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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* (*Hphoc1*Δ) did not generate any detectable changes in *N*-glycosylation, the null mutant strains of *HpOCH1* (*Hpoch1*Δ) and *HpOCR1* (*Hpocr1*Δ) displayed a remarkable reduction in hypermannosylation. Although the apparent phenotypes of *Hpocr1*Δ were most similar to those of *S. cerevisiae och1* mutants, the detailed structural analysis of *N*-glycans revealed that the major defect of *Hpocr1*Δ is not in the initiation step but rather in the subsequent step of outer chain elongation by α-1,2-mannose addition. Most interestingly, *Hpocr1*Δ 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 $Man_8GlcNAc_2$ was completely blocked in *Hpoch1*Δ despite the comparable growth of its wild type under normal growth conditions. The complementation of the *S. cerevisiae och1* null mutation by the expression of *HpOCH1* and the lack of *in vitro* α-1,6-mannosyltransferase activity in *Hpoch1*Δ provided supportive evidence that *HpOCH1* is the functional orthologue of *ScOCH1*. The engineered *Hpoch1*Δ strain with the targeted expression of *Aspergillus saitoi* α-1,2-mannosidase in the endoplasmic reticulum was shown to produce human-compatible high mannose-type $Man_5GlcNAc_2$ oligosaccharide as a major *N*-glycan.

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),[2] where an identical $Glc_3Man_9GlcNAc_2$ 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 ($Man_8GlcNAc_2$). Glycoproteins containing $Man_8GlcNAc_2$ are then collected into transport vesicles and delivered to the Golgi apparatus. It is in the Golgi that the diversity of *N*-glycan structures is generated by a series of glycosidases 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 $Man_8GlcNAc_2$ 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 mannoses, highly branched by the addition of α-1,2-linked mannoses and terminally capped by α-1,3-linked mannoses (4). The mannosyltransferase activity and the substrate specificity of the *S. cerevisiae* Och1p are well characterized and shown to require the intact structure of $Man_8GlcNAc_2$ for efficient mannose 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 mannose 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

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF540063, AF490971, AY502025, DQ249343, DQ249344, DQ249345, and DQ249346.

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[2] The abbreviations used are: ER, endoplasmic reticulum; ORF, open reading frame; HPLC, high pressure liquid chromatography; PNGase F, peptide:*N*-glycosidase F; M-Pol, mannan polymerase; HA, hemagglutinin; PA, 2-aminopyridine; GOD, glucose oxidase; rGOD, recombinant glucose oxidase.

suppress an *och1* mutation (9). The *S. cerevisiae HOC1* gene product (ScHoc1p) was found to reside in the M-Pol II complex, but whether ScHoc1p directly contributes to the α-1,6-mannosyltransferase activity is not yet proven (2). At present, the function of ScHoc1p is unclear, although the *S. cerevisiae hoc1* mutants show phenotypes associated with glycosylation defects, including sensitivity to Calcofluor White and hygromycin B. ScHoc1p 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 ($Man_{8-12}GlcNAc_2$) and that they are mainly branched by α-1,2-linkages without hyper-immunogenic terminal α-1,3-linked mannose residues. More interestingly, the outer chains of *H. polymorpha N*-glycans were shown to have very short α-1,6-extensions, mainly composed of single α-1,6-linked mannose (14). To identify *H. polymorpha* genes coding for glycosyltransferases involved in the first step of α-1,6-linked mannose addition in *N*-linked outer chain biosynthesis, we isolated and characterized three *H. polymorpha* genes, *HpHOC1, HpOCH1,* and *HpOCR1* (*OCH1*-related), that share significant homology with *S. cerevisiae HOC1* and *OCH1* genes. Here we report that *HpOCH1* codes for the key enzyme responsible for initiating *N*-linked outer chain biosynthesis by adding the first α-1,6-mannose residue onto the core oligosaccharide, whereas the functions of *HpHOC1* and *HpOCR1* 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 och1*Δ (*MAT**a** leu2 ura3 trp1 ade2 his3 och1*Δ::*TRP1*) and *S. cerevisiae* TOY137 (*MAT**a** leu2 ura3 trp1 ade2 his3 och1*Δ::*hisG mnn1*Δ::*hisG mnn4*Δ::*hisG*) were derived from *S. cerevisiae* W303-1A.[3] The vector pDLMOX-GOD(H) (14) was used for the secretory expressions of *Aspergillus niger* glucose oxidase tagged with 6 residues of histidine (GOD-His) in *H. polymorpha*. pMOX-YPS1ct-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 saitoi* 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 NcoI restriction site), using pGAMH1 as a template (16). The resulting PCR product was digested with EcoRI and NcoI and used to substitute the 1.8-kb EcoRI/NcoI 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 peptide:*N*-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 an 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* (*HpOCH1*) 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 AY502025. The other members of *H. polymorpha OCH1* family, named *HpOCR2*,

[3] Y. Shimma, unpublished results.
[4] E. J. Kim and H. A. Kang, unpublished results.
[5] K. Nakayama and Y. Jigami, unpublished results.

HpOCR3, *HpOCR4*, and *HpOCR5*, were also isolated by PCR from *H. polymorpha* DL1-L genomic DNA using the primer sets, ORF288F/ORF288R, ORF580F/ORF580R, ORF100F/ORF100R, and ORF576F/ORF576R, respectively, which were designed based on information obtained from the whole genome sequence of *H. polymorpha*. The nucleotide sequences of the resulting PCR product were submitted to GenBank™ under accession numbers DQ249343, DQ249344, DQ249345, and DQ249346, respectively. The sequences of the primers used for the gene cloning are available on request.

Deletion of the H. polymorpha HOC1, OCR1, and OCH1 Genes—Deletional disruptions of the *HpHOC1*, *HpOCR1*, and *HpOCH1* genes were carried out using fusion PCR and *in vivo* recombination as described previously (18, 19) with slight modification. Briefly, in the fusion PCR stage, two DNA fragments containing the promoter (H1N) and terminator (H1C) regions of *HpHOC1*, respectively, were amplified by using two pairs of primers, HNfor/HNrev and HCfor/HCrev. The PCR primers HNrev and HCfor were 20-bp oligonucleotides and corresponded to the upstream and downstream sequences of the directed repeat region of the *HpURA3* pop-out cassette in plasmid pLacUR3.[6] The N- and C-terminal fragments (UR3N and UR3C) of the *HpURA3* pop-out cassette containing the overlapped internal sequence of *HpURA3* (100 bp) were amplified from pLacUR3 by PCR using two pairs of primers UNfor/UNrev and UCfor/UCrev. Subsequently, the UR3N and UR3C fragments were fused with the H1N and H1C fragments by PCR using two pairs of primers (HNfor/UNrev and UCfor/HCrev) to generate the fusion products HN-UR3N and HC-UR3C, respectively. In the *in vivo* recombination stage, these two PCR fusion products were introduced into *H. polymorpha* DL-LdU (*leu2 ura3*Δ::*lacZ*) and converted into one linear gene disruption cassette via *in vivo* homologous recombination at the overlapping sequence. Ura^+ colonies were selected and screened for the *Hphoc1*Δ::*HpURA3* strain, which was generated by the double homologous crossover of the disruption cassette at the *HpHOC1* gene locus. Gene disruption was confirmed by PCR and Southern hybridization analysis (data not shown). Disruptions of the genomic copies of *HpOCR1* and *HpOCH1* were performed using the same procedure. To construct double deletion *Hphoc1*Δ*Hpocr1*Δ or *Hphoc1*Δ*Hpoch1*Δ mutant strains, the *HpURA3* pop-out cassette was removed from the *Hphoc1*Δ::*HpURA3* strain to recover the Ura^- auxotrophic marker by cultivation on YPD plates containing 5-fluoroorotic acid (0.5 mg/ml), generating the DL-LdUdH1 (*leu2 ura3*Δ::*lacZ hoc1*Δ::*lacZ*) strain. Subsequent gene disruptions of *HpOCR1* and *HpOCH1* were performed in the DL-LdUdH1 strain using the same procedure described above. The sequences of the primers used in this study are available on request.

HPLC Analysis of N-Linked Oligosaccharides—*N*-Linked oligosaccharides were released from 200 μg of purified rGOD or HpYps1p by PNGase F treatment, and the oligosaccharides obtained were labeled with 2-aminopyridine (PA) using the commercially available reagent kit (Takara Shuzo Co., Shiga, Japan). After pyridylamination, the samples were purified using Sephadex G-15 spin columns (Amersham Biosciences) to remove residual PA. Size fractionation HPLC was performed using a Shodex Asahipak NH2P-50 column (Showa Denko K. K., Tokyo, Japan, 0.46 × 25 cm) at a flow rate of 1.0 ml/min (6). After sample injection, the proportion of solvent B was increased linearly up to 45% over 42 min. PA-oligosaccharides were detected by fluorescence (λ_{ex} = 320 nm and λ_{em} = 400 nm) using a Waters 2475 fluorescence detector. PA-oligosaccharides were digested sequentially with α-1,2-mannosidase (from *A. saitoi*, Seikagaku Corp., Tokyo) and α-1,6-mannosidase (from *Xanthomonas manihotis*, New England Biolab, Beverly, MA) according to the manufacturer's instructions.

Preparation of Membrane Proteins and α-1,6-Mannosyltransferase Activity Assay—Yeast membrane fractions were obtained as described previously (5). Yeast cells were cultivated in YPD medium and harvested at mid-logarithmic phase (A_{600} = 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 $MnCl_2$, 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 $MnCl_2$, 1 mM GDP-mannose, 0.5 mM 1-deoxymannojirimycin, and 100 pmol of $Man_8GlcNAc_2$-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 cutoff; Millipore, Bedford, MA), and the filtrate was submitted for HPLC.

O-Glycosylation Analysis of Chitinase—Native chitinases from supernatants of saturated cultures 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 Na_2HPO_4, 2 mM KH_2PO_4 (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 $Man_6GlcNAc_2$-PA at 37 °C for 30 min and analyzed by reversed-phase HPLC using a TSK-GEL ODS-80Ts column (Tosoh, Tokyo, Japan, 0.46 × 25 cm^3) 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 HpHOC1, HpOCR1, and HpOCH1 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 $Man_8GlcNAc_2$ in *H. polymorpha*, we had initially isolated two *H. polymorpha* genes, designated *HpHOC1* and *HpOCR1* (*OCH1*-related gene 1), using sequence information from random sequenced tags of *H. polymorpha* (22). Sequence analysis revealed that the *HpHOC1* gene product (HpHoc1p) showed 40 and 24% overall identities with the *S. cerevisiae HOC1* and *OCH1* gene products, respectively. On the other hand, the *HpOCR1* gene product (HpOcr1p) showed relatively low sequence identity with both the *ScHOC1* and *ScOCH1* gene products (21 and 22% identity, respectively). A hydropathy analysis performed using PSORT II (www.psort.org/; see Ref. 23) predicted that HpHoc1p and HpOcr1p

[6] J. H. Bae, unpublished observations.

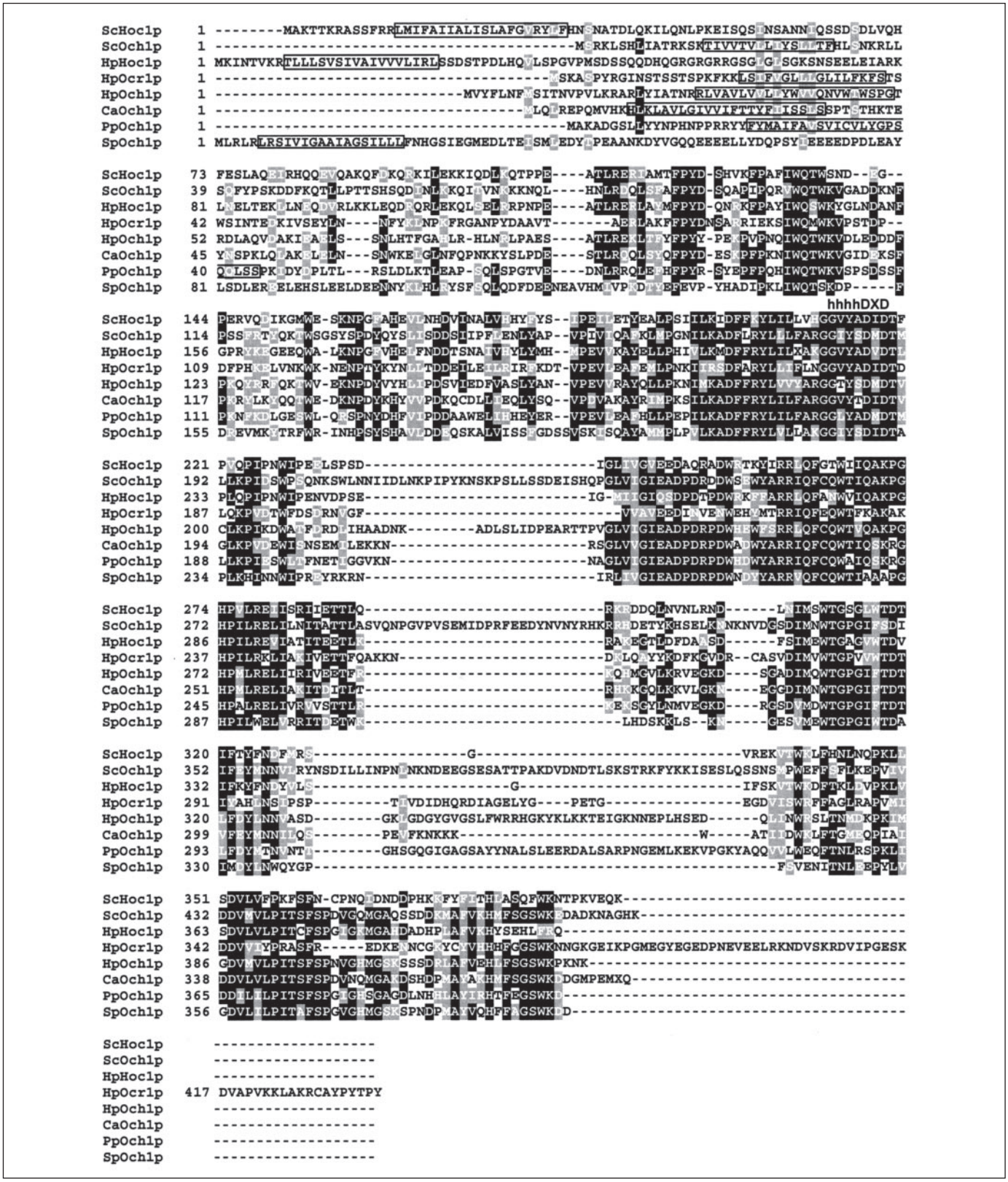

FIGURE 1. **Overall sequence comparison of Och1p homologues in yeast.** The amino acid sequences of gene products of *S. cerevisiae HOC1* (ScHoc1p, NCBI protein accession number NP_012609), *S. cerevisiae OCH1* (ScOch1p, NCBI protein accession number NP_011477), *H. polymorpha HOC1*, *OCR1*, and *OCH1* (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 D*X*D motif is shown above a sequence as *hhhhDXD*.

TABLE 1

Sequence identities and similarities between the Och1p homologs in *S. cerevisiae* and *H. polymorpha*

The degree of relatedness between protein pairs is shown as the percentage identity and similarity calculated from pairwise sequence alignments with gaps to maximize homology.

	ScHoc1p (396 aa)	ScOch1p (480 aa)	HpHoc1p (402 aa)	HpOcr1p (428 aa)	HpOch1p (436 aa)	HpOcr2p (414 aa)	HpOcr3p (362 aa)	HpOcr4p (425 aa)	HpOcr5p (369 aa)
ScHoc1p		20	40	21	23	18	19	18	17
ScOch1p	36		24	22	37	21	18	17	15
HpHoc1p	63	40		19	28	18	21	17	16
HpOcr1p	36	36	34		22	22	32	21	19
HpOch1p	41	54	45	39		21	21	20	17
HpOcr2p	35	36	34	40	40		21	51	33
HpOcr3p	34	36	35	48	39	40		20	19
HpOcr4p	34	33	33	37	36	66	38		32
HpOcr5p	30	31	30	33	32	50	33	47	

Identity (upper triangle) / **Similarity** (lower triangle)

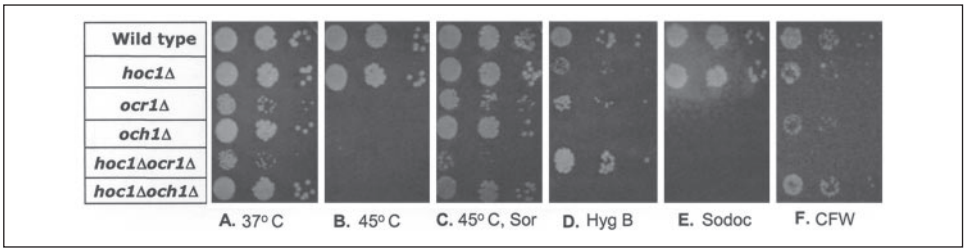

FIGURE 2. **Phenotypic analysis of the *Hphoc1*Δ, *Hpocr1*Δ, *Hpoch1*Δ, *Hphoc1*Δ*Hpocr1*Δ, and *Hphoc1*Δ*Hpoch1*Δ mutant strains.** Yeast cells were grown on YPD plates incubated at 37 °C (*A*) and 45 °C, (*B*) on YPD supplemented with 1 M sorbitol at 45 °C (*C*), on YPD supplemented with 40 μg/ml hygromycin B (*D*), 0.4% sodium deoxycholate (*E*), or 7 mg/ml Calcofluor White (*F*) 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.

possess a single potential transmembrane-spanning region, which acts as a signal-anchor domain (24) near the N terminus of the proteins, suggesting that they are type II membrane proteins. In addition, they possess a D*X*D motif well conserved in many glycosyltransferase families (25). An alignment of amino acid sequences of HpHoc1p and HpOcr1p with Och1p homologues from other yeast species reveals that several conserved regions are shared by Och1p homologues, although N-terminal portion is largely unique to each protein (Fig. 1). Most interestingly, HpOcr1p has a long C-terminal region that is absent in other Och1p homologues.

While we were analyzing the functions of *HpHOC1* and *HpOCR1*, the entire genome of *H. polymorpha* was completely sequenced (17). Thus we searched this *H. polymorpha* whole genome data base to identify other possible *H. polymorpha* ORFs showing significant similarity to the *S. cerevisiae OCH1* and *HOC1* genes. In addition to the ORFs identified as *HpHOC1* and *HpOCR1*, at least five other ORFs were found to encode putative mannosyltransferases with significant sequence identities to ScOch1p, ranging from 15 to 37% (Table 1). These additional ORFs were also predicted to have a potential transmembrane domain at the N-terminal region and a conserved region encompassing a D*X*D motif. Of these, an ORF with the highest homology to *ScOCH1* was designated *HpOCH1,* and its functions in cell growth and *N*-linked glycosylation were investigated.

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

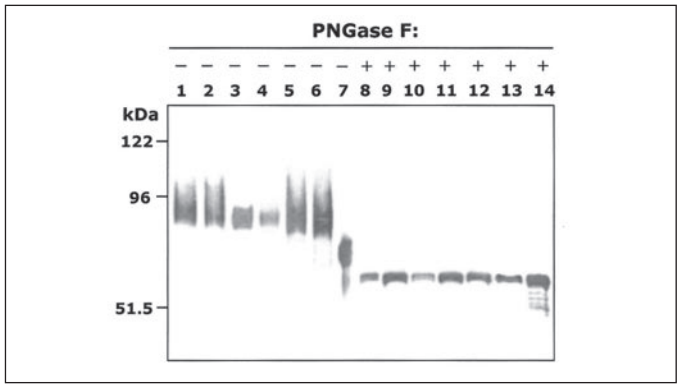

FIGURE 3. **Effects of the *HpHOC1*, *HpOCR1*, and *HpOCH1* mutations on protein *N*-glycosylation.** Western blot analysis of rGOD secreted from *H. polymorpha* wild type (*lanes 1* and *8*), *Hphoc1*Δ (*lanes 2* and *9*), *Hpocr1*Δ (*lanes 3* and *10*), *Hphoc1*Δ*Hpocr1*Δ (*lanes 4* and *11*), *Hpoch1*Δ (*lanes 5* and *12*), *Hphoc1*Δ*Hpoch1*Δ (*lanes 6* and *13*), and authentic GOD from *A. niger* (*lanes 7* and *14*). *Lanes 8–14*, culture supernatants treated with PNGase F.

results indicate that HpHoc1p, HpOcr1p, and HpOch1p are required for the maintenance of cell wall integrity, and that the function of HpHoc1p appears either to partially overlap or to be closely related with that of HpOcr1p. We could not obtain the double deletion strain of *HpOCR1* and *HpOCH1* even in the presence of osmotic stabilizers, possibly because of a severe growth defect in the absence of both genes.

The effect of the deletions of the *HpHOC1, HpOCR1,* and *HpOCH1* genes on protein *N*-glycosylation was preliminarily investigated by analyzing the electrophoretic mobility of a recombinant *A. niger* glucose oxidase (rGOD) protein secreted by *H. polymorpha* wild type, *Hphoc1*Δ, *Hpocr1*Δ, *Hphoc1*Δ*Hpocr1*Δ, *Hpoch1*Δ, and *Hphoc1*Δ*Hpoch1*Δ mutant strains (Fig. 3). The *Hphoc1*Δ mutant strain secreted heterogeneous forms of rGOD with high molecular weights similar to those of rGOD secreted by the wild type, whereas the *Hpocr1*Δ mutant strain produced much less heterogeneous forms of rGOD, which had a higher electrophoretic mobility than that of the wild type (Fig. 3, *lanes 1-3*). Slightly more homogeneous forms of rGOD were detected in the *Hphoc1*Δ*Hpocr1*Δ double mutant strain, although their mobility was still slower than that of authentic GOD from *A. niger* (Fig. 3, *lanes 4* and *7*). The electrophoretic mobility of rGOD secreted by the *Hpoch1*Δ mutant was also increased compared with rGOD from the wild type strain, even though a certain extent of heterogeneity still existed (Fig. 3, *lane 5*). No difference in the mobility of rGODs was detected between the *Hpoch1*Δ single and the *Hphoc1*Δ*Hpoch1*Δ double mutant strains (Fig. 3, *lane 5 versus lane 6*). These results suggest that the functions of HpOcr1p and HpOch1p are strongly associated with the *N*-linked glycosylation of rGOD, whereas HpHoc1p appears to play a minor role in mannose outer chain elongation.

*Structural Analysis of N-Linked Oligosaccharides Produced in the Hphoc1*Δ*, Hpocr1*Δ*, Hphoc1*Δ*Hpocr1*Δ*, and Hpoch1*Δ *Strains*—To gain more detailed information on the size and structure of *N*-glycans attached to rGOD expressed by the *Hphoc1*Δ, *Hpocr1*Δ, *Hphoc1*Δ*Hpocr1*Δ, or *Hpoch1*Δ strains, the *N*-linked oligosaccharides of rGOD secreted by these mutant strains were analyzed by size-fractionation HPLC (Fig. 4*A*). Comparisons of the oligosaccharide profiles of the wild type and of the deletion mutant strains revealed a dramatic reduction in the sizes of major oligosaccharides assembled on the proteins secreted by the mutant strains, except the *Hphoc1*Δ single deletion mutant strain. In the case of oligosaccharides derived from the wild type, four major peaks of $Man_{8-11}GlcNAc_2$ were detected with several minor peaks of $Man_{12-15}GlcNAc_2$ (Fig. 4*A*, *panel a*). An almost identical oligosaccharide profile to that of the wild type, but with a slight decrease in the portion of oligosaccharides larger than $Man_8GlcNAc_2$, was observed in the *Hphoc1*Δ single mutant (Fig. 4*A*, *panel d*). However, oligosaccharides from *Hpocr1*Δ, *Hphoc1*Δ*Hpocr1*Δ, or *Hpoch1*Δ mutant cells were mainly composed of a single predominating species, corresponding to the core-glycosylated form $Man_8GlcNAc_2$, although larger structures of $Man_{9-14}GlcNAc_2$ were present as minor components (Fig. 4*A*, *panels g, j,* and *m*). In the *Hphoc1*Δ*Hpocr1*Δ mutant strain, the relative proportions of the oligosaccharides larger than $Man_8GlcNAc_2$ appeared to be reduced *versus* the *Hpocr1*Δ strain. These results indicate that mannose outer chain elongation is significantly inhibited in the *Hpocr1*Δ, *Hphoc1*Δ*Hpocr1*Δ, and *Hpoch1*Δ mutant strains, although small fractions of oligosaccharides ($Man_{9-14}GlcNAc_2$) larger than $Man_8GlcNAc_2$ were observed.

To investigate in more detail the structure of the oligosaccharide species produced by the mutant strains, sequential digestion experiments with α-1,2- and α-1,6-mannosidases were carried out. After digestion of the oligosaccharides with α-1,2-mannosidase from *A. saitoi*, which is highly specific for nonreducing terminal α-1,2-mannose linkages, most of the oligosaccharides species from the wild type strain were shifted to two major species, *i.e.* $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$ (Fig. 4*A*, *panel b*). Subsequent digestion with α-1,6-mannosidase from *X. manihotis*, which is highly specific for terminal α-1,6-linked mannose residues that are linked to a nonbranched sugar, converted all the $Man_6GlcNAc_2$ oligosaccharide species generated by α-1,2-mannosidase digestion into $Man_5GlcNAc_2$ (Fig. 4*A*, *panel c*). Our previous study indicated that $Man_5GlcNAc_2$ (M5) is the final product of specific α-1,2-mannosidase digestion of the core oligosaccharide $Man_8GlcNAc_2$ or of the larger oligosaccharide species extended with only α-1,2-mannose linkages, whereas $Man_6GlcNAc_2$ (M6) oligosaccharide is the final product of the large oligosaccharides elongated by a single α-1,6-linked mannose addition and branched with a variable number of α-1,2-linked mannose units (14). After digestion with α-1,2-mannosidase, the oligosaccharides from the *Hphoc1*Δ, *Hpocr1*Δ, and *Hphoc1*Δ*Hpocr1*Δ strains were shifted to two major species, *i.e.* M5 and M6, like the wild type (Fig. 4*A*, *panels e*, *h,* and *k*). Moreover, subsequent digestion with α-1,6-mannosidase converted all M6 oligosaccharide species into M5 (Fig. 4*A*, *panels f, i,* and *l*). These results indicate that a significant portion of large oligosaccharides from *Hphoc1*Δ, *Hpocr1*Δ, and *Hphoc1*Δ*Hpocr1*Δ strains contain an additional α-1,6-linked mannose attached to the core oligosaccharide, which is believed to be mediated by the α-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*Δ and to *Hphoc1*Δ*Hpocr1*Δ cells, our results strongly suggest that the major roles of HpHoc1p and HpOcr1p are not in the addition of the first α-1,6-linked mannose. However, in contrast to the *N*-glycans from the wild type, *Hphoc1*Δ, *Hpocr1*Δ, and *Hphoc1*Δ*Hpocr1*Δ strains, all oligosaccharide species from the *Hpoch1*Δ strain were converted to M5 just after α-1,2-mannosidase treatment (Fig. 4*A*, *panel n*), indicating that the α-1,6-linked mannose addition to the core oligosaccharide was completely blocked in the *Hpoch1*Δ strain. This result strongly suggests that HpOch1p plays a key role as α-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, *HpYPS1* protein, which is a glycosylphosphatidylinositol-anchored aspartic protease with four potential *N*-linked glycosylation sites (Fig. 4*B*). The secreted form of HpYps1p was obtained by deleting a C-ter-

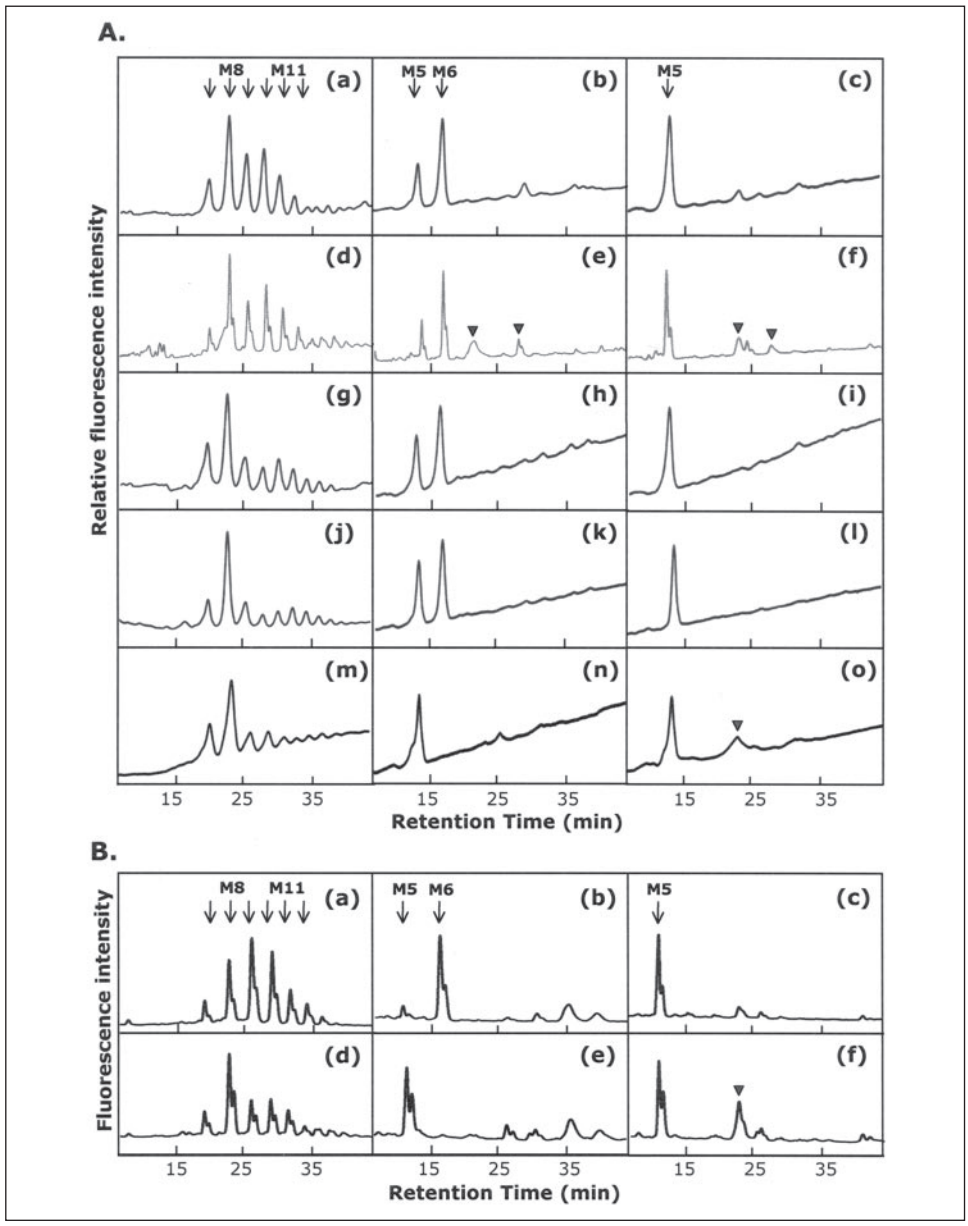

FIGURE 4. **HPLC analyses of *N*-linked oligosaccharides assembled on glycoproteins secreted by *H. polymorpha* mutant strains.** *A*, analysis of *N*-glycans attached to rGOD secreted by *H. polymorpha* wild type (*panels a–c*), *Hphoc1*Δ (*panels d–f*), *Hpocr1*Δ (*panels g–i*), *Hphoc1*Δ*Hpocr1*Δ (*panels j–l*), and *Hpoch1*Δ (*panels m–o*) strains. Chromatograms of the *N*-glycan profiles released from rGOD before any treatment (*panels a, d, g, j,* and *m*), after α-1,2-mannosidase treatment (*panels b, e, h, k,* and *n*), and after subsequent α-1,6-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 *Hpoch1*Δ (*panels d–f*) strains. Chromatogram of the *N*-glycans released from HpYps1p before any treatment (*panels a* and *d*), after α-1,2-mannosidase treatment (*panels b* and *e*), and after subsequent α-1,6-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 $Man_5GlcNAc_2$-PA (*M5*), $Man_6GlcNAc_2$-PA (*M6*), and $Man_8GlcNAc_2$-PA (*M8*) of known structure (indicated by *arrows*). The unidentified peak (indicated by *arrowhead*) is detected in some samples during the HPLC analysis. We observed that the peak is resistant to digestion by jack bean (α-1,2-, α-1,3-, and α-1,6-) 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 *Hpoch1*Δ 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 ($Man_{9-10}GlcNAc_2$) were present as predominant species attached to HpYps1p (Fig. 4*B, panel a*). After digestion with α-1,2-mannosidase, most of the oligosaccharide species were converted to M6, which was then completely converted to M5 by subsequent digestion with α-1,6-mannosidase (Fig. 4*B, panels b* and *c*). This indicates that most oligosaccharides assembled on HpYps1p contained a single α-1,6-linked mannose attached to the core oligosaccharide in the wild type strain. In contrast, in the *Hpoch1*Δ mutant strain, core form oligosaccharide $Man_8GlcNAc_2$ predominated; oligosaccharides larger than $Man_8GlcNAc_2$ were detected as minor fractions (Fig. 4*B, panel d*). Moreover, digestion with α-1,2-mannosidase converted all *Hpoch1*Δ strain-derived oligosaccharides to M5 (Fig. 4*B, panel e*). These results clearly indicate that a defective addition of α-1,6-mannose residue onto the core oligosaccharide $Man_8GlcNAc_2$ is a general phenotype of the *Hpoch1*Δ strain and not a protein-specific phenotype.

Involvement of HpOCR1 in O-Glycosylation—To examine whether *HpHOC1*, *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, *Hphoc1*Δ, *Hpocr1*Δ, *Hpoch1*Δ, *Hphoc1*Δ*Hpocr1*Δ, and *Hphoc1*Δ*Hpoch1*Δ mutant strains (Fig. 5). The *Hpoch1*Δ, *Hphoc1*Δ, and *Hphoc1*Δ*Hpoch1*Δ 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 *Hpoch1*Δ 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-

[7] E. J. Kim and H. A. Kang, unpublished results.

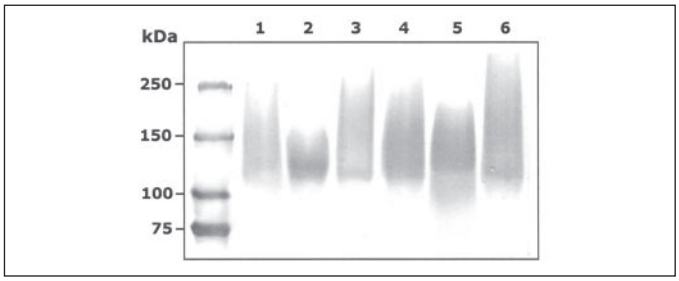

FIGURE 5. **SDS-PAGE analysis of endogenous chitinase secreted by *H. polymorpha* mutant strains.** Chitinases were isolated from the culture mediums of *H. polymorpha* wild type (*lane 1*), *Hpocr1*Δ (*lane 2*), *Hpoch1*Δ (*lane 3*), *Hphoc1*Δ (*lane 4*), *Hphoc1*Δ*Hpocr1*Δ (*lane 5*), and *Hphoc1*Δ*Hpoch1*Δ (*lane 6*) by binding to chitin and analyzed by SDS-PAGE, as described under "Experimental Procedures."

nificantly homogeneous forms of chitinase were detected in the *Hpocr1*Δ and *Hphoc1*Δ*Hpocr1*Δ 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 (*Scoch1*Δ) mutant with the plasmid YEp352GAPII-HpOCH1 carrying *HpOCH1* under the control of the *S. cerevisiae GAPDH* promoter. The *Scoch1*Δ mutant strain transformed with the null vector YEp352GAPII showed temperature sensitivity, but the *Scoch1*Δ strain transformed with YEp352GAPII-HpOCH1 grew well at 30 °C (Fig. 6*A, row 2 versus row 5*), indicating that the temperature-sensitive growth defect of *Scoch1*Δ was recovered by the expression of *HpOCH1*. In contrast, *Scoch1*Δ cells transformed with YEp352GAPII-HpHOC1 or YEp352GAPII-HpOCR1 displayed the same temperature-sensitive phenotype as the *Scoch1*Δ mutant (Fig. 6*A, rows 3* and *4*). Moreover, the electrophoretic mobility of invertase secreted from the *Scoch1*Δ cells transformed with the *HpOCH1* expression plasmid decreased to the same extent as that observed from the *Scoch1*Δ cells transformed with the *ScOCH1* expression plasmid (Fig. 6*B, lanes 5* and *6*). Together with the observed complementation of the temperature-sensitive growth defect, the restoration of the hyperglycosylation defect of *Scoch1*Δ 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 (*Scoch1*Δ*mnn1*Δ*mnn4*Δ), but harboring YEp352GAPII, YEp352GAPII-HpOCH1, or YEp352GAPII-ScOCH1 plasmids, and these were used as enzyme sources for α-1,6-mannosyltransferase assays (Fig. 6*C*). The *Scoch1*Δ*mnn1*Δ*mnn4*Δ strain was shown to be defective in adding mannose residues to *N*-linked core oligosaccharide (16). PA-labeled $Man_8GlcNAc_2$, 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, $Man_8GlcNAc_2$-PA (M8), was not converted into any other form by the membrane fraction of *Scoch1*Δ*mnn1*Δ*mnn4*Δ transformant harboring the null vector (Fig. 6*C, panel a*), a peak corresponding to $Man_9GlcNAc_2$-PA (M9) was detected as a reaction product in the membrane fraction of *Scoch1*Δ*mnn1*Δ*mnn4*Δ transformants harboring YEp352GAPII-HpOCH1 or YEp352GAPII-ScOCH1 (Fig. 6*C, panels b* and *c*). These results strongly indicate that *HpOCH1*, like *ScOCH1*, encodes the initiation-specific α-1,6-mannosyltransferase acting on the

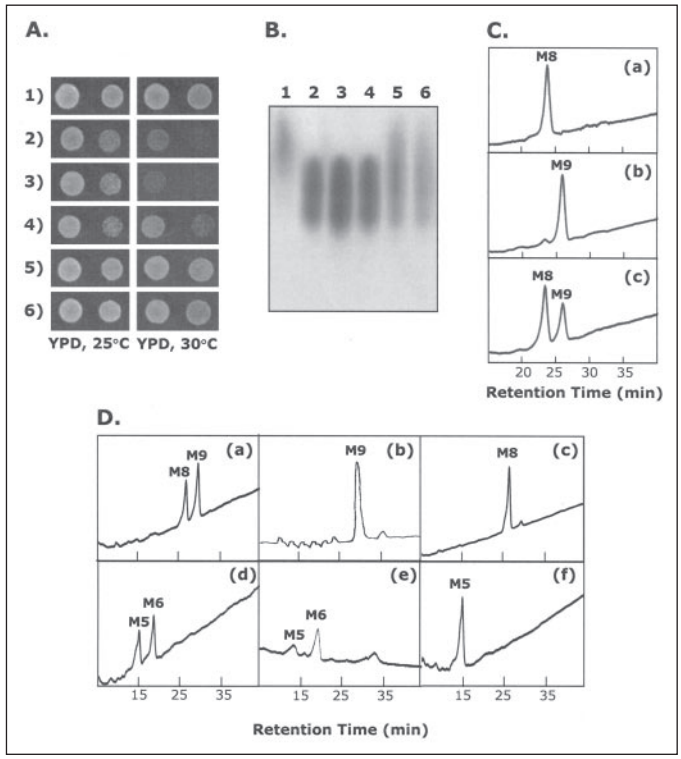

FIGURE 6. **Functional complementation and *in vitro* activity analysis of *H. polymorpha OCH1* gene product.** *A*, spotting analysis of growth phenotype. Yeast cultures were spotted onto YPD plates, which were then incubated at 25 and 30 °C for 3 days. *S. cerevisiae* wild type strain (*row 1*) and *Scoch1*Δ mutant strains transformed with the control vector YEp352GAPII (*row 2*), YEp352GAPII-HpHOC1 (*row 3*), YEp352GAPII-HpOCR1 (*row 4*), YEp352GAPII-HpOCH1 (*row 5*), and YEp352GAPII-ScOCH1 (*row 6*). *B*, activity staining of invertase, carried out by the method of Gabriel and Wang (35). Analyzed samples are in the same order as in *A*. *C*, analysis of α-1,6-mannosyltransferase activity in *S. cerevisiae* triple null strains *Scoch1*Δ*mnn1*Δ*mnn4*Δ carrying YEp352GAPII (*panel a*), YEp352GAPII-HpOCH1 (*panel b*), or YEp352GAPII-ScOCH1 (*panel c*). Solubilized membrane fractions were prepared as enzyme sources, and $Man_8GlcNAc_2$-PA was used as an acceptor. The reaction products were analyzed by HPLC. *D*, analysis of α-1,6-mannosyltransferase activity in *H. polymorpha* wild type, *Hpocr1*Δ, and *Hpoch1*Δ mutant strains. Reaction products by the membrane fractions of the wild type (*panel a*), *Hpocr1*Δ (*panel b*), and *Hpoch1*Δ mutant (*panel c*) strains, and the reaction products of (*panels a–c*) treated with α-1,2-mannosidase (*panels d–f*, respectively) were analyzed by HPLC.

core oligosaccharide. Solubilized membrane fractions prepared from the *H. polymorpha* wild type, *Hpocr1*Δ, and *Hpoch1*Δ mutant strains were also analyzed for α-1,6-mannosyltransferase activity (Fig. 6*D*). The M9 peak corresponding to $Man_9GlcNAc_2$-PA was generated by the reaction between the acceptor oligosaccharide and the membrane fractions from the wild type and *Hpocr1*Δ mutant strains but was hardly detected in the reaction with the membrane fraction of the *Hpoch1*Δ mutant (Fig. 6*D, panels a* and *b versus panel c*). After α-1,2-mannosidase digestion, the M9 product was converted to $Man_6GlcNAc_2$-PA (M6), whereas the M8 core oligosaccharide was converted to $Man_5GlcNAc_2$-PA (M5) (Fig. 6*D, 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 Hpoch1*Δ—The *Hpoch1*Δ 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 *msdS* gene cassette fused with the signal sequence

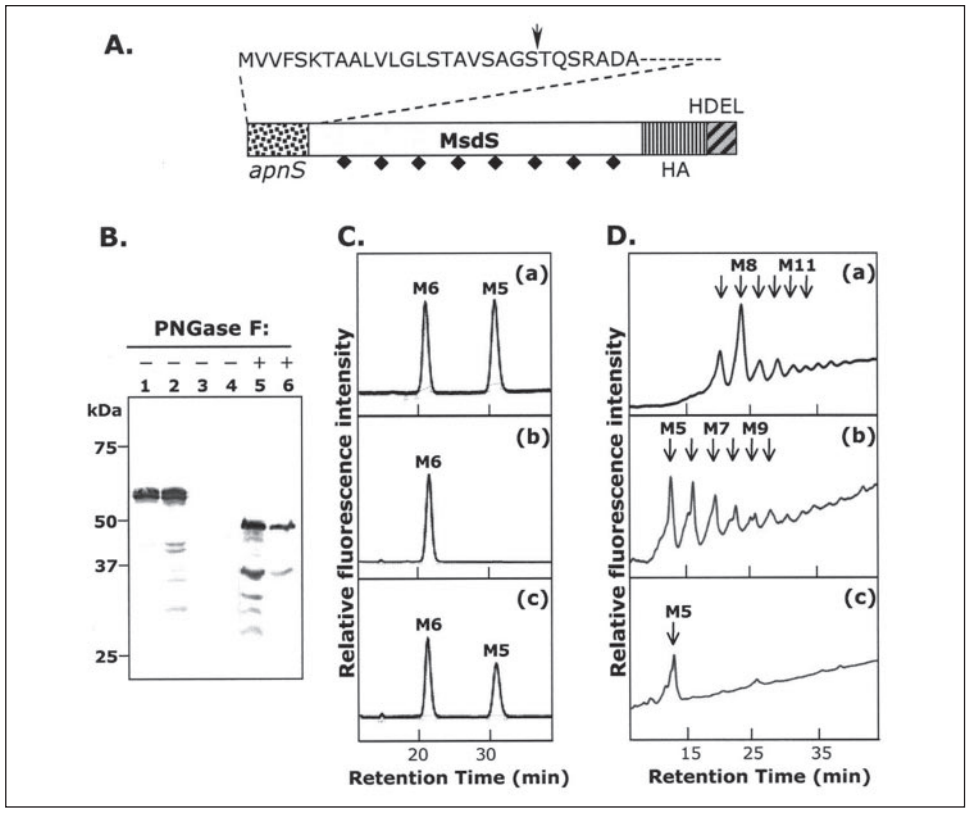

FIGURE 7. **Expression of ER-targeted α-1,2-mannosidase in *Hpoch1*Δ mutant strain.** *A*, construct of the gene cassette for *A. saitoi* α-1,2-mannosidase (MsdSp) tagged with the HA epitope and the ER retention signal HDEL. The signal sequence of aspergillopepsin I (*apnS*), which was used to direct secretion of MsdSp in yeast, and 8 potential *N*-linked glycosylation sites of MsdSp (36) are shown. The *arrowhead* indicates the fusion site between apnS and MsdSp. *B*, immunoblot analysis of *A. saitoi* α-1,2-mannosidase expressed in *H. polymorpha*. Total cell lysates (corresponding to 0.5 A_{600}, *lanes 1* and *2*) and the culture supernatant (16 μl, *lanes 3* and *4*) were fractionated on 10% polyacrylamide gel and analyzed using antibody raised against HA. *Lanes 5* and *6* contain cell lysates treated with PNGase F. *C, in vitro* assay for *A. saitoi* α-1,2-mannosidase activity in *H. polymorpha*. The standard $Man_6GlcNAc_2$-PA and $Man_5GlcNAc_2$-PA (*panel a*), the reaction products by the extracts prepared from *H. polymorpha* cells transformed with the null vector (*panel b*) and with the α-1,2-mannosidase-HA-HDEL expression plasmid (*panel c*) were analyzed by reversed-phase HPLC. *D*, *N*-glycan profiles of the *Hpoch1*Δ mutant strain expressing *A. saitoi* α-1,2-mannosidase. *N*-glycans obtained from the rGOD secreted by the *Hpoch1*Δ strain transformed with the null vector (*panel a*) and from *Hpoch1*Δ transformed with α-1,2-mannosidase-HA-HDEL expression plasmid (*panel b*) were analyzed by HPLC. The *N*-glycans of *b* were further treated with α-1,2-mannosidase (*panel c*).

of aspergillopepsin and the ER retention signal, HDEL (16), was further modified at its C terminus to contain the HA sequence to monitor its expression (Fig. 7*A*). 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. 7*B*). Cell extracts of recombinant *H. polymorpha* containing the α-1,2-mannosidase-HA-HDEL construct were able to convert $Man_6GlcNAc_2$-PA into $Man_5GlcNAc_2$-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 (Fig. 7*C*). The result 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 *Hpoch1*Δ mutant strain, we analyzed the structures of *N*-glycans on rGOD. Compared with the oligosaccharides synthesized in the *Hpoch1*Δ mutant without α-1,2-mannosidase-HA-HDEL, the oligosaccharides produced in the recombinant *Hpoch1*Δ mutant strain expressing the active α-1,2-mannosidase-HA-HDEL were much shorter in length (Fig. 7*D, panels a* and *b*). The fraction corresponding to $Man_5GlcNAc_2$, the smallest structure of human-compatible type high mannose oligosaccharide, was detected as a major component in the recombinant *Hpoch1*Δ 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 $Man_5GlcNAc_2$ after *in vitro* α-1,2-mannosidase treatment (Fig. 7*D, panel c*). The incomplete trimming to $Man_5GlcNAc_2$ in the recombinant *Hpoch1*Δ 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-mannosyltransferases in the Golgi might have subsequently added α-1,2-linked mannoses to $Man_5GlcNAc_2$, 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 $Man_8GlcNAc_2$ 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*, *HOC1*, 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 *HpHOC1, HpOCR1,* and *HpOCH1* 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*, *HOC*, *OCR*, and *SUR* groups. As expected from relatively high sequence homologies with those of *S. cerevisiae*, *HpOCH1* and *HpHOC1* are classified into *OCH* and *HOC* groups, respectively. Interestingly, the other members of the *H. polymorpha OCH1* gene family are grouped together remotely from the *OCH* and *HOC* groups, constituting their own subfamily (*HpOCR* group). The

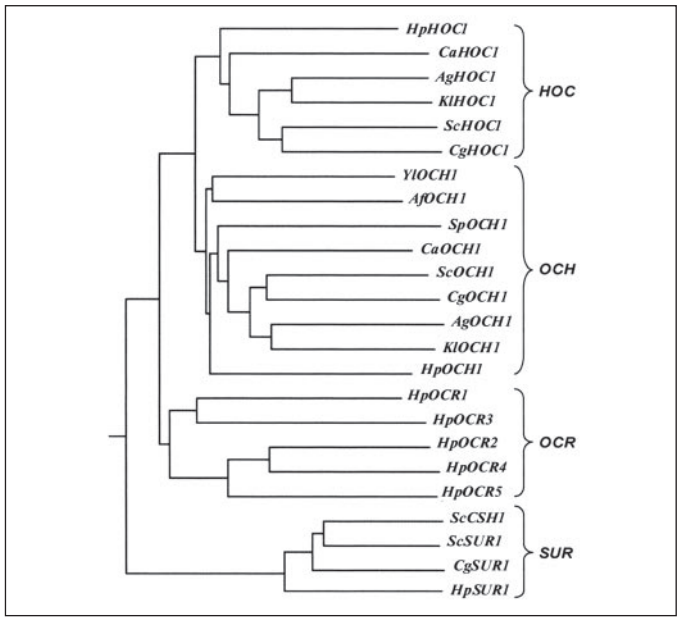

FIGURE 8. **Neighbor-joining phylogenetic tree of the *OCH1* family and homologues from yeasts and fungi.** The sequences of the *OCH1* family and homologues were collected by Blast searches with *S. cerevisiae OCH1* sequence. *C. albicans HOC1*, *OCH1* (*CaHOC1*, NCBI protein accession number XP_716693; *CaOCH1*, XP_716632), *Ashbya gossypii HOC1*, *OCH1* (*AgHOC1*, AAS53806; *AgOCH1*, AAS53836), *Kluveromyces lactis HOC1*, *OCH1* (*KlHOC1*, XP_452168; *KlOCH1*, XP_456072), *S. cerevisiae HOC1*, *OCH1*, *CSH1*, and *SUR1* (*ScHOC1*, NP_012609; *ScOCH1*, NP_011477; *ScCSH1*, NP_009719; *ScSUR1*, NP_015268), *Candida glabrata HOC1*, *OCH1*, and *SUR1* (*CgHOC1*, XP_445987; *CgOCH1*, XP_444841; *CgSUR1*, XP_449590), *Y. lipolytica OCH1* (*YlOCH1*, CAD91643), *Aspergillus fumigatus OCH1* (*AfOCH1*, XP_753779), *S. pombe OCH1* (*SpOCH1*, 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 hypersensitivities 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*Δ and *Hphoc1*Δ 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 HpOcr1 and HpOch1 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 *Hphoc1*Δ*Hpocr1*Δ double deletion mutant showed a more marked *N*-glycosylation defect than the single *Hpocr1*Δ mutant strain (Figs. 3 and 4), suggesting that HpHoc1p might be partly involved in *N*-glycosylation in a redundant fashion with HpOcr1p.

Although the *Hpocr1*Δ mutant strain displays apparent phenotypes quite similar to those of the *Scoch1*Δ 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-mannose addition to the core oligosaccharide was not impaired in the *Hpocr1*Δ mutant strain (Fig. 4*A, 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 *Hpocr1*Δ 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 *Hpoch1*Δ mutant strain were shown to be devoid of additional α-1,6-mannose residues attached on the core oligosaccharide $Man_8GlcNAc_2$ (Fig. 4, *A , panel n,* 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 $Man_8GlcNAc_2$. 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. 6*A*) and the hyperglycosylation defect (Fig. 6*B*) of *Scoch1*Δ 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 *Scoch1*Δ mutant (Fig. 6) and in *Hpoch1*Δ 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 (*YlOCH1*), which led to speculation that either *YlOCH1* 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 *Hpocr1*Δ 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 *Hpocr1*Δ 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 mannoses. However, little homology (below 8% sequence identity) exists between *HpOCR1* and *S. cerevisiae KTR* (Kre-Two-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* Mnn9p, 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.

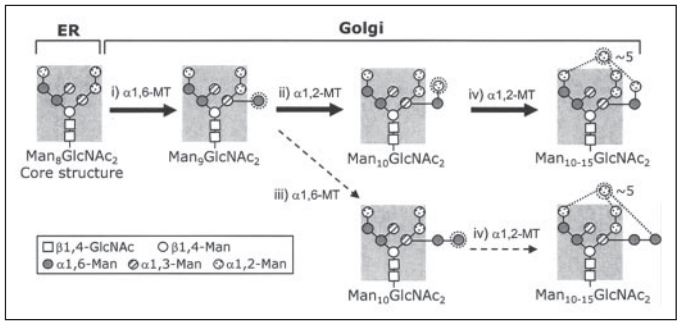

FIGURE 9. **A proposed pathway of the outer chain biosynthesis of *N*-linked oligosaccharides in *H. polymorpha*.** Core oligosaccharide $Man_8GlcNAc_2$, departing the ER, is extended with a single α-1,6-mannose by the *HpOCH1* gene product, an initiation specific α-1,6-mannosyltransferase (*i*), and then takes either of two pathways in the Golgi apparatus. The major pathway in *H. polymorpha* appears to involve the formation of core-type oligosaccharides by successive α-1,2-mannose addition on $Man_9GlcNAc_2$, which appears to be mediated by the *HpOCR1* gene product (*ii*) rather than the formation of mannan-type oligosaccharides with extensive α-1,6-mannose elongations, mediated by the *HpMNN9* gene product (*iii*). *H. polymorpha* outer chains are generally elongated only by α-1,2-mannose linkages without terminal α-1,3-linked mannose addition (*iv*). *α1,6-MT*, α-1,6-mannosyltransferase; *α1,2-MT,* α-1,2-mannosyltransferase.

As in the case of ScHoc1p (9), the function of HpHoc1p is unclear. The deletion of *HpHOC1* generates cell wall defect, which is characterized by the sensitivity to hygromycin B and Calcofluor White. However, *HpHOC1* could not complement the hypersensitivity of the *Schoc1*Δ strain to hygromycin B (data not shown). The deletion effect of *HpHOC1* on *N*-glycosylation became more manifest in the absence of HpOcr1p, indicating that the function of HpHoc1p 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 *HpHOC1* 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 *HpHOC1, HpOCR1*, and other members of the *HpOCR1* 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 $Man_8GlcNAc_2$ originating from a dolichol-linked $Glc_3Man_9GlcNAc_2$ is elongated at the α-1,3-branch by the addition of an α-1,6-linked mannose unit by an initiating α-1,6-mannosyltransferase, a process that is mainly catalyzed by HpOch1p. In *S. cerevisiae*, after the addition of the first α-1,6-mannose, the outer chain backbone is intensively elongated by the action of Mnn9p-containing M-Pol I and M-Pol II complexes, generating a mannan-type structure (2). *S. cerevisiae* strains mutated at *MNN9* show severe underglycosylation and osmotic fragility (33). However, most surprisingly, the deletion of the *MNN9* homologue in *H. polymorpha* (34) did not lead to a serious alteration in growth phenotype and caused no apparent hypermannosylation reduction,[8] indicating that the activity of HpMnn9p is not critical for outer chain elongation in *H. polymorpha*. This is in a good agreement with our previous report (14) and present data (Fig. 4) on the structures of *N*-glycans attached to the recombinant GOD, the cell wall mannoproteins, and the HpYps1 protein obtained from *H. polymorpha*, which reveal that the outer chains of *H. polymorpha N*-linked oligosaccharides have very short α-1,6-extensions and are mainly elongated by α-1,2-mannose addition. Therefore, in this methylotrophic yeast, the activities of α-1,2-mannosyltransferases appear to out-compete those of α-1,6-mannosyltransferases 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 terminal 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 *Hpoch1*Δ strain, despite its defect in outer chain initiation on the core glycan $Man_8GlcNAc$, did not show severe growth retardation under normal conditions, unlike the *Hpocr1*Δ and *Scoch1*Δ mutant strains, although it displayed a temperature-sensitive growth phenotype. Differing from the outer chains of *S. cerevisiae N*-glycan 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 *HpOCH1* deletion on cell growth of *H. polymorpha*. On the contrary, the deletion of *HpOCR1,* which generated dramatic defects in both *N*- and *O*-linked glycosylation, would cause severe growth retardation. The wild type comparable growth of the *Hpoch1*Δ 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 *Hpoch1*Δ strain with the targeted expression of *A. saitoi* α-1,2-mannosidase in the ER was able to synthesize $Man_5GlcNAc_2$ as a major *N*-glycan (Fig. 7*D*). 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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[8] S. Y. Kim, unpublished results.

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