. Yeast cultures at the early exponential stage were diluted serially by 10-fold (from left to right) and spotted onto each plate.

In addition to the ORFs identified showing significant similarity to the \( S. \text{cerevisiae} \text{OCH1} \) and \( \text{HOC1} \) genes, the \( \text{ScOch1p} \) homologues ranging from 15 to 37% (Table 1). These additional ORFs were also predicted to have a potential transmembrane domain at the \( \text{N} \)-terminal region and a conserved region encompassing a DXD motif. Of these, an ORF with the highest homology to \( \text{ScOch1p} \), designated \( \text{HpOCH1} \), and its functions in cell growth and \( \text{N} \)-linked glycosylation were investigated.

Effect of \( \text{HpHOC1}, \text{HpOCR1}, \) and \( \text{HpOCH1} \) Deletion on Cell Growth and \( \text{N} \)-Glycosylation—To investigate the effect of \( \text{HpHOC1}, \text{HpOCR1}, \) or \( \text{HpOCH1} \) gene deletion on cell growth and \( \text{N} \)-linked glycosylation,
oligosaccharide profile to that of the wild type, but with a slight decrease in the portion of oligosaccharides larger than Man$_6$GlcNAc$_2$, was observed in the Hphoc1$\Delta$ single mutant (Fig. 4A, panel d). However, oligosaccharides from Hpocr1$\Delta$, Hphoc1$\Delta$Hpocr1$\Delta$, or Hphoch1$\Delta$ mutant cells were mainly composed of a single predominating species, corresponding to the core-glycosylated form Man$_6$GlcNAc$_2$, although larger structures of Man$_9$GlcNAc$_2$ were present as minor components (Fig. 4A, panels g, j, and m). In the Hphoc1$\Delta$Hpocr1$\Delta$ mutant strain, the relative proportions of the oligosaccharides larger than Man$_6$GlcNAc$_2$ appeared to be reduced versus the Hpocr1$\Delta$ strain. These results indicate that mannose outer chain elongation is significantly inhibited in the Hpocr1$\Delta$, Hphoc1$\Delta$Hpocr1$\Delta$, and Hphoch1$\Delta$ mutant strains, although small fractions of oligosaccharides (Man$_9$-14GlcNAc$_2$) larger than Man$_6$GlcNAc$_2$ were observed.

To investigate in more detail the structure of the oligosaccharide species produced by the mutant strains, sequential digestion experiments with $\alpha$-1,2- and $\alpha$-1,6-mannosidases were carried out. After digestion of the oligosaccharides with $\alpha$-1,2-mannosidase from A. saitoi, which is highly specific for nonreducing terminal $\alpha$-1,2-mannose linkages, most of the oligosaccharides species from the wild type strain were shifted to two major species, i.e. Man$_5$GlcNAc$_2$ and Man$_6$GlcNAc$_2$ (Fig. 4A, panel b). Subsequent digestion with $\alpha$-1,6-mannosidase from X. manihotis, which is highly specific for terminal $\alpha$-1,6-linked mannose residues that are linked to a nonbranched sugar, converted all the Man$_n$GlcNAc$_2$ oligosaccharide species generated by $\alpha$-1,2-mannosidase digestion into Man$_5$GlcNAc$_2$ (Fig. 4A, panel c). Our previous study indicated that Man$_5$GlcNAc$_2$ (M5) is the final product of specific $\alpha$-1,2-mannosidase digestion of the core oligosaccharide Man$_n$GlcNAc$_2$, or of the larger oligosaccharide species extended with only $\alpha$-1,2-mannose linkages, whereas Man$_6$GlcNAc$_2$ (M6) oligosaccharide is the final product of the large oligosaccharides elongated by a single $\alpha$-1,6-linked mannose addition and branched with a variable number of $\alpha$-1,2-linked mannose units (14). After digestion with $\alpha$-1,2-mannosidase, the oligosaccharides from the Hphoc1$\Delta$, Hpocr1$\Delta$, and Hphoc1$\Delta$Hpocr1$\Delta$ strains were shifted to two major species, i.e. M5 and M6, like the wild type (Fig. 4A, panels e, h, and k). Moreover, subsequent digestion with $\alpha$-1,6-mannosidase converted all M6 oligosaccharide species into M5 (Fig. 4A, panels f, i, and l). These results indicate that a significant portion of large oligosaccharides from Hphoc1$\Delta$, Hpocr1$\Delta$, and Hphoc1$\Delta$Hpocr1$\Delta$ strains contain an additional $\alpha$-1,6-linked mannose attached to the core oligosaccharide, which is believed to be mediated by the $\alpha$-1,6-initiating mannosyltransferase activity of S. cerevisiae Och1p homologue. Even though the relative proportions of M6 to M5 appeared to gradually decrease from the wild type to Hpocr1$\Delta$ and to Hphoc1$\Delta$Hpocr1$\Delta$ cells, our results strongly suggest that the major roles of Hphoc1p and Hpocr1p are not in the addition of the first $\alpha$-1,6-linked mannose. However, in contrast to the N-glycans from the wild type, Hphoc1$\Delta$, Hpocr1$\Delta$, and Hphoc1$\Delta$Hpocr1$\Delta$ strains, all oligosaccharides species from the Hpoch1$\Delta$ strain were converted to M5 just after $\alpha$-1,2-mannosidase treatment (Fig. 4A, panel n), indicating that the $\alpha$-1,6-linked mannose addition to the core oligosaccharide was completely blocked in the Hpoch1$\Delta$ strain. This result strongly suggests that HpOch1p plays a key role as $\alpha$-1,6-mannosyltransferase during the first step of outer chain biosynthesis.

The effect of HpOCH1 gene deletion on the structure of oligosaccharides attached to secreted glycoproteins was further analyzed using the N-glycans released from the endogenous *H. polymorpha* glycoprotein, *Hypys1* protein, which is a glycosylphosphatidylinositol-anchored aspartic protease with four potential N-linked glycosylation sites (Fig. 4B). The secreted form of Hypys1p was obtained by deleting a C-ter-
H. polymorpha OCH1 Mannosyltransferase Family

Involvement of HpOCR1 in O-Glycosylation—To examine whether HpOCR1, HpOCR1, and HpOCH1 gene products are involved in O-linked glycosylation, we analyzed the electrophoresis mobility of an endogenous O-modified glycoprotein, H. polymorpha chitinase, that is reported to lack N-linked oligosaccharides (21). secreted by the wild type, Hpocr1Δ, Hpoc1Δ, Hpoch1Δ, Hpocr1ΔHpoc1Δ, and Hpocr1ΔHpoch1Δ mutant strains (Fig. 5). The Hpoch1Δ, Hpocr1Δ, and Hpocr1ΔHpoch1Δ strains secreted heterogeneous forms of chitinase with high molecular weights similar to those of chitinase secreted by the wild type (Fig. 5, lanes 1, 3, 4, and 6). Most interestingly, compared with the wild type strain, the Hpoch1Δ strains showed a rather increased extent of O-glycosylation in chitinase (Fig. 5, lanes 3 and 6 versus lane 1), probably reflecting a kind of compensatory mechanism to maintain the cell integrity caused by the loss of HpOch1p function. In contrast, sig-

FIGURE 4. HPLC analyses of N-linked oligosaccharides assembled on glycoproteins secreted by H. polymorpha mutant strains. A, analysis of N-glycans attached to rGOD secreted by H. polymorpha wild type (panels a–c), Hpocr1Δ (panels d–f), Hpoc1Δ (panels g–i), Hpocr1ΔHpoc1Δ (panels j–l), and Hpoch1Δ (panels m–o) strains. Chromatograms of the N-glycan profiles released from rGOD before any treatment (panels a, d, g, j, and m), after α1,2-mannosidase treatment (panels b, e, h, k, and n), and after subsequent α1,6-mannosidase treatment (panels c, f, i, l, and o) are shown. The HPLC column was equilibrated with 80% of solvent A (200 mM acetic acid/triethylamine (pH 7.3), acetonitrile 1:9) and 20% of solvent B (200 mM acetic acid/triethylamine (pH 7.3), acetonitrile 9:1). B, analysis of N-glycans assembled on Yps1p secreted by H. polymorpha wild type (panels a–c) and Hpoch1Δ (panels d–f) strains. Chromatogram of the N-glycans released from HpYps1p before any treatment (panels a and d), after α1,2-mannosidase treatment (panels b and e), and after subsequent α1,6-mannosidase treatment (panels c and f). The HPLC column was equilibrated with 75% of solvent A and 25% of solvent B. The elution times of peaks were compared with those of authentic ManαGlcNAc-PA (M5), ManαGlcNAc-PA (M6), and ManαGlcNAc-PA (M8) of known structure (indicated by arrows). The unidentified peak (indicated by arrow/heading) is detected in some samples during the HPLC analysis. We observed that the peak is resistant to digestion by jack bean (α1,2-, α1,3-, and α1,6-) mannosidase and becomes more dominant in the old samples compared with the fresh samples after PA labeling.

minal region containing a glycosylphosphatidylinositol-anchoring motif.7 Oligosaccharide profiles of HpYps1p secreted by the wild type and Hpoch1Δ mutant strains displayed greater differences in terms of the relative proportions of M5 to M6 oligosaccharides than in those of rGOD secreted by these two strains. In the wild type strain, oligosaccharides containing 9–10 mannoses (Manα-Mannosidase, most of the oligosaccharide species were converted to M6, which was then completely converted to M5 by subsequent digestion with α1,6-mannosidase (Fig. 4B, panels b and c). This indicates that most oligosaccharides assembled on HpYps1p contained a single α1,6-linked mannose attached to the core oligosaccharide in the wild type strain. In contrast, in the Hpoch1Δ mutant strain, core form oligosaccharide ManαGlcNAc predominated; oligosaccharides larger than ManαGlcNAc were detected as minor fractions (Fig. 4B, panel d). Moreover, digestion with α1,2-mannosidase converted all Hpoch1Δ strain-derived oligosaccharides to M5 (Fig. 4B, panel e). These results clearly indicate that a defective addition of α1,6-mannose residue onto the core oligosaccharide ManαGlcNAc is a general phenotype of the Hpoch1Δ strain and not a protein-specific phenotype.

7 E. J. Kim and H. A. Kang, unpublished results.
nificantly homogeneous forms of chitinase were detected in the Hpocr1Δ and Hphoc1Δ Hpocr1Δ strains, indicating that the O-glycosylation of extracellular chitinase appeared to be severely impaired in the absence of HpOcr1p (Fig. 5, lanes 2 and 5). The results strongly implied that HpOcr1p is involved in O-linked chain elaboration in addition to N-linked outer chain biosynthesis.

HpOCH1 as a Functional Homologue of ScOCH1 Encoding an Initiating α-1,6-Mannosyltransferase—To investigate whether HpOCH1 is a functional homologue of ScOCH1, we transformed an S. cerevisiae och1 null (Scoh1Δ) mutant with the plasmid YEp352GAPII-HpOCH1 carrying HpOCH1 under the control of the S. cerevisiae GAPDH promoter. The Scoh1Δ mutant strain transformed with the null vector YEp352GAPII showed temperature sensitivity, but the Scoh1Δ strain transformed with YEp352GAPII-HpOCH1 grew well at 30 °C (Fig. 6A, row 2 versus row 5), indicating that the temperature-sensitive growth defect of Scoh1Δ was recovered by the expression of HpOCH1. In contrast, Scoh1Δ cells transformed with YEp352GAPII-HpHOC1 or YEp352GAPII-HpOCR1 displayed the same temperature-sensitive phenotype as the Scoh1Δ mutant (Fig. 6A, rows 3 and 4). Moreover, the electrophoretic mobility of invertase secreted from the Scoh1Δ cells transformed with the HpOCH1 expression plasmid decreased to the same extent as that observed from the Scoh1Δ cells transformed with the ScOCH1 expression plasmid (Fig. 6B, lanes S and 6). Together with the observed complementation of the temperature-sensitive growth defect, the restoration of the hyperglycosylation defect of Scoh1Δ suggests that HpOCH1 encodes a functional homologue of ScOch1p that plays a key role in the initiation of outer chain elongation.

To measure the α-1,6-mannosyltransferase activity of HpOch1p, solubilized membrane fractions were prepared from the S. cerevisiae mutant strains with OCH1, MNN1, and MNN4 deletions (Scoh1Δ mnn1Δ mnn4Δ), but harboring YEp352GAPII, YEp352GAPII-HpOCH1, or YEp352GAPII-ScOCH1 plasmids, and these were used as enzyme sources for α-1,6-mannosyltransferase assays (Fig. 6C). The Scoh1Δ mnn1Δ mnn4Δ strain was shown to be defective in adding mannose residues to N-linked core oligosaccharide (16). PA-labeled Man6GlcNAc2, which has the structure of the core oligosaccharide formed in the ER of S. cerevisiae (3), was used as an acceptor, and the reaction products were analyzed by HPLC. Although the acceptor oligosaccharide, Man6GlcNAc2-PA (M6), was not converted into any other form by the membrane fraction of Scoh1Δ mnn1Δ mnn4Δ transformant harboring the null vector (Fig. 6C, panel a), a peak corresponding to Man6GlcNAc2-PA (M9) was detected as a reaction product in the membrane fraction of Scoh1Δ mnn1Δ mnn4Δ transformants harboring YEp352GAPII-HpOCH1 or YEp352GAPII-ScOCH1 (Fig. 6C, panels b and c). These results strongly indicate that HpOCH1, like ScOCH1, encodes the initiation-specific α-1,6-mannosyltransferase acting on the core oligosaccharide. Solubilized membrane fractions prepared from the H. polymorpha wild type, Hpocr1Δ, and Hphoc1Δ mutant strains were also analyzed for α-1,6-mannosyltransferase activity (Fig. 6D). The M9 peak corresponding to Man6GlcNAc2-PA was generated by the reaction between the acceptor oligosaccharide and the membrane fraction from the wild type and Hpocr1Δ mutant strains but was hardly detected in the reaction with the membrane fraction of the Hphoc1Δ mutant (Fig. 6D, panels a and b versus panel c). After α-1,2-mannosidase digestion, the M9 product was converted to Man6GlcNAc2-PA (M6), whereas the M9 core oligosaccharide was converted to Man6GlcNAc2-PA (M5) (Fig. 6D, panels d–f). These results consistently support the notion that HpOch1p is a major key enzyme in the initiation of outer chain biosynthesis, i.e. in the addition of α-1,6-mannose on the lower arm of the core oligosaccharide, as reported for ScOch1p.

Expression of the ER-targeted α-1,2-Mannosidase in Hphoc1Δ—The Hpocr1Δ mutant strain, with a defect in the yeast-specific outer chain initiation step, was further evaluated as a starting strain for the genetic engineering of the N-linked glycosylation pathway to produce human-type sugars in H. polymorpha. To trim off the core oligosaccharide by removing α-1,2-mannose residues as in mammalian cells, we heterologously expressed ER-targeted A. saitoi α-1,2-mannosidase in H. polymorpha. Briefly, the mds5 gene cassette fused with the signal sequence...
of aspergillopeptin and the ER retention signal, HDEL (16), was further modified at its C terminus to contain the HA epitope to monitor its expression (Fig. 7A). Western blot analysis using HA antibody showed that the recombinant α,1,2-mannosidase-HA-HDEL was primarily expressed as core-glycosylated forms and retained inside H. polymorpha cells. No secreted form of the recombinant α,1,2-mannosidase was detected in the culture supernatant, which strongly indicated the proper localization of the recombinant α,1,2-mannosidase in the ER (Fig. 7B). Cell extracts of recombinant H. polymorpha containing the α,1,2-mannosidase-HA-HDEL construct were able to convert Man₉GlcNAc₂-PA into Man₃GlcNAc₂-PA in the in vitro α,1,2-mannosidase activity assay for A. saitoi α,1,2-mannosidase (16), whereas no such activity was detected in extracts of recombinant H. polymorpha transformed with the null vector (panel b) and with the α,1,2-mannosidase-HA-HDEL expression plasmid (panel c). The results of this in vitro assay indicated that α,1,2-mannosidase-HA-HDEL was expressed as an active form in H. polymorpha.

To monitor N-glycan modification by the targeted expression of A. saitoi α,1,2-mannosidase in the ER of the Hpoch1Δ mutant strain, we analyzed the structures of N-glycans on rGOD. Compared with the oligosaccharides synthesized in the Hpoch1Δ mutant without α,1,2-mannosidase-HA-HDEL, the oligosaccharides produced in the recombinant Hpoch1Δ mutant strain expressing the active α,1,2-mannosidase-HA-HDEL were much shorter in length (Fig. 7D, panels a and b). The fraction corresponding to Man₃GlcNAc₂, the smallest structure of human-compatible type high mannose oligosaccharide, was detected as a major component in the recombinant Hpoch1Δ mutant strain expressing the active α,1,2-mannosidase-HA-HDEL. However, larger structures containing up to 10 mannoses were also detected as minor components, and all of these were converted into Man₃GlcNAc₂ after in vitro α,1,2-mannosidase treatment (Fig. 7D, panel c). The incomplete trimming to Man₃GlcNAc₂ in the recombinant Hpoch1Δ strain might have been due to the low expression level or activity of α,1,2-mannosidase-HA-HDEL. Alternatively, the activity of endogenous α,1,2-mannosidases in the Golgi might have subsequently added α,1,2-linked mannoses to Man₃GlcNAc₂ to generate larger oligosaccharides extended with α,1,2-mannose linkages.

**DISCUSSION**

The yeast-specific outer chain biosynthesis of N-glycans is initiated by the addition of α,1,6-linked mannose to the Man₃GlcNAc₂ core-oligosaccharide in the Golgi apparatus, and this process is mediated by the activity of the OCH1 gene product in S. cerevisiae. The three genes OCH1, HOCl, and SUR1 comprise the OCH1 gene family in S. cerevisiae, implying that one interesting feature of yeast Golgi glycosyltransferases is their redundancy in terms of function and structure (4). Seven ORFs, showing significant sequence homologies to ScOCH1 and the topologic characteristics of glycosyltransferases, were identified from the H. polymorpha genome sequence (Table 1). All seven members of the H. polymorpha OCH1 gene family, including HpHOCl, HpOCR1, and HpOCR2, analyzed during this study, were predicted to encode type II membrane proteins with a short cytoplasmic N-terminal domain, a single membrane-spanning region, and a C-terminal catalytic domain, suggesting their function as glycosyltransferases localized in the Golgi lumen (data not shown). To clarify sequence relationships and to functionally group the different members of the H. polymorpha OCH1 family, phylogenetic analysis was carried out together with the OCH1 family genes and homologues from other yeasts and fungi (Fig. 8). The tree shows that the OCH1 family genes would be grouped into several subfamilies such as OCH, HOCl, OCR, and SUR groups. As expected from relatively high sequence homologies with those of S. cerevisiae, HpOCH1 and HpHOCl are classified into OCH and HOCl groups, respectively. Interestingly, the other members of the H. polymorpha OCH1 gene family are grouped together remotely from the OCH and HOCl groups, constituting their own subfamily (HpOCR group).
**H. polymorpha OCH1 Mannosyltransferase Family**

![Image](49x504 to 300x733)

**FIGURE 8.** Neighbor-joining phylogenetic tree of the OCH1 family and homologues from yeasts and fungi. The sequences of the OCH1 family and homologues were collected by Blast searches with S. cerevisiae OCH1 sequence. C. albicans HOC1, OCH1 (CahOCH1, NCBI protein accession number XP_716693; CaOCH1, XP_716632), Ashbya gossypii OCH1, OCH1 (AgOCH1, AAS53806; AgOCH1, AAS53836), Kluyveromyces lactis HOC1, OCH1 (KlHOC1, XP_552168; KlOCH1, XP_556072), S. cerevisiae OCH1, OCH1 (SCHO1, NP_012609; ScOCH1, NP_011477; ScCSH1, NP_009719; ScSUR1, NP_015268), Candida glabrata HOC1, OCH1, and SUR1 (CgHOC1, XP_449987; CgOCH1, XP_444841; CgSUR1, XP_449590), Y. lipolytica OCH1 (YIOCH1, CAD91463), Aspergillus fumigatus OCH1 (AfOCH1, XP_753779; A. fumigatus OCH1 (AfOCH1, CAD24818), and H. polymorpha SUR1 (HpSUR1) were included for phylogenetic analysis, and the result was graphically presented using ClustalW (align.genome.jp).

HpOCR subfamily appears to be evolved from the common origin of the OCH1 and HOC1 genes before their split occurred. The genes belonging to HpOCR group were named as HpOCR2, HpOCR3, HpOCR4, and HpOCR5, in which the numbering was made based on their homologies to H. polymorpha OCH1 (Table 1). None of the HpOCR subfamily was able to complement the defects of S. cerevisiae OCH1 and HOC1 mutation (data not shown), which is consistent with their relatively remote relationship with the OCH1 and HOC1 subfamily.

As generally observed in glycosylation defective mutant strains of S. cerevisiae (26), the deletion of HpHOC1, HpOCR1, or HpOCH1 resulted in characteristic phenotypic defects in cell wall integrity, such as hyper-sensitivity to hygromycin B or sodium deoxycholate, thus indicating that the functions of HpHOC1, HpOCR1, and HpOCH1 are associated with cell wall biosynthesis (Fig. 2). In particular, the null mutant strain of HpOCR1 had a slow growing and temperature-sensitive phenotype as was observed in the deletion strain of ScOCH1, whereas the HpOCH1 and HpHoc1Δ mutant strains showed growth rates that were comparable with that of the wild type under normal growth conditions. Analysis of the recombinant glycoprotein GOD expressed in the deletion mutants of HpOCR1 and HpOCH1 revealed a significant reduction in the size of N-linked oligosaccharides (Fig. 4), indicating that HpOcr1p and HpOch1p proteins have major roles in H. polymorpha-specific outer chain biosynthesis. No apparent defect in N-glycosylation was detected on disrupting HpHOC1, as is the case for S. cerevisiae HOC1 (9). However, the HpHoc1ΔHpOcr1Δ double deletion mutant showed a more marked N-glycosylation defect than the single Hpocr1Δ mutant strain (Figs. 3 and 4), suggesting that HpHoc1p might be partly involved in N-glycosylation in a redundant fashion with HpOcr1p.

Although the Hpocr1Δ mutant strain displays apparent phenotypes quite similar to those of the Scoch1Δ strain (5), i.e. a retarded growth rate and a dramatic reduction in the size of N-glycans, detailed structural analysis revealed that the initiation step of α-1,6-mannoside addition to the core oligosaccharide was not impaired in the Hpocr1Δ mutant strain (Fig. 4A, panels h and i). Rather, the noticeable reduction in large oligosaccharide species branched with α-1,2-linked mannose units led us to speculate that the severe defect of outer chain biosynthesis shown by the Hpocr1Δ mutant strain might be due to a defect of outer chain extension by α-1,2-linked mannose addition. In contrast, most N-oligosaccharide species derived from the recombinant GOD and the endogenous glycoprotein HpYps1p secreted by the Hpoch1Δ mutant strain were shown to be devoid of additional α-1,6-mannose residues attached on the core oligosaccharide Manα1Glcnac2α, (Fig. 4A, A, panel a, and B, panel e), suggesting that HpOch1p functions as a critical element during the first addition of an α-1,6-mannose to the core oligosaccharide Manα1Glcnac2α. In addition, the physiological role of HpOch1p as a functional homologue of ScOch1p was further confirmed by its ability to complement the temperature-sensitive growth phenotype (Fig. 6A) and the hyperglycosylation defect (Fig. 6B) of Scoch1Δ cells. Furthermore, the result of in vitro assay on α-1,6-mannosyltransferase activity showed that HpOch1p has an initiating α-1,6-mannosyltransferase activity (Fig. 6, C and D). Taken together, our data provide evidence that HpOch1p is a key α-1,6-mannosyltransferase, which is responsible for the first step of outer chain biosynthesis in H. polymorpha. At present, only single α-1,6-mannosyltransferase has been found to be responsible for adding initiating α-1,6-mannose to the core oligosaccharide in outer chain biosynthesis in S. cerevisiae, S. pombe, and P. pastoris (5, 7, 28). The overexpressions of HpOCR1 and HpHOC1 in Scoch1Δ mutant (Fig. 6) and in Hpoch1Δ mutant cells (data not shown) did not complement the temperature-sensitive growth and N-glycosylation defects of these mutant strains, indicating that HpOcr1p and HpHoc1p cannot substitute for the function of ScOch1p and HpOch1p as an initiating α-1,6-mannosyltransferase. Interestingly, no direct N-glycosylation defect was reported in the null mutation of Yarrowia lipolytica OCH1 homologue (YIOCH1), which led to speculation that either YIOCH1 has a different role in the N-linked glycosylation pathway in Y. lipolytica (29) or that redundant genes may encode a functional homologue of Scoch1p in Y. lipolytica. At present, the functions of HpOcr1p and HpHoc1p have yet to be defined, although they appear to be involved in the elongation step of outer chain biosynthesis. The apparent decrease of α-1,2-mannose extension in the Hpocr1Δ mutant strain strongly suggests that the function of HpOcr1p is closely related to α-1,2-mannosyltransferase activity. Moreover, the drastic decrease in the extent of O-glycosylation in the Hpocr1Δ mutant strain (Fig. 5) strongly indicates that HpOcr1p might encode α-1,2-mannosyltransferase, because a large class of glycan additions that are found on both O- and N-linked oligosaccharides are α-1,2-linked mannosides. However, little homology (below 8% sequence identity) exists between HpOCR1 and S. cerevisiae KTR (KreTwo-Related) family, which encodes α-1,2-mannosyltransferase involved in both N- and O-linked protein glycosylation in S. cerevisiae (30), excluding the possibility that HpOcr1p could be classified as a member of the KRE2/MNT1 family. Thus, we propose HpOCR1 as a novel member of the OCH1 family that is implicated in both N- and O-linked glycosylation. As suggested for S. cerevisiae Mnt9p, which was shown to have α-1,2-mannosyltransferase activity in addition to α-1,6-mannosyltransferase activity (31), it could be that HpOcr1p has α-1,2-mannosyltransferase activity and acts on N-glycans in which the first α-1,6-mannose was added by HpOch1p. The question of whether HpOcr1p is an enzyme directly involved in N- and O-linked oligosaccharide elaboration has to be addressed by further biochemical analysis.
addition. Therefore, in this methyloptotic yeast, the activities of α-1,2-
mannosyltransferases appear to out-compete those of α-1,6-mannosyl-
transferases for outer chain elongation, to generate mainly core-type glycans that lack the extended α-1,6-mannose backbone structure (Fig. 9, ii versus iii). Moreover, α-1,2-linked mannoses are exposed as termi-
nal residues without further decoration with α-1,3-linked mannoses in H. polymorpha N-glycans (Fig. 9, iv), which is different from S. cerevisiae N-glycans in which the addition of α-1,3 mannoses acts as a stop signal for further extension.

It is noteworthy that the HpoOH1 strain, despite its defect in outer chain initiation on the core glycan Man9GlcNAc2, did not show severe growth retardation under normal conditions, unlike the HpoOCR1 and Scoch1Δ mutant strains, although it displayed a temperature-sensitive growth phenotype. Differing from the outer chains of S. cerevisiae N-glycans with extensive α-1,6-extensions, those of H. polymorpha N-glycans were shown to have very short α-1,6-extensions, mainly composed of single α-1,6-linked mannose. This might explain the mild effect of HpoOH1 deletion on cell growth of H. polymorpha. On the contrary, the deletion of HpoOCR1, which generated dramatic defects in both N- and O-linked glycosylation, would cause severe growth retardation. The wild type comparable growth of the HpoOH1Δ strain under normal growth conditions warrants that this strain can be developed as a starting strain for the production of recombinant glycoproteins mimicking humanized N-glycans in H. polymorpha. In addition, the lack of the immunogenic terminal α-1,3-mannose linkage and the extremely low level of phosphomannose residues in H. polymorpha N-glycans (14) represent additional advantages of this yeast over S. cerevisiae in terms of glycan engineering with a view toward glycoprotein production. The engineered HpoOH1Δ strain with the targeted expression of A. saitoi α-1,2-mannosidase in the ER was able to synthesize Man9GlcNAc2 as a major N-glycan (Fig. 7D). These results demonstrate that H. polymorpha has the potential to be developed as a host for the production of therapeutic glycoproteins containing humanized oligosaccharides, although further optimization of mannose removal and the addition of other sugars are required to generate complex N-glycans of therapeutic value.

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REFERENCES

1. Helenius, A., and Aebi, M. (2001) Science 291, 2364–2369
2. Munro, S. (2001) FEBS Lett. 498, 223–227
3. Nakayama, K., Shindo, Y., Nakayama, K., Tanaka, A., Toda, Y., and Jigami, Y. (1993) J. Biol. Chem. 268, 26338–26345
4. Dean, N. (1999) Biochim. Biophys. Acta 1426, 309–322
5. Nakayama, K., Nagasu, T., Shimma, Y., Kuromitsu, J., and Jigami, Y. (1992) EMBO J. 11, 2511–2519
6. Nakayama, K., Nakashima, Shindo, Y., Tanaka, A., Haga-Toda Y., and Jigami, Y. (1997) EMBO J. 16, 547–550
7. Yoko-o, T., Tsukahara, K., Watanabe, T., Haga-Sugi, N., Yoshimatsu, K., Nagasu, T., and Jigami, Y. (2001) FEBS Lett. 489, 75–80
8. Murakami, K., and Sugio, N. (January 7, 1997) Japan Patent JP 1997000261-A1
9. Neiman, A. M., Mhasikar, V., Manus, V., Galibert, F., and Dean, N. (1997) Genetics 145, 637–645
10. Gelissen, G., and Veennhuis, M. (2001) Yeast 18, i–ii
11. Kang, H. A., and Gelissen, G. (2005) in Production of Recombinant Proteins (Gelissen, G., ed) pp. 111–142, Wiley-VCH, Weinheim, Germany
12. Rodriguez, L., Narciandi, R. E., Roca, H., Cremata, J., Montesinos, R., Rodriguez, E., Grillo, J. M., Muzio, V., Herrera, L. S., and Delgado, J. M. (1996) Yeast 12, 815–822
13. Kang, H. A., Sohn, J. H., Choi, E. S., Chung, B. H., Yu, M. H., and Rhee, S. K. (1998) Yeast 14, 371–381

As in the case of ScHoc1p (9), the function of HpHoc1p is unclear. The deletion of HpoOH1 strain generates cell wall defect, which is characterized by the sensitivity to hygromycin B and Calcofluor White. However, HpoOH1 could not complement the hypersensitivity of the Schoc1 strain to hygromycin B (data not shown). The deletion effect of HpoOH1 on N-glycosylation became more manifest in the absence of HpOcr1p, indicating that the function of HpoOH1 might be partially overlapped with HpOcr1p. Our recent study on transcriptome analysis using a H. polymorpha partial genome microarray (32) showed a significant induction of HpoOH1 after administering the superoxide-generating drug, menadione, which suggests that the function of HpHoc1p might be associated with stress response. The elucidation of the specific roles of HpoOH1, HpoOCR1, and other members of the HpoOCR1 subfamily in the N-glycosylation pathway and other cellular processes in H. polymorpha remains an intriguing issue, which requires further genetic and biochemical studies.

Based on structural information of the N-linked oligosaccharides of H. polymorpha and the data presented in this study, we propose a putative N-linked outer chain biosynthetic pathway in H. polymorpha, as shown in Fig. 9. As reported in other yeasts, the core oligosaccharide of Man9GlcNAc2 originating from a dolichol-linked Glc3Man9GlcNAc2 is elongated at the α-1,3-branch by the addition of an α-1,6-linked manno-

Note: The image contains a diagram of the proposed pathway of the outer chain biosynthesis of N-linked oligosaccharides in H. polymorpha. The diagram illustrates the core structure, followed by steps involving the Golgi apparatus, with specific enzymatic activities and structures labeled. The pathway includes the formation of core-type oligosaccharides by successive reactions, the attachment of terminal α-1,6-extensions, and the role of HpMnn9p-containing M-Pol I and M-Pol II complexes, among other processes.
H. polymorpha OCH1 Mannosyltransferase Family

14. Kim, M. W., Rhee, S. K., Kim, J. Y., Shimma, Y., Chiba, Y., Jigami, Y., and Kang, H. A. (2004) Glycobiology 14, 243–251
15. Kang, H. A., Sohn, J. H., Agaphonov, M. O., Choi, E. S., Ter-Avanesyan M. D., and Rhee, S. K. (2002) in Hansenula polymorpha-Biology and Applications (Gellissen, G., ed) pp. 124–146, Wiley-VCH, Weinheim, Germany
16. Chiba, Y., Suzuki, M., Yoshida, S., Yoshida, A., Ikenaga, H., Takeuchi, M., Jigami, Y., and Ichishima, E. (1998) J. Biol. Chem. 273, 26298–26304
17. Ramezani-Rad, M., K. P., Lauber, J., Wedler, H., Griess, E., Wanger, C., Albermann, K., Hani, I., Fiontek, M., Dahlem, U., and Gellissen, G. (2003) FEBS Lett. 527, 207–215
18. Lorenz, M. C., Muir, R. S., Lim, E., McElver, J., Weber, S. C., and Heitman, J. (1995) Gene (Amst.) 158, 113–117
19. Storici, F., Lewis, I. K., and Resnick, M. A. (2001) Nat. Biotechnol. 19, 773–776
20. Nakajima, T., and Ballou, C. E. (1974) J. Biol. Chem. 249, 7679–7684
21. Agaphonov, M., Sokolov, S. S., Romanova, N. V., Sohn, J.-H., Kim, S.-Y., Kalebina, T. S., Choi, E.-S., and Ter-Avanesyan, M. D. (2005) Yeast 22, 1037–1047
22. Blandin, G., Llorente, B., Malpertuy, A., Wincker, P., Artiguenave, F., and Dujon, B. (2000) FEBS Lett. 487, 76–81
23. Nakai, K., and Horton, P. (1999) Trends Biochem. Sci. 24, 34–36
24. Wickner, W. T., and Lodish, H. F. (1985) Science 230, 400–407
25. Wiggins, C. A., and Munro, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7945–7950
26. Dean, N. (1995) Proc. Natl. Acad. Sci. U. S. A. 15, 3209–3212
27. Ram, A. F., Wolters, A., Ten Hoopen, R., and Klin, F. M. (1994) Yeast 10, 1019–1030
28. Choi, B. K., Bobrowicz, P., Davidson, R. C., Hamilton, S. R., Kung, D. H., Li, H., Miele, R. G., Nett, J. H., Wildt, S., and Gerngross, T. U. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5022–5027
29. Barnay-Verdier, S., Boisrame, A., and Beckerich, J.-M. (2004) Microbiology 150, 2185–2195
30. Lussier, M., Sdicu A.-M., and Bussey, H. (1999) Biochim. Biophys. Acta 1426, 323–334
31. Stolz, J., and Munro, S. (2002) J. Biol. Chem. 277, 44801–44808
32. Oh, K. S., Kwon, O., Oh, Y. W., Sohn, M. J., Jung, S., Kim, Y. K., Kim, M.-G., Rhee, S. K., Gellissen, G., and Kang, H. A. (2004) J. Microbiol. Biotechnol. 14, 1239–1248
33. Jungmann, J., and Munro, S. (1998) EMBO J. 17, 423–434
34. Kim, S. Y., Sohn, J.-H., Kang, H. A., and Choi, E.-S. (2001) Yeast 18, 455–461
35. Gabriel, O., and Wang, S. F. (1969) Anal. Biochem. 27, 545–554
36. Inoue, T., Yoshida, T., and Ichishima, E. (1995) Biochim. Biophys. Acta 1253, 141–145