Identification of Novel α,1,3-Galactosyltransferase and Elimination of α-Galactose-containing Glycans by Disruption of Multiple α-Galactosyltransferase Genes in Schizosaccharomyces pombe*

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The oligosaccharides from fission yeast Schizosaccharomyces pombe contain large amounts of D-galactose (Gal) in addition to α-linked oligosaccharides via α1,2- or α1,3-linkages. Previously we constructed and characterized a septuple α-galactosyltransferase disruptant (7GalTΔ) anticipating a complete lack of α-Gal residues. However, the 7GalTΔ strain still contained oligosaccharides consisting of α1,3-linked Gal residues, indicating the presence of at least one more additional unidentified α1,3-galactosyltransferase. In this study we searched for unidentified putative glycosyltransferases in S. pombe genome sequence and identified three novel genes, named otg1+–otg3+. α1,3-galactosyltransferases belong to glycosyltransferase gene family 8 in the Carbohydrate Active EnZymes (CAZY) database. Gal-recognizing lectin blotting and HPLC analyses of pyridylaminated oligosaccharides after deletion of these three additional genes from 7GalTΔ strain demonstrated that the resultant disruptant missing 10 α-galactosyltransferase genes, 10GalTΔ, exhibited a complete loss of galactosylation. In an in vitro galactosylation assay, the otg2+ gene product had Gal transfer activity toward a pyridylaminated Manα1,2GlcNAc2 oligosaccharide and pyridylaminated Manα1,2-Manα1,2-Man oligosaccharide. In addition, the otg3+ gene product exhibited Gal transfer activity toward the pyridylaminated Manα1,GlcNAc2 oligosaccharide. Generation of an α1,3-linkage was confirmed by HPLC analysis, α-galactosidase digestion analysis, 1H NMR spectroscopy, and LC-MS/MS analysis. These results indicate that Otg2p and Otg3p are involved in α1,3-galactosylation of S. pombe oligosaccharides.

The fission yeast Schizosaccharomyces pombe is a promising host for expression of therapeutic glycoproteins because it shares greater similarity to higher animals than the budding yeast Saccharomyces cerevisiae in regard to splicing mechanisms, cell division control, transcription-initiation mechanisms, and post-translational modifications (1, 2). To date our group has developed S. pombe protein-production systems that have been useful for producing many types of heterologous proteins from various organisms including humans (3). However, for the production of therapeutic glycoproteins intended for human use, yeasts including S. pombe are currently less useful due to their inability to modify proteins with human-compatible glycan structures. To overcome this problem, glycoengineering has been attempted in several yeast species (4). Recently, the secretory production of erythropoietin containing sialylated biantennary glycan was demonstrated in the methylotrophic yeast Pichia pastoris by deletion of yeast-specific glycosyltransferases and introduction of many other genes responsible for 1) processing of high mannose-type glycans to a precursor of complex-type glycan, 2) conversion of the complex-type precursor glycan to complex-type glycan, 3) human-like sugar nucleotide synthesis, and 4) their transport from the cytosol to the Golgi lumen (5). With the increasing importance of yeast species other than P. pastoris as alternative hosts for production of therapeutic glycoproteins, glycoengineering targeting human-compatible glycans has received significant attention in such species.

S. pombe N- and O-linked glycans contain large amounts of α-linked D-galactose (Gal) in addition to α-linked D-mannose.

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**TABLE 1**

List of *S. pombe* strains used in this study

| Strains | Genotype | Reference or source |
|---------|----------|---------------------|
| ARC039  | gms1Δ    | Asahi Glass Co. Ltd. |
| 7Ga1Δ   | h− leu1-2 ura4-C190T | (14) |
| 9GalΔ   | 7Ga1Δ gms1Δ gna1Δ gna2Δ gmh1Δ gmh2Δ gmh3Δ gmh4Δ gmh5Δ::ura4− gmh6Δ | (23) |
| 10GalΔ  | 9GalΔ::otg2-otg3::ura4− | This study |
| och1Δ   | 7GalΔ::otg2-otg3::ura4− | This study |
| 10GalΔ och1Δ | ARC039 och1::ura4− | (21) |
| otg1::otg2-otg3Δ | ARC039 och1::ura4− | This study |

(Man) unlike *S. cerevisiae* or *P. pastoris* (6). O-Linked oligosaccharides in *S. pombe* consist of Galβ1-2Manα1-3 structures, which have an α1,2-linked Man backbone decorated with α1,2-linked and/or α1,3-linked Gal residues (7). N-Linked oligosaccharides in *S. pombe* comprise a ManαNε1Nε2Manα1-3 structures, which have an α1,6-linked Man backbone decorated with α1,6-linked Gal residues (10). Some of these α1,2-linked Gal residues are further modified with pyruvylated β1,3-linked Gal residues (11–13). Such yeast-specific outer chain structures and the fission yeast-specific α-linked Gal residues may cause rapid removal of the corresponding glycoproteins from the blood stream or may provoke an immune response in humans (14) and, therefore, must be eliminated.

To this end we have been analyzing the precise glycan structures in *S. pombe* glycosylation mutants to allow use of this organism as an alternative glycoprotein-producing host (15–23). Although we have succeeded in completely eliminating both the outer chain structures and α-linked Gal residues by construction of a *gms1Δoch1Δ* deletion mutant, the *gms1*Δ gene encoding a Golgi-localized UDP-Gal transporter is required for β1,4-galactosylation processes that produce biantennary complex-type oligosaccharides in a subsequent humanization step in *S. pombe*. Recently, we constructed a septuple α-galactosyltransferase-related gene disruptant (7GalΔ) as an alternative approach for eliminating α-galactosylation and analyzed resultant glycan structures (23). Although the 7GalΔ strain was expected to have lost α-galactosylation, glycan structural analysis revealed that it still had α1,3-linked Gal residues, indicating the presence of unidentified α1,3-galactosyltransferase(s). Therefore, to construct the desired *S. pombe* galactosyltransferase null mutant, identification of the α1,3-galactosyltransferase(s) is required.

Here we describe the identification and characterization of the novel α1,3-galactosyltransferase genes. By searching for putative glycosyltransferases in the *S. pombe* genome, we found three novel uncharacterized genes, *otg1−otg3* (α one, three-galactosyltransferase). Disruption of these three genes in the 7GalΔ mutant resulted in a strain in which 10 α-galactosyltransferase genes had been deleted (10GalΔ) and caused a complete loss of α-galactosylation in its glycan. Moreover, *in vitro* enzymatic assay revealed that Otg2p had α-Gal transfer activity toward both N- and O-linked glycans, whereas Otg3p had α-Gal transfer activity toward N-glycans.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Genetic Methods**—The fission yeast strains used in this study are listed in Table 1. Standard rich medium (YES (yeast extract/supplement) and YPD (yeast extract/peptone/dextrose)) and synthetic minimal medium for growing *S. pombe* were used as described (24). *S. pombe* cells were transformed by the lithium acetate method (25, 26). Standard genetic methods have been described (27). *Escherichia coli* XL1-Blue (Stratagene) was used for all cloning procedures.

**Gene Disruptions**—In a previous study the 7GalΔotg1Δ strain (8GalΔ) had already been constructed because *otg1*Δ gene is located between *gmh1*Δ and *gmh2*Δ genes, and these genes were disrupted simultaneously (23). For construction of the 10GalΔ strain, the *S. pombe ura4Δ* marker was used for disruptions in the 8GalΔ strain was recovered by FOA treatment (28). Because *otg2*Δ and *otg3*Δ are located adjacent to each other and are about 3 kbp in length, they were disrupted simultaneously. The plasmid used to disrupt *otg2*Δ and *otg3*Δ was constructed as follows. A 3.8-kbp fragment carrying the *otg2*Δ and *otg3*Δ genes was amplified from chromosomal DNA by PCR using the Ex Taq DNA polymerase (Takara Co. Ltd., Kyoto, Japan) and the following oligonucleotides: sense (5’-GGTTCTTCGCCTGCTAATGTTGCTGTCGG-3’) and antisense (5’-CAGCACGAAGATAGGGTAAGTCCTCTTCGG-3’). The 3.8-kbp fragments were recovered and ligated into pGEM-T Easy vector. The HindIII–Xhol sites within the cloned *otg2*Δ−*otg3* fragment were digested, and a 1.6-kbp *ura4Δ* containing fragment was inserted.

The *otg1Δotg2Δotg3Δ* triple disruptant strain was constructed by replacing the internal *otg1*Δ gene and *otg2*Δ−*otg3*Δ fragments with the *ura4Δ* marker. For the *otg1*Δ locus, a DNA fragment carrying the *otg1*Δ gene was amplified from chromosomal DNA by PCR using Ex Taq DNA polymerase, and the following oligonucleotides were used: sense (5’-AAGCTTCTT- TCTGTGTGGTTCACACTAATTTGC-3’) and antisense (5’-TAGCTCTTTATTTGGCATTGTGGTTGTTGG-3’). A 3.0-kbp fragment was recovered and ligated into a pGEM-T Easy vector (Promega). Replacement of an internal *otg1*Δ gene fragment with *ura4Δ* marker was conducted by the in-fusion method (29). Preparation of a linearized pGEM-T Easy vector carrying only flanking regions of *otg1*Δ was performed by inverse PCR using PrimeSTAR HS DNA polymerase (Takara) and the following oligonucleotide primers, which contained overlapping 15-bp extensions homologous to regions on the

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2The abbreviations used are: 7GalΔ, septuple α-galactosyltransferase-related gene disruption mutant; 10GalΔ, disruptant missing 10 α-galactosyltransferase-related gene; hTF, human transferrin; PA, pyridylamino; PNA, peanut agglutinin; GT8, glycosyltransferase family 8.
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ura4+ marker fragment used to replace the otg1+ ORF: sense (5′-CTATAGTGCACCTCTATATTAAAGGATATAC-
C-3′) and antisense (5′-GTAGTGTACCTAACAGGAAG-
ATTATCTCAGG-3′). A DNA fragment carrying ura4+
marker fragment was amplified from a pGEM-T Easy vector
carrying the ura4+ marker by PCR using PrimeSTAR HS DNA
polymerase and the following primers: sense (5′-TGA-
TATAGTGTCACCTACTCATTAAAGGAGGAATAC-
3′) and antisense (5′-GTGAGTCGTATTACAGATAAGA-
3′). The two DNA fragments were then joined by an In-Fusion enzyme reac-
tion (Clontech).

The 9GalTΔ strain in which only Otg1p is present was con-
structed from the 10GalTΔ strain by using of integrating plas-
mid, pJK148-otg1+, as described (23). The 10GalTΔoch1Δ
strain was constructed from the 10GalTΔ strain by replacing an
internal och1+ fragment with the ura4+ marker as described
(30).

Plasmid Constructs.—To tag the C terminus of Otg1p-Otg3p
with green fluorescent protein (GFP), the otg1+–otg3+ ORFs
were amplified by PCR using PrimeSTAR DNA polymerase
(Takara), genomic DNA from S. pombe as template, and the
following primers with added restriction sites underlined: otg1+
5′-GGTTTCTCGAGATGCTCAATTTCCATCTTC-
3′ (XhoI) and 5′-GGTTTTGCGGCCGCTAGATTTCCATA-
CTTAGG-3′ (NotI); otg2+, 5′-GGTTTTCTCGAGATGGC-TT
TATTTATTGC-3′ (XhoI) and 5′-GGTTTTGCGGCCGCTA-
CATATAATCAGCATTGC-3′ (NotI); otg3+, 5′-GGTTTT-
AGATCTATGAATTTCTTTAAGGCGG-3′ (BgIII) and 5′-GT-
TTTTGCGGCCCTTCTACACATATGCAGT-3′ (NotI). PCR
products were digested with XhoI-NotI or BglII-NotI
and cloned into equivalent site of pREP1. The
otg1+, otg2+, and otg3+ fragments with the
otg1+–otg3+ ORF were amplified by PCR using PrimeSTAR HS DNA
polymerase and the following oligonucleotide primers: sense
5′-TAGGTTGAACATCTAGAAGAATCTCAGG-3′ and antisense
5′-CATATGAATTTCTTTAAGGCCG-3′. The two
DNA fragments were then joined by an In-Fusion enzyme reac-
tion (Clontech).

Preparation of Pyridylaminated (PA) Glycans from Cell Sur-
face Glycoproteins—Cells were cultivated in YES medium at
30 °C and harvested in early stationary phase. Cell surface glyco-
proteins were extracted by autoclaving at 121 °C for 90 min in
citrate buffer (20 mM citrate-NaOH, pH 7.0) followed by pre-
cipitation with methanol. The precipitates were dissolved in
hot water, dialyzed, and lyophilized. Oligosaccharides were le-
culated from galactomannoproteins by hydrazinolysis followed
by N-acetylation. The reducing ends of the liberated oligo-
saccharides were pyridylaminated as described (32, 33).

Glycan Structural Analysis.—Separation and structural iden-
tification of the PA-oligosaccharides were carried out using size-fractionation or reversed-phase high performance liquid chromatography as described (23).

Glycosidase Digestions—PA-oligosaccharides were digested with 250 milliunits of coffee bean α-galactosidase (Sigma) in 50
mM NaH2PO4-NaOH, pH 6.5, for 24 h at 30 °C.

Protein Assay.—The amount of protein was determined using BCA protein assay kit (Thermo Scientific, Rockford, IL)
according to the manufacturer’s instructions with bovine serum albumin as a standard.

Preparation of Solubilized Microsomal Proteins.—Solubilized
enzymes from various strains were prepared as described (39).
Briefly, cells were grown in minimal medium at 30 °C, har-
vested in early stationary phase, washed with ice-cold water,
and suspended in 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.25
m sucrose, and Complete EDTA-free protease inhibitor mixture
(Roche Applied Science). Cells were lysed with glass beads by
vortexing for 30 s and then holding on ice for 30 s for a total of
15 repeated cycles. Glass beads, unbroken cells, and large cell
debris were removed by centrifugation at 1000 × g at 4 °C. The
resultant supernatant was centrifuged at 100,000 × g for 1 h at
4 °C. The membrane pellet was resuspended in 50 mM Tris-
HCl, pH 7.4, 5 mM MgCl2, 0.25 m sucrose, and 2% Triton X-100
to a protein concentration of 4 mg/ml and incubated on ice for
30 min. The resultant mixture was then used as the source of
solubilized enzyme for the galactosyltransferase assay.
Galactosyltransferase Assay—The galactosyltransferase assay was carried out as described (39). The reaction mixture in a total volume of 30 μl contained 60 μg of solubilized enzyme, 100 mM HEPES-NaOH, pH 7.0, 0.1 M sucrose, 1 mM MnCl₂, 1 mM UDP-Gal, 10 pmol PA-oligosaccharides, and was incubated at 37 °C for 12 h. The reaction was terminated by boiling for 5 min. Reaction products were analyzed by size-fractionation HPLC.

NMR Spectroscopy—Sample was exchanged several times in D₂O followed by lyophilization. Finally, the lyophilized sample was dissolved in 35 μl of D₂O. The ¹H NMR spectrum was recorded on a Varian Inova-600 NMR spectrometer equipped with NANO probe (Agilent Technologies, Inc., Wilmington, DE).

Liquid Chromatography-Tandem Mass Spectrometry—LC-MS/MS was performed with an Agilent Technologies 1200 series instrument (Agilent Technologies, Santa Clara, CA) equipped with HCT plus software (Bruker Daltonics, Bremen, Germany) as described (49).

RESULTS

Identification of a Family of Putative S. pombe α,1,3-Galactosyltransferase Genes—We previously constructed a septuple α-galactosyltransf erase mutant and showed that α,1,3-linked Gal-containing oligosaccharides were still present, indicating an unidentified residual α,1,3-galactosyltransferase activity (23). To identify undiscovered α,1,3-galactosyltransferase gene(s), we searched for glycosyltransferases in the S. pombe genome sequence. Among the predicted glycosyltransferases, three genes (SPAC5H10.12c, SPBC4C3.08, and SPBC4C3.09, designated otg1⁺, otg2⁺, and otg3⁺ for α one, three-galactosyltransferase, respectively) with unknown functions were identified and exhibited 31–48% identity with each other at the amino acid level (Fig. 1A). The proteins encoded by the otg genes belong to glycosyltransferase family 8 (GT8) of the Carbohydrate-Active EnZymes (CAZy) database (35) and possess a DXD motif that is involved in divalent cation binding necessary for sugar-nucleotide binding. The Otg proteins also have the typical type II architecture of Golgi glycosyltransferases with the prediction of a small N-terminal cytoplasmic domain, a transmembrane domain, and a C-terminal catalytic domain. To predict the function of the genes, we conducted a BLASTp search that revealed greater amino acid sequence similarity of Otg proteins to many GT8 proteins, which included Arabidopsis myo-inositol α,1,3-galactosyltransferase (galactinol synthase) and E. coli lipopolysaccharide α,1,3-galactosyltransferase. Therefore, we speculated that these genes might be responsible for α,1,3-galactosylation in S. pombe. A phylogenetic analysis of these GT8 proteins indicated that the Otg proteins comprised a novel branch relative to GT8 proteins with known activities (Fig. 1B). This branch was relatively close to a branch consisting of pathogenic fungal putative GT8 proteins that may share similar enzymatic functions. These observations are consistent with the possibility that the Otg proteins are α,1,3-galactosyltransferases. To explore this directly, we disrupted the three genes from the septuple mutant to construct a strain lacking 10 presumptive α-galactosyltransferase genes (10GalΔ).

Phenotypic Characterization of the 10GalΔ Mutant—Light microscopic observation using Nomarski optics showed that wild-type cells displayed a normal cylindrical morphology. The morphology of 10GalΔ cells was an unusual in that the center swelled abnormally or was rounded, similar to gms1Δ and the 7GalΔ cells (Fig. 2A). The peanut agglutinin PNA, a lectin that recognizes the Gal residues, reacted with wild-type and 7GalΔ cells, whereas it did not react with 10GalΔ cells or with the gms1Δ cells, which served as a negative control (Fig. 2B). The 7GalΔ cells stained less intensely than wild-type cells, presumably due to the remaining α,1,3-linked Gal residues. In addition, the gms1Δ, the 7GalΔ, and the 10GalΔ cells were temperature- and hygromycin B-sensitive (Fig. 2C). Interestingly, 7GalΔ and 10GalΔ cells grew slower than gms1Δ cells on YES agar plates. The doubling times for the wild type and gms1Δ, the 7GalΔ, and 10GalΔ strains were 2.70, 4.03, 4.48, and 4.52 h, respectively.

Intracellular Localization of Otg Proteins—To determine the localization of the Otg proteins, we expressed C-terminal GFP fusion Otg in wild-type cells and observed fluorescence by fluorescence microscopy. Cells expressing Otg1-GFP, Otg2-GFP, and Otg3-GFP exhibited punctate fluorescent patterns, which are characteristic of Golgi localization and which overlapped with signals for the Golgi marker protein Gms1-RFP (Fig. 3) (17). However, some Otg-GFP signals did not appear to colocalize with Gms1-RFP. This might reflect differential intracellular dynamics of these proteins for Golgi retention as described in the case of Mnn9p or Vrg4p and Och1p (37, 38, 50). These results indicate that all three Otg-GFP fusion proteins localize to the Golgi apparatus.

Otgs Proteins Are Involved in α,1,3-Galactosylation of O- and N-Linked Glycans—To examine the effects of deleting the otg1⁺–otg3⁺ genes, the O-linked glycans were released from glycoproteins from wild-type, gms1Δ, 7GalΔ, and 10GalΔ cells, fluorescently labeled by 2-aminopyridine, and were analyzed by size-fractionation HPLC (Fig. 4). As shown previously, in the chromatograms of wild-type and 7GalΔ cells, peaks corresponding to Galα1,2(Galα1,3)Manα1,2Man-PA and Manα1,2(Galα1,3)Manα1,2Man-PA were detected near where tetra-oligosaccharides typically eluted, respectively, whereas no Gal-containing peaks were detected from gms1Δ cells (19, 21, 23). The 10GalΔ cells lacked α,1,3-Gal-containing tetra-oligosaccharide but contained mannotetraose as well as the gms1Δ cells. The O-glycan structures of otg1Δotg2Δotg3Δ triple disruption strain were also analyzed. In this strain, the peaks corresponding to tetrasaccharides containing an α,1,3-Gal residue, such as Galα1,2(Galα1,3)Manα1,2Man-PA or Manα1,2(Galα1, 3)Manα1,2Man-PA, were absent, but peaks corresponding to di- and tri-saccharides containing an α,1,2-Gal residue, such as Galα1,2Manα1,2Man-PA or Galα1,2Manα1,2Man-PA, were still present. These results indicate that Otg proteins are involved in α,1,3-galactosylation of O-linked glycans.

hTF, which is a secreted glycoprotein of ~80 kDa with 20 disulfide bonds and two N-glycosylation sites, was used as a heterologous protein with N-linked glycans. Western blot analysis of recombinant hTF secreted into the culture medium of wild-type cells showed a smeared band with a higher molecular weight.
mass, ~100–120 kDa, whereas that from gms1Δ cells migrated faster (Fig. 5A), presumably due to these glycosa-
ylation defects. The mobility of recombinant hTF from 10GalTΔ cells was almost the same as that from gms1Δ and och1Δ cells (Fig. 5A), indicating that the size of N-linked glycan on hTF from gms1Δ and 10GalTΔ cells was almost identical. The smaller bands below 80 kDa seen in gms1Δ, och1Δ, and 10GalTΔ cells were degradation products as described in the case of the recombinant human growth hormone (52). Silver staining after SDS-PAGE analysis was conducted as a loading control (Fig. 5B).

To analyze N-linked core glycans, we deleted the och1Δ gene from the 10GalTΔ strain to generate a 10GalTΔoch1Δ mutant and analyzed its N-linked glycans by HPLC (Fig. 5C). Major peaks ranging from Hexα1,5GlcNAc2-PA were detected in the 10GalTΔoch1Δ cells as well as in gms1Δoch1Δ cells but differed from the peaks observed in the 7GalTΔoch1Δ strain (Hexα1,5GlcNAc2-PA). These results indicate that Otg proteins are involved in α,1,3-galactosylation of both O-linked and N-linked glycans.

**Otg2p Exhibits in Vitro α,1,3-Galactosyltransferase Activity**

To assess enzymatic activity of Otg proteins, they were expressed in 10GalTΔ cells. Solubilized membrane preparations from 10GalTΔ cells containing the otg1Δ, otg2Δ, and otg3Δ expression plasmid pREP1-otg1Δ, pREP1-otg2Δ, and pREP1-otg3Δ, respectively, were used as the enzyme source. Assays carried out with UDP-Gal as the donor substrate revealed that Otg2p had galactosyltransferase activity toward Man9GlcNAc2-PA (Fig. 6A) and Man1,2Man1,2Man-PA (Fig. 6B) and that Otg3p had activity toward Manα1,3-Galactosyltransferase (Manα1,3-Galactosyltransferase (HumanGXYLT1 and HumanGXYLT2), and mammalian α,1,3-galactosyltransferases from rat, mouse, pig, and bovine (Rat, Mouse, Pig, and Bovine).
glycan structure by size-fractionation HPLC. HPLC profile of O-linked glycan structures in both of the 9GalT/H9004 and 10GalT/H9004 strains were quite similar (data not shown). Furthermore the proteins from the 9GalT/H9004 strains did not react with PNA (data not shown). These data suggest that Otg1p does not have galactosyltransferase activity. Furthermore, all Otg proteins were inactive toward Man-PA, Man/H92511,2Man-PA, and Gal/H92511,2Man-PA as acceptor substrates (data not shown). In addition, the linkage formed by Otg2p was investigated. The enzymatic product (fraction a in Fig. 6B) obtained from a reaction with Man/H92511,2Man/H92511,2Man-PA was collected and subjected to α-galactosidase digestion assays (data not shown). After α-galactosidase digestion, the elution position of fraction a shifted toward that of Manα-Manα1,2-Man-PA, indicating that the Gal residue was attached by an α-linkage. Further structural determination of the enzymatic product was carried out by 1H NMR spectroscopy (Fig. 6C) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Fig. 6D). Three anomeric signals at 5.16, 5.22, and 5.23 were detected in 1H NMR spectrum. The 1H NMR spectrum of the enzymatic product was not identical to that of Manα1,2-(Galα1,3-)Manα1,2-Man as described in the previous report (7), although we pre-
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**FIGURE 5. Structural analysis of N-linked glycans.** A, a Western blot analysis of secreted recombinant hTF is shown. After 60 h of cultivation, 5 ml of culture supernatant from wild-type, gms1Δ, och1Δ, and 10GalTΔ strains expressing hTF were precipitated with acetone (50%, v/v) and dissolved in SDS-PAGE sample buffer. The dissolved protein samples (corresponding to 200 μl culture medium in each lane) were subjected to 7% SDS-PAGE and analyzed by Western blot with anti-FLAG. B, SDS-PAGE analysis of secreted recombinant hTF (silver staining) is shown. The same samples used above for Western blot were analyzed by SDS-PAGE. Sample amounts applied to each lane corresponded to 4 ml of culture supernatant. C, N-linked glycans from gms1Δ, och1Δ, 7GalTΔ, och1Δ, and 10GalTΔ mutants were pyridydilaminated and analyzed by size-fractionation HPLC. The vertical bars and numbers on the chromatogram indicate the elution positions of the standards M5-, M6-, M7-, M8-, and M9-PA and Galα1,2Man, GlcNAcβ1,2-PA identified in och1Δ cells (20).

dicted that the Otg2p enzymatic product would be Manα1,2-(Galα1,3-)Manα1,2-Man. The signal at 5.16 could be assigned to a 3-substituted α1,2-linked Man residue with a downfield shift due to an α-Gal attachment (20). This appeared to be quite unlike the 2-substituted α1,2-linked Man residue, whose anomic signal was reported to be around 5.26 (7). The other two anomic signals (δ 5.22 and 5.23) are thought to correspond to 2-substituted α1,2-linked Man and the terminal α1,3-linked Gal residues, whose signals were reported to be detected around 5.26 and 5.28 (7), respectively. Although we attempted to determine these signal assignments by two-dimensional correlation spectroscopy analysis, no signals were found to correlate with the C2-H resonances (data not shown), likely due to limited amounts of the sample. The negative mode MS spectra indicated a precursor ion of m/z 743.7 corresponding to the calculated mass of Hex14-PA (m/z = 743.3) (data not shown). In LC-MS/MS analysis, the presence of the α3X2-H2O/Y3 ion at m/z 472.1 indicated a 2- or 3-substituted penultimate α1,2-linked Man. Furthermore, the E2 ion at m/z 305.0 allowed exclusion of a 1,2-substitution on the penultimate α1,2-linked Man. These results demonstrated that the terminal α-Gal residue was attached to the penultimate α1,2-linked Man by 1,3-linkage. From these results, the structure of the Otg2p product was deduced to be Galα1,3-Manα1,2-Manα1,2-Manα1,2-PA. Collectively, these results indicate that Otg2p acts on N- and O-linked glycans and that Otg3p acts on N-linked glycan (Fig. 7).

**DISCUSSION**

The glycans of *S. pombe* are known to contain large amounts of Gal residues attached to N- and O-linked glycans via α1,2- and α1,3-linkages (6). Although α1,2-galactosyltransferases have been identified and partially characterized (23, 39–41), before this study, none of the α1,3-galactosyltransferases had yet been identified or characterized. We first sought for genes responsible for α1,3-galactosylation in the *S. pombe* genome database to delete them from the 7GalTΔ strain that still contained α1,3-Gal residues in its glycans. Elimination of α1,3-Gal residues is required to produce human-type complex-type glycans in *S. pombe*. The genome-wide search detected only three genes (*otg1*, *otg2*, and *otg3*) of unknown function. These genes were originally annotated as *N*-acetylglucosaminyltransferases based on weak sequence similarity to *ScGNT1* (34). However, amino acid sequence alignment revealed that ScGnt1p had an extra unshared sequence and a relatively large number of amino acid residues that were not conserved in the SpOtg proteins (Fig. 1A). Furthermore, ScGnt1p were found to be phylogenetically distinct from the Otg proteins (Fig. 1B). Therefore, we assumed that the SpOtg proteins might not have *N*-acetylglucosaminyltransferase activity but other glycosyltransferase activities instead. The *otg* genes were then deleted from wild-type and from the 7GalTΔ strain to generate *otg1Δotg2Δotg3Δ* and 10GalTΔ, respectively, and glycan structures from the latter strains were analyzed (Figs. 4 and 5). The observed loss of α1,3-Gal residues suggested that at least one of the Otg proteins was involved in α1,3-galactosylation. The chromatogram of glycan structures from the *otg1Δotg2Δotg3Δ* mutant shows that α1,3-Gal-containing tetrasaccharides were completely eliminated. Although the residual peak could be an α1,2-Gal-containing glycan such as Galα1,2Manα1,2Manα1,2Man-PA, the precise structure has not been determined.

Mammalian-type α1,3-galactosyltransferases, which are involved in anti-Gal epitope and ABO blood group B synthesis and belong to the GT6 family have been well characterized (14). However, the mammalian-type α1,3-galactosyltransferases and SpOtg proteins share limited amino acid sequence similarity and comprise a distinct clade (Fig. 1B), suggesting that the SpOtg proteins comprise a new family. To determine whether the novel *S. pombe* Otg proteins have homologs in other organisms, BLASTp searches were conducted against public genome databases. Interestingly, homologous proteins were found to be putative GT8 proteins and mostly found among nematode-, plant-, or human-pathogenic fungal species (e.g. *Arthrobotrys oligospora*, *Leptosphaeria maculans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*) (Fig. 1B and Refs 42–45), suggesting that these species may synthesize glycan containing α1,3-Gal residues. However, to our knowledge, no reports have documented the presence of such residues in these species. It would be intriguing if such residues were related to pathogenicity.

We have been investigating mechanisms of galactosylation in *S. pombe*, which consists of UDP-Gal biosynthesis in the cytosol (46), its transport into the lumen of the Golgi apparatus (16), and the transfer of Gal residues to glycans by α-galactosyltransferases (23, 39–41). From our previous results, Gma12p, Gmh2p, and Gmh6p were shown to be involved in galactosylation of both of N- and O-linked glycans, and Gmh3p was shown to be involved in galactosylation of N-linked glycan. From our present in vitro enzymatic analysis, Otg2p and Otg3p have the galactosyltransferase activity toward M9-PA whereas Otg2p acts on Manα1,2-Manα1,2-Man-PA (Fig. 6). Analysis of the products of the enzymatic reactions using the combination of
reversed-phase HPLC, α-galactosidase digestion, $^1$H NMR analysis, and LC-MS/MS analysis (Fig. 6) indicated that Otg2p was able to transfer α1,3-Gal to Manα1,2-Manα1,2-Man-PA to generate Galα1,3-Manα1,2-Manα1,2-Man-PA (Fig. 6C). Glycans with a lone α1,3-Gal terminus have never been detected in *S. pombe* O-linked glycan. This might be because α1,2-galactosyltransferases such as Gma12p or putative α1,2-mannosyltransferases (presumably Omh proteins) immediately transfer the α1,2-Gal or Man residues onto this type of glycan after transfer of α1,3-Gal residues. Although we tested Man-PA, Manα1,2Man-PA, and Galα1,2Man-PA as a potential substrate, no Otg proteins were able to transfer α1,3-Gal to these PA-O-linked glycans, possibly due to structural differences between the PA-glycan and native glycan. For example, in these PA-O-linked glycans, the manno pyranose ring at the reducing ends are open due to pyridylamination, which might prevent the Otg enzymes from recognizing them and transferring the α1,3-Gal moiety to them. Among the Otg proteins, only Otg1p has never shown any enzymatic activities. Therefore, there are still possibilities that Otg1p has other additional glycosyltransferase activities other than α1,3-galactosyltransferase activity (e.g. N-acetylgalcosaminyltransferase activity). We are currently attempting to overexpress and purify soluble forms of the Otg proteins to better characterize their enzymatic activities.

**FIGURE 6. In vitro enzymatic assay of Otg proteins.** A, galactosyltransferase activity was assayed as described under “Experimental Procedures”. The size-fractionation HPLC profiles of the reaction mixtures using the acceptor substrate Man$_9$GlcNAc$_2$-PA and solubilized enzyme extracts from 10GalTΔ cells with empty vector pREP1 (10GalTΔ control) and 10GalT cells expressing pREP1-otg1$,^5$, pREP1-otg2$,^5$, or pREP1-otg3$^5$ are shown. The vertical bars and numbers on the chromatogram indicate the elution positions of the standards M9- and M10-PA. B, the size-fractionation HPLC profiles of the reaction mixtures using the acceptor substrate Manα1,2Manα1,2Man-PA and solubilized enzyme extracts from 10GalTΔ cells with empty vector pREP1 (10GalTΔ control) and 10GalT cells expressing pREP1-otg1$,^5$, pREP1-otg2$,^5$, or pREP1-otg3$^5$ are shown. The vertical bars on the chromatograms indicate the elution positions of the oligosaccharide standard peaks as described in the Fig. 4 legend. C, $^1$H NMR spectrum of purified fraction a by reversed-phase HPLC is shown. D, a LC-MS/MS spectrum of purified fraction a (see Ref. 51 for nomenclature) is shown.
It has been reported that α-galactosylation in *S. pombe* is required for sexual and nonsexual flocculation (18, 47, 48). The mating process was significantly affected in the *gms1Δ* cells during nutritional starvation because cells were incapable of sexual flocculation (18). Recently the *gfs2* gene, encoding a flocculin that binds to Gal residues located on cell surface glycans, was identified (48). Nonsexual flocculation and filamentous invasive growth was tightly controlled by the *gms1* protein (47, 48). The 1,3-galactosyltransferases will provide a more complete understanding of galactosylation pathways in *S. pombe* and how to manage them for glycoengineering therapeutics and industrial purposes.

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