Characterization of two different types of UDP-glucose/-galactose 4-epimerase involved in galactosylation in fission yeast

Shotaro Suzuki,¹ Tomohiko Matsuzawa,¹ Yayoi Nukigi,¹ Kaoru Takegawa² and Naotaka Tanaka¹

¹Department of Applied Biological Sciences, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan
²Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Fukuoka 812-8581, Japan

Schizosaccharomyces species are currently the only known organisms with two types of genes encoding UDP-glucose/-galactose 4-epimerase, uge1⁺ and gal10⁺. A strain deleted for uge1⁺ exhibited a severe galactosylation defect and a decrease in activity and in UDP-galactose content when grown in glucose-rich medium (2 % glucose), indicating that Uge1p is a major UDP-glucose/-galactose 4-epimerase under these growth conditions. In contrast, gal10⁺ was efficiently expressed and involved in galactosylation of cell-surface proteins in low-glucose medium (0.1 % glucose and 2 % glycerol), but not in galactose-containing medium. In a uge1⁰gal10Δ strain, the galactosylation defect was suppressed and UDP-galactose content restored to wild-type levels in galactose-containing medium. Disruption of gal7⁺, encoding galactose-1-phosphate uridylyltransferase, in the uge1⁰gal10Δ strain reversed suppression of the galactosylation defect and reduced levels of UDP-galactose, indicating that galactose is transported from the medium to the cytosol and is converted into UDP-galactose via galactose 1-phosphate by Gal7p in Sch. pombe.

INTRODUCTION

Glycoproteins in the fission yeast Schizosaccharomyces pombe contain a large amount of galactose in addition to both N- and O-linked mannan (Manners & Meyer, 1977; Moreno et al., 1985; Ikeda et al., 2009; Ohashi et al., 2009), indicating that Sch. pombe is equipped with mechanisms for glycoprotein galactosylation like animal cells. Research has demonstrated that the outer chain structure of galactomannan has an α-1,2-linked galactose or pyruvylated galactose attached to a poly-α-1,6-linked mannose backbone (Gemmill & Trimble, 1998), and that the pyruvylated galactose epitope bears remarkable structural resemblance to the N-acetylneuraminic acid-linked galactose epitope found in mammalian cells (Gemmill & Trimble, 1996). These galactose residues are enzymically modified by galactosyltransferases using UDP-galactose as substrate (Andreishcheva et al., 2004; Chappell et al., 1994; Yoko-o et al., 1998). UDP-galactose is transported to the Golgi lumen from the cytosol by the Golgi-localized UDP-galactose transporter (Gms1p) (Tabuchi et al., 1997; Tanaka & Takegawa, 2001). In Sch. pombe, the precise manner in which UDP-galactose is synthesized in the cytosol has been unclear. In the cytosol, the key enzyme for UDP-galactose synthesis is UDP-galactose/-glucose 4-epimerase, which catalyses the interconversion of UDP-galactose and UDP-δ-glucose. Interestingly, Schizosaccharomyces species have two types of UDP-glucose/-galactose 4-epimerase, while other organisms have only one. It is not known why two types of epimerase are needed and how they are regulated in Sch. pombe.

In Saccharomyces cerevisiae, Gal10p is the sole UDP-glucose/-galactose 4-epimerase, which consists of a UDP-glucose/-galactose 4-epimerase domain and a galactose mutarotase domain (Majumdar et al., 2004; Scott & Timson, 2007; Thoden & Holden, 2005), and which catalyses mutarotation of α-galactose to β-galactose. Expression of the GAL10 gene is regulated by Gal4p, a DNA-binding transcription factor required for activation of the GAL genes in cells growing on galactose as sole carbon source (De Robichon-Szulmajster, 1958; Platt & Reece, 1998; Lohr et al., 1995). In contrast to Sch. pombe, UDP-galactose is not used as a glycosylation substrate by glycosyltransferases because secreted glycoproteins are mannosylated in Sac. cerevisiae. Therefore, in Sac. cerevisiae, UDP-galactose and UDP-glucose/-galactose 4-epimerase function solely in galactose metabolism.

Abbreviations: HRP-PNA, horseradish peroxidase-conjugated peanut lectin.

Received 4 October 2009
Revised 19 November 2009
Accepted 24 November 2009
The metabolism of galactose in most organisms occurs via the Leloir pathway (Frey, 1996; Holden et al., 2003), comprising galactokinase (EC 2.7.1.6), galactose-1-phosphate uridyltransferase (EC 2.7.7.12) and UDP-glucose-galactose 4-epimerase (EC 5.1.3.2) (see Fig. 7b). In the first step of this pathway, cytosolic α-D-galactose is phosphorylated by ATP-dependent galactokinase to yield galactose 1-phosphate (Gal-1-P). Next, Gal-1-P uridyltransferase transfers UMP from UDP-glucose (UDP-Glc) to Gal-1-P via a two-step ping-pong mechanism, releasing glucose 1-phosphate (Glc-1P) and forming UDP-galactose. Finally, NAD⁺-dependent UDP-galactose-4-epimerase converts UDP-galactose to UDP-Glc. The glucose 1-phosphate is used in glycolysis and the UDP-galactose is utilized in mammals for galactosylation of glycoproteins.

In Sch. pombe, the metabolism of galactose is poorly understood because cells are not able to grow on galactose as a sole carbon source. This phenotype makes it difficult to analyse the Leloir pathway, despite the fact that genes encoding galactokinase, Gal-1-P uridylyltransferase and UDP-glucose-galactose epimerase are present in the Sch. pombe genome.

In the present study, we describe the role of two types of UDP-galactose-glucose 4-epimerase in Sch. pombe, Uge1p and Gal10p.

### METHODS

#### Strains, medium and genetic methods.

The wild-type Sch. pombe strain ARCO39 (h+ leu1-32 ura4-C190T) was used. The gms1A strain was constructed as described by Tabuchi et al. (1997). Standard rich medium (YES) and synthetic minimal medium (MM) for growing Sch. pombe were used as described by Moreno et al. (1991). Sch. pombe cells were transformed by the lithium acetate method or by electroporation (Morita & Takegawa, 2004; Okazaki et al., 1990; Suga & Hatakeyama, 2001; Suga et al., 2000). Escherichia coli XL1-blue (Strategene) was used for all cloning procedures. Standard genetic methods have been used for all cloning procedures (Ahfa et al., 1993).

#### Gene disruptions.

The uge1+ locus (SPBC365.14) was disrupted in the wild-type Sch. pombe strain by replacing an internal uge1+ gene fragment with the Sch. pombe uge1+ gene. To amplify the uge1+ gene by PCR, the following oligonucleotides were used: sense, 5'-GTAGTGGCATGAGCTGTGGTG-3', and antisense, 5'-ACCTAGCTGCTGAGCCG-3'. A 1.97 kb fragment was recovered and ligated into pGEM-T Easy vector (Promega). A HindIII site within the cloned uge1+ open reading frame was digested and a 1.6 kb urad+ gene was inserted. To disrupt the gal10+ locus (SPBBB2B2.12C), the following oligonucleotides were used: sense, 5'-CATCTGGTGTCATTTTCTGGTGCCC-3', and antisense, 5'-GACAAAAAGATGTCAATGCCTGGG-3'. A HindIII site within the cloned gal10+ open reading frame was digested and the urad+ gene was inserted. To disrupt the gal7+ locus (SPBBB2B2.10C), the following oligonucleotides were used: sense, 5'-GTAGAAGCC-3', and antisense, 5'-GGAGCTT-3'. A HindIII site within the cloned gal7+ open reading frame was digested and the urad+ gene was inserted. Linearized DNA fragments carrying the disrupted uge1+, gal10+ and gal7+ genes, respectively, were used to transform the wild-type haploid ARCO39 strain, and urad+ transformants were selected. To confirm that the corresponding individual genes had been disrupted, urad+ transformants were analysed by Southern blotting and PCR to verify correct integration of the deletion constructs.

#### Lectin staining.

Horseradish peroxidase-conjugated peanut lectin (HRP-PNA) was used to detect galactose residues on the cell surface as described previously (Takegawa et al., 1996). Briefly, cells were grown to stationary phase in YES or MM at 30 °C. Harvested cells were washed twice with distilled water and then resuspended in distilled water. Cells were fractured using glass beads to yield a crude protein extract. Extracted protein was spotted onto filter paper and dried. The filters were then washed twice in 10 mM Tris/HCl buffer (pH 7.4), 0.15 M NaCl and 0.05 % Tween 20 at room temperature for 5 min, followed by addition of HRP-PNA at room temperature and incubation for 1 h with continuous shaking. The filter paper was incubated in 0.03 % 3,3'-diaminobenzidine and 0.003 % H2O2 in PBS until spots were visible.

#### Acid phosphatase staining.

Glycosylation of acid phosphatase was analysed as described by Huang & Snider (1995), with minor modifications. Briefly, cells were grown in 5 ml MM medium to mid-exponential phase at 30 °C. To induce acid phosphatase, cells were centrifuged, washed and resuspended in 5 ml phosphate-free MM medium, and incubated for 12 h at 30 °C. Cells were then collected by centrifugation, washed once in 62.5 mM Tris/HCl (pH 6.8), and suspended in 240 μl lysis buffer (62.5 mM Tris/HCl pH 6.8, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol and 10 % glycerol, pH 6.8). A cell lysate were prepared using 0.5 mm glass beads in a Mini BeadBeater (BioSpec Products). The lysate was recovered and centrifuged at 15,000 g for 10 min at 4 °C, and the supernatant was loaded onto a 6 % native polyacrylamide gel. Electrophoresis and staining of acid phosphatase activity were performed as described by Schweingruber et al. (1986).

#### Northern-blot analysis.

Total RNA from cultures with an OD600 of 0.8–1.0 was extracted by the glass bead method described above (Sambrook et al., 1989). An aliquot (20 μg) of total RNA was separated on a 1 % (w/v) agarose gel containing 20 % formaldehyde. Subsequently, RNA was blotted onto Hybond-N membranes in 10 × SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and hybridized with gene-specific probes. The probes were obtained by PCR using the following primers. 5'-GGTTGTATTTGGTGTAGAGGC-3' and 5'-CCAGGTTCTAATGTTGACG-3', constitutive endogenous control); 5'-GCGAGGTGTCTATCATGTCGACTCGG-3' and 5'-TTACCT-ATTGTCCTGTATGTTGAGCCTG-3' for uge1+, and 5'-AATTTAAGACGACTAAATGCTATT-3' and 5'-TTAATGTTAATCTCCACATTTGATGACG-3' for gal10+. These primers amplified 502 bp probes, which were subsequently labelled using the AlkPhos Direct Labelling and Detection system (GE Healthcare).

#### Assay of epimerase activity.

Cells were cultured to the exponential phase in MM. A sample (100 OD600 units) of cells was resuspended in 600 μl ice-cold-buffer consisting of 0.1 M glycine/KOH (pH 8.7) and 2 mM EDTA. To lead to glucose repression of the cells, the cells were collected by centrifugation, and incubated for 20 min in 0.1 % glucose (2 % galactose) as the carbon source. Epimerase was extracted by the glass beads method from the resuspended cells. Epimerase activity was measured by a two-step assay (Brahma & Bhattacharyya, 2004). Briefly, extracted epimerase (4 μl) and 0.5 mM UDP-galactose (1 μl) were mixed with 75 μl ice-cold buffer. The mixture was then incubated for 30 min at 30 °C and boiled for 10 min, after which ice-cold-buffer containing 18 mM NAD⁺ and 12 millilitres UDP-glycoside dehydrogenase (Sigma) was added. A30 values were measured immediately for half of the sample volume (zero time) and for the other half volume after 30 min at 30 °C. One unit of UDP-galactose-4-epimerase catalyses the reaction.
conversion of 1 µmol UDP-galactose to UDP-glucose, and thus the formation of 2 µmol NADPH, per minute. Each nanomole of UDP-galactose converted to UDP-glucose corresponds to an \( A_{340} \) change of 0.02.

**Analysis of UDP-galactose levels.** Samples for analysis of UDP-galactose were prepared as described by Huang & Snider (1995). The UDP-galactose level was measured with the Amplex Red Galactose/Galactose Oxidase Assay kit (Invitrogen) according to the manufacturer’s instructions.

**Plasmid constructs.** For overexpression of gal10\(^+\), the cDNA was amplified by PCR using the following primers: 5'-GGTTCCATATGG-CGGTATACGGGAATAACA-3' (including a Nde I site) and 5'-GTTTGGATCCATATAGTATTCTCCACTA-3' (including a BamHI site). Derivatives of the thiamine-repressible expression vectors pREP1 and pREP41 were used to overexpress gal10\(^+\) (Nakamura et al., 2001).

**RESULTS**

**Sch. pombe has two types of UDP-glucose/-galactose 4-epimerase**

Proteins that shared significant homology with human GALE and with Sac. cerevisiae GAL10 were identified through BLAST searches of the Sch. pombe genome and were designated uge1\(^+\) (SPBC365.14c) and gal10\(^+\) (SPBPB2B2.12c), respectively (Fig. 1, Table 1). The Uge1 protein was characterized by the presence of only an epimerase domain. GALE and Sch. pombe Uge1p are similar in size (348 and 355 aa, respectively) and share more than 50% amino acid sequence identity (Fig. 1). On the other hand, the Gal10 protein was characterized by the presence of a mutarotase domain fused to the epimerase domain, which were also similar in size (699 and 713 aa, respectively) and also shared more than 50% amino acid sequence identity with Sc Gal10p (Fig. 1). Table 1 shows UDP-glucose/-galactose epimerase homologues from various organisms. It is interesting to note that only Schizosaccharomyces species have two types of UDP-glucose/-galactose 4-epimerase, while other yeast species have only the Sc Gal10 type (epimerase and mutarotase domains). This might be related to the fact that unlike other yeast species, Schizosaccharomyces species secrete galactomannoproteins and possess galactosylation mechanisms mediated by galactosyltransferases, and have UDP-galactose transporters (Chappell et al., 1994; Ohashi et al., 2009; Tabuchi et al., 1997; Umeda et al., 2000). Therefore, like mammalian cells, Schizosaccharomyces species must produce UDP-galactose constitutively from UDP-glucose via UDP-glucose/-galactose 4-epimerase as a substrate for galactosyltransferases and UDP-galactose transporters.

**A uge1Δ strain exhibits a severe galactosylation defect in rich medium**

In order to determine which UDP-glucose/-galactose epimerases are functional in Sch. pombe, we constructed uge1\(^+\) and gal10\(^+\) gene disruptants. To confirm the extent of the glycosylation defect, we examined acid phosphatase as a representative highly glycosylated glycoprotein (Dibenedetto & Cozzani, 1975), whose migration on native gel electrophoresis depends on the size and number of N-linked glycans (Schweingruber et al., 1986; Tanaka et al., 2001). The analysis revealed that acid phosphatase prepared from the uge1Δ strain migrated faster than that from wild-type strains, but similarly to that from a gms1Δ strain lacking the UDP-galactose transporter and which is known to contain secreted glycoproteins lacking galactose (Fig. 2a) (Tanaka et al., 2001). Acid phosphatase from the gal10Δ strain had almost the same mobility as that from the wild-type strain, while that from the uge1Δgal10Δ disruptant migrated similarly to acid phosphatase from the gms1Δ strain. This result indicates that the oligosaccharide content is reduced in acid phosphatase prepared from the uge1Δ strain. To analyse the effect of cell-surface galactosylation, galactose-specific HRP-PNA staining was used to detect quantitative differences in the galactosylation of cell-surface proteins. Like the gms1Δ mutant, neither the uge1Δ strain nor the uge1Δgal10Δ disruptant migrated similarly to acid phosphatase from the gms1Δ strain. This result indicates that the oligosaccharide content is reduced in acid phosphatase prepared from the uge1Δ strain. To analyse the effect of cell-surface galactosylation, galactose-specific HRP-PNA staining was used to detect quantitative differences in the galactosylation of cell-surface proteins. Like the gms1Δ mutant, neither the uge1Δ nor the uge1Δgal10Δ strain reacted with PNA (Fig. 2b). In addition, the uge1Δ and the uge1Δgal10Δ strains exhibited sensitivity to hygromycin B (Fig. 2c), which is an aminoglycoside antibiotic used for isolating glycosylation mutants (Dean, 1995; Takegawa et al., 1996; Yoko-o et al., 2001). These results suggest that Uge1p is the predominant enzyme involved in galactosylation of glycoproteins in Sch. pombe.

**gal10\(^+\) is efficiently expressed in low-glucose medium but not in galactose-containing medium**

To test UDP-glucose/-galactose epimerase activity in the disruptants, the two-step assay was used (Brahma & Bhattacharyya, 2004) (Fig. 3a). In glucose-rich medium (2% glucose), the uge1Δ and the uge1Δgal10Δ double mutant had about 20% activity compared to that of wild-type. On the other hand, epimerase activity of the gal10Δ mutant showed little change. These results indicate that Uge1p is responsible for most epimerase activity in rich medium containing 2% glucose.
In *Sac. cerevisiae*, transcription of *GAL10* is regulated by the Gal4p transcription factor, a positive regulator of gene expression for galactose-induced genes such as *GAL1*, *GAL2*, *GAL7*, *GAL10* and *MEL1*, which play important roles in galactose metabolism (Hashimoto *et al.*, 1983; Keegan *et al.*, 1986; Lohr *et al.*, 1995). Gal4p recognizes a 17 bp sequence (5′-CGGRNNRCYNYNCNCCG-3′) in the upstream activating sequence (UAS<sub>GAL</sub>) of these genes (Lohr *et al.*, 1995; Fedor & Kornberg, 1989). Interestingly, the *Sch. pombe* genome lacks a gal4<sup>+</sup> homologue, and the upstream sequences of *uge1<sup>+</sup>* and *gal10<sup>+</sup>* also lack a typical UAS<sub>GAL</sub> as far as we can ascertain. Therefore, we checked the mRNA levels of *uge1<sup>+</sup>* and *gal10<sup>+</sup>* in cells growing in galactose-containing medium (Fig. 3b). Northern analysis indicated the presence of *uge1<sup>+</sup>* mRNA in cells growing in 2% glucose medium, but no increase was observed in cells grown in medium containing 2% glucose and 2% galactose. These results indicate that *uge1<sup>+</sup>* was expressed constitutively and was not induced by galactose. On the other hand, *gal10<sup>+</sup>* mRNA was expressed weakly regardless of the presence of galactose. These findings indicate that *uge1<sup>+</sup>* encodes a major UDP-glucose/-galactose epimerase in cells growing in rich medium containing 2% glucose.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Name of homologue (and accession number)</th>
<th>Epimerase domain only</th>
<th>Epimerase + mutarotase domain</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td></td>
<td>Gal10 (NP_009575)</td>
<td></td>
</tr>
<tr>
<td><em>Vanderwaltozyma polyspora</em> DSM 70294</td>
<td></td>
<td>Hydrophilic protein (XP_00164714)</td>
<td></td>
</tr>
<tr>
<td><em>Lachancea thermotolerans</em> CBS 6340</td>
<td></td>
<td>Hydrophilic protein (XP_00255154)</td>
<td></td>
</tr>
<tr>
<td><em>Zygosaccharomyces rouxii</em> CBS 732</td>
<td></td>
<td>Hydrophilic protein (XP_00249455)</td>
<td></td>
</tr>
<tr>
<td><em>Kluyveromyces lactis</em> NRRR Y-1140</td>
<td></td>
<td>Gall10 bimuralatase protein (XP_00545642)</td>
<td></td>
</tr>
<tr>
<td><em>Candida tropicalis</em> MYA-3404</td>
<td></td>
<td>Gall10 bimuralatase protein (XP_002550320)</td>
<td></td>
</tr>
<tr>
<td><em>Candida dubliniensis</em> CD36</td>
<td></td>
<td>Gall10 bimuralatase protein (XP_002416873)</td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em> SC5314</td>
<td></td>
<td>Gall10 (XP_001383537)</td>
<td></td>
</tr>
<tr>
<td><em>Debaryomyces hansenii</em> CBS767</td>
<td></td>
<td>Hydrophilic protein (XP_057787)</td>
<td></td>
</tr>
<tr>
<td><em>Pichia guilliermondii</em> ATCC 6260</td>
<td></td>
<td>Hydrophilic protein (XP_001482100)</td>
<td></td>
</tr>
<tr>
<td><em>Pichia stipitis</em> CBS 6054</td>
<td></td>
<td>Gall10 (XP_001383357)</td>
<td></td>
</tr>
<tr>
<td><em>Lodderomyces elongisporus</em> NRRR YB-4239</td>
<td></td>
<td>Gall10 bimuralatase protein (XP_001526820)</td>
<td></td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td></td>
<td>Uge1 (NP_596043)</td>
<td>Gall10 bimuralatase protein (XP_001526820)</td>
</tr>
<tr>
<td><em>Schizosaccharomyces japonicus</em> yFS275</td>
<td></td>
<td>Hydrophilic protein (XP_00273024)</td>
<td>Hydrophilic protein (XP_002174949)</td>
</tr>
<tr>
<td><em>Schizosaccharomyces octopus</em> yFS286</td>
<td></td>
<td>Hydrophilic protein (SOCH_04717.2)</td>
<td>Hydrophilic protein (SOCH_02640.2)</td>
</tr>
<tr>
<td><em>Yarrowia lipolytica</em> CLIB122</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Botryotinia fuckeliana</em> B05.10</td>
<td></td>
<td>Hydrophilic protein (XP_001550259)</td>
<td></td>
</tr>
<tr>
<td><em>Sclerotinia sclerotiorum</em> 1980</td>
<td></td>
<td>Gal10 (XP_000590586)</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td></td>
<td>Hydrophilic protein (XP_001401007)</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus terreus</em> NIH2624</td>
<td></td>
<td>UDP-glucose 4-epimerase (XP_001212625)</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em> AT935</td>
<td></td>
<td>UDP-glucose 4-epimerase (XP_753568)</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em> RIB40</td>
<td></td>
<td>Hydrophilic protein (XP_001827449)</td>
<td></td>
</tr>
<tr>
<td><em>Talaromyces stipitatus</em> ATCC 10500</td>
<td></td>
<td>UDP-glucose 4-epimerase (XP_002479094)</td>
<td></td>
</tr>
<tr>
<td><em>Magnaporthe grisea</em> 70-15</td>
<td></td>
<td>Hydrophilic protein (XP_362429)</td>
<td></td>
</tr>
<tr>
<td><em>Penicillium marneffei</em> ATCC 18224</td>
<td></td>
<td>UDP-glucose 4-epimerase (XP_002146810)</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus clavatus</em> NRRR 1</td>
<td></td>
<td>UDP-glucose 4-epimerase (XP_001274365)</td>
<td></td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em> Wisconsin 54-1255</td>
<td></td>
<td>UDP-glucose 4-epimerase (XP_002560870)</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em> FGSC A4</td>
<td></td>
<td>Hydrophilic protein (XP_662331)</td>
<td></td>
</tr>
<tr>
<td><em>Neurospora crassa</em> OR74A</td>
<td></td>
<td>Gall10 bimuralatase protein (XP_957519)</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium cellulolyticum</em> H10</td>
<td></td>
<td>UDP-glucose 4-epimerase (YP_002504732)</td>
<td></td>
</tr>
<tr>
<td><em>Arthrobacter aurescens</em> TC1</td>
<td></td>
<td>UDP-glucose 4-epimerase (YP_948505)</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> subsp. subtilis strain 168</td>
<td></td>
<td>UDP-glucose 4-epimerase (NP_391765)</td>
<td>UDP-glucose 4-epimerase (ACA78806)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 8739</td>
<td></td>
<td>UDP-glucose 4-epimerase (ACA78806)</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em> strain Challis substrain CH1</td>
<td></td>
<td>UDP-glucose 4-epimerase (XP_001450301)</td>
<td></td>
</tr>
<tr>
<td><em>Dictyostelium discoideum</em> AX4</td>
<td></td>
<td>UDP-glucose 4-epimerase (XP_643834)</td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td></td>
<td>UGE1 (NP_172738)</td>
<td></td>
</tr>
<tr>
<td><em>Oryza sativa</em></td>
<td></td>
<td>Os09g032000 (NP_001062869)</td>
<td></td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td></td>
<td>GALE (NP_542961)</td>
<td></td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td></td>
<td>GALE (NP_848476)</td>
<td></td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td></td>
<td>GALE (NP_00108217)</td>
<td></td>
</tr>
</tbody>
</table>
In most organisms, glucose plays important regulatory roles in the expression of many genes, including those encoding proteins that function in respiration and glycolysis, utilization of alternative carbon sources, glucose transporters, and others (Johnston, 1999; Rolland et al., 2001, 2002). In Sch. pombe, however, these control mechanisms have not been analysed in detail (Tanaka et al., 1998; Hoffman, 2005; Kig et al., 2005), especially for enzymes needed for utilization of galactose as an alternative carbon source. Interestingly, Sch. pombe is not able to use galactose as a sole carbon source. Therefore, we tested whether uge1+ mRNA and gal10+ mRNA were induced in glucose-repressed (0.1% glucose, 2% galactose) medium. uge1+ mRNA was detected at the same level as in cells growing in 2% glucose medium, indicating that uge1+ mRNA is expressed constitutively and is not induced as a consequence of glucose repression (Fig. 3b). gal10+ mRNA was detected at the same level as uge1+ mRNA in cells grown in 0.1% glucose, 2% galactose medium, indicating that gal10+ is induced during glucose repression (Fig. 3b).

We next tested whether the UDP-glucose-/galactose epimerase activity in the uge1Δ strain was elevated in cells grown in the 0.1% glucose/2% galactose medium. The uge1Δ strain grown in 0.1% glucose, 2% galactose medium showed a rise of UDP-glucose-/galactose epimerase activity compared to the wild-type strain grown in 2% glucose medium.

Fig. 3. Enzymic activity of UDP-glucose-/galactose 4-epimerase and expression level of uge1+ and gal10+. (a) UDP-glucose-/galactose 4-epimerase activity was observed in each disruptant. Wild-type and mutant strains were cultured in MM containing 2% glucose or 0.1% glucose and 2% galactose as carbon sources. UDP-glucose 4-epimerase was extracted from the cultures and activity was monitored by measuring A340. Values are means ± SEM, n=3. (b) Northern blot analysis of uge1+ and gal10+ mRNA. Total RNA was extracted from the wild-type cells grown in glucose-containing (2% glucose) medium, or in medium containing both 2% glucose and 2% galactose, or under glucose-repressing (0.1% glucose, 2% galactose) conditions. Each RNA sample (20 μg) was separated on agarose gel in the presence of formamide, blotted onto a nylon membrane and hybridized to uge1+, gal10+ and leu1+ probes.
ase activity while no detectable increase in activity was observed in the uge1Δgal10Δ strain. These results indicate that gal10Δ is efficiently expressed in cells grown in 0.1% glucose medium and Gal10p has UDP-glucose/-galactose epimerase activity in Sch. pombe.

**Under glucose-repressing conditions, Gal10p is involved in galactosylation of cell-surface proteins**

We demonstrated that the uge1Δ and the uge1Δgal10Δ strains were not reactive with HRP-PNA and were sensitive to hygromycin B (Fig. 2b, c) due to insufficient galactosylation of secreted cell-surface glycoproteins. To verify the role of Gal10p in galactosylation, we confirmed suppression of the galactosylation defect in the uge1Δ strain under glucose-repressing conditions (Fig. 4). The hygromycin B sensitivity of the uge1Δ strain was overcome under preculture conditions in YES (0.1% Glc, 2% glycerol) preculture medium, but not in YES (2% Glc) preculture medium (Fig. 4a). Further, the HRP-PNA staining showed that galactosylation of the cell surface of the uge1Δ mutant was restored in the YES (0.1% Glc, 2% glycerol) medium (Fig. 4b). These results indicate that Gal10p can replace loss of Uge1p in the uge1Δ mutant in YES (0.1% Glc, 2% glycerol) medium, which is consistent with the activity (Fig. 3a) and expression (Fig. 3b) data.

In complementary studies, we next investigated the effect of gal10Δ overexpression on galactosylation in the uge1Δ strain (Fig. 5) using the pREP1 plasmid containing the thiamine-repressible wild-type promoter (Maundrell, 1990). The induced level is about 80× greater than the repressed level. pREP41 has a 6× lower induced level and a 15× lower repressed level than the wild-type promoter (Basi et al., 1993; Maundrell, 1993; Forsburg & Rhind, 2006). As expected, HRP-PNA staining showed that galactosylation of the cell surface of the uge1Δ strain was restored in an expression-dependent manner (Fig. 5a), as was the amount of acid phosphatase-associated oligosaccharides (Fig. 5b).

![Fig. 4. Growth and galactosylation phenotypes of uge1Δ and gal10Δ strains in glucose-repressing medium.](http://mic.sgmjournals.org)

(a) Wild-type and each disruptant were pre-cultured to exponential phase in YES medium containing 2% glucose or 0.1% glucose and 2% glycerol. Cells were then streaked onto YES plates (0.1% glucose, 2% glycerol and 20 μg HB ml⁻¹), and incubated for 3 days at 30 °C. (b) The dot-spot HRP-PNA staining assay. Wild-type and each disruptant were cultured to exponential phase in YES medium containing 2% glucose or 0.1% glucose and 2% glycerol. Each cell lysate was stained with HRP-PNA.

![Fig. 5. Phenotype of the uge1Δ strain overexpressing gal10+ under the nmt promoters.](http://mic.sgmjournals.org)

Each strain was incubated in MM containing 2% glucose for 16 h. The uge1Δ strain overexpressed gal10+ either under the pREP1 or pREP41 nmt promoters. Cell lysates of each strain were stained with HRP-PNA (a), and subjected to gel electrophoresis followed by staining for acid phosphatase activity (b).
Gal7p is associated with UDP-galactose biosynthesis in Sch. pombe

As documented above, Sch. pombe cannot grow on galactose-containing medium with galactose as sole carbon source. This phenotype has greatly complicated analysis of the Leloir pathway in this yeast. Therefore, we wanted to determine whether galactose was transported from the medium to the cytosol and whether it was converted into UDP-galactose via Gal-1-P. The uge1Δgal10Δ strain is useful for assessing this pathway in Sch. pombe. If galactosylation of the cell surface is recovered on galactose-containing medium in the uge1Δgal10Δ strain, this indicates that the galactose residues in the glycoproteins were synthesized using UDP-galactose formed from Gal-1-P by Gal-1-P uridylyltransferase. Fig. 6(a) shows that HRP-PNA staining of the uge1Δ and uge1Δgal10Δ strains was indeed restored in the galactose-containing medium.

In Sch. pombe, a search for homologue(s) of Sac. cerevisiae GAL7, which encodes Gal-1-P uridylyltransferase, identified SPBPB2B2.10c, designated gal7+ in this study. The predicted amino acid sequences of the Sc GAL7 and gal7+ gene products share 63 % identity. To examine whether gal7+ is involved in the recovery of HRP-PNA staining, we constructed a uge1Δgal10Δgal7Δ triple disruptant (Fig. 6a). HRP-PNA staining was not observed in either the uge1Δgal10Δgal7Δ strain or the uge1Δgal10Δgms1Δ triple disruptant in galactose-containing medium. These results indicate that galactose is transported into the cytosol from the medium and is converted into UDP-galactose via Gal-1-P by Gal7p in Sch. pombe.

We next determined the UDP-galactose content of the cytosol in these strains (Fig. 6b). All the disruptants except gal10Δ had reduced levels of cytosolic UDP-galactose when grown in 2 % glucose medium. After incubation in medium containing 1 % galactose, the uge1Δ and uge1Δgal10Δ strains had increased amounts of cytosolic UDP-galactose. In contrast, increased UDP-galactose content in the uge1Δgal10Δgal7Δ strain was not detected in cells grown in the 1 % galactose-containing medium. These results indicate that Gal7p is involved in UDP-galactose biosynthesis in Sch. pombe.

**DISCUSSION**

Glycoproteins in Sch. pombe contain a large amount of galactose in addition to both N-linked and O-linked mannans (Manners & Meyer, 1977; Moreno et al., 1985; Ikeda et al., 2009; Ohashi et al., 2009). The galactose residues are modified by galactosyltransferases using UDP-galactose as substrate (Andreyishcheva et al., 2004; Chappell et al., 1994; Yoko-o et al., 1998). UDP-galactose is transported to the Golgi lumen from the cytosol by a Golgi-localized UDP-galactose transporter (Gms1p) (Tabuchi et al., 1997; Tanaka & Takegawa, 2001). In Sch. pombe, biosynthesis of cytosolic UDP-galactose is poorly characterized. UDP-galactose/glucose 4-epimerase is the key enzyme involved in UDP-galactose synthesis in the cytosol, where it catalyses the interconversion of UDP-glucose/galactose and UDP-D-glucose. Interestingly, *Schizosaccharomyces* species have two types of UDP-glucose/galactose 4-epimerase homologues, although other organisms have only one kind. It is not known why two types of epimerase are needed and how they are regulated in Sch. pombe.

Here we show (1) that Sch. pombe has two genes encoding functional epimerases (named uge1+ and gal10+); (2) that Uge1p is a major UDP-glucose/galactose epimerase expressed constitutively in cells grown in glucose-rich medium (2 % glucose, Fig. 7a); (3) that gal10+ is efficiently expressed and involved in galactosylation of cell surface proteins in low-glucose medium (0.1 % glucose, Fig. 7a), but not in galactose-containing medium; and (4) that galactose is transported into the cytosol and is converted into UDP-galactose via Gal-1-P by Gal7p in Sch. pombe (Fig. 7b).

In this study, we shed new light on the Leloir pathway from the standpoint of UDP-galactose synthesis in Sch. pombe (Fig. 7). It is interesting to note that *Schizosaccharomyces*
species are the only organisms known to have two types of UDP-glucose/-galactose 4-epimerase (Figs 1 and 7, Table 1). To our knowledge, other yeast species only have the Sc Gal10 type of epimerase, which has both epimerase and mutarotase domains. A key difference between these yeast species and Sh. pombe is that Sh. pombe can modify galactose for use in synthesizing glycoproteins, while other yeasts modify mannose for the same purpose (Gemmill & Trimble, 1999). This might suggest that the Uge1 type of epimerase with only an epimerase domain could have evolved for...
efficient UDP-galactose production for galactosylation of glycoproteins in Sch. pombe. We previously reported that disruption of the gns1+ gene led to a complete loss of protein galactosylation, due to a defect in transport of UDP-galactose from the cytosol to the lumens of the Golgi apparatus as a substrate for galactosyltransferase (Tabuchi et al., 1997; Tanaka et al., 2001). The gns1Δ strain exhibited aberrant cell morphology and increased sensitivity to digestion with β-glucanase and to various drugs, such as hygromycin B, sodium orthovanadate and calciofluor white. Further, the gns1Δ strain was incapable of sexual conjugation during nutritional starvation and of non-sexual flocculation (Tanaka et al., 1999, 2001). The uge1Δ strain also had a severe galactosylation defect and a decrease in epimerase activity and UDP-galactose content when grown in glucose-rich medium (2% glucose) (Figs 2, 3 and 6b), indicating that UGel1p is a major UDP-glucose/galactose epimerase expressed constitutively in 2% glucose medium (Fig. 7a).

In Sac. cerevisiae, all the galactose-inducible genes (GAL1, GAL10, GAL7 and GAL2) are coordinately regulated at the level of transcription in response to galactose by Gal4p, Gal80p and Gal3p (De Robichon-Szulmajster, 1958; Lohr et al., 1995; Platt & Reece, 1998). In the absence of galactose, binding of Gal80p to Gal4p effectively limits active transcription of the GAL genes. In the presence of galactose, Gal3p–Gal80p interactions are triggered in the cytoplasm, resulting in redistribution of Gal80p from the nucleus to the cytoplasm, thereby relieving inhibition of Gal4p and resulting in GAL gene expression (Peng & Hopper, 2002). However, the Sch. pombe genome has no homologues of Gal4p, Gal80p or Gal3p. Further, the promoter sequences of uge1+, gal10+ and gal7+ also lack typical Gal4p-binding sequences [UASGal: 5′-CGGRNN-RCYNNCNNCG-3′ (Fedor & Kornberg, 1989; Lohr et al., 1995)]. In this study, we found that gal10+ is efficiently expressed and involved in galactosylation of cell-surface proteins in cells grown in low (0.1%) glucose medium, but not in galactose-containing medium (Figs 3b, 4 and 7a). We previously reported that the inv1+ gene that encodes invertingase is repressed in the presence of glucose (Tanaka et al., 1998). The transcription of inv1+ is regulated by Scr1p, which is required for glucose repression of inv1+ (Tanaka et al., 1998) and frp1+ (Neely & Hoffman, 2000). Similarly, Scr1p may have repressed the transcription of gal10+ in 2% glucose medium.

Sch. pombe is not able to grow on galactose-containing medium with galactose as sole carbon source. This phenotype makes it difficult to analyse the Leloir pathway in Sch. pombe, despite the presence of the three relevant enzymes, galactokinase, Gal1-P uridylyltransferase and UDP-glucose/galactose epimerase. To address this problem, we used a uge1Δgal10Δ strain. Recovery of galactosylation in the uge1Δgal10Δ strain indicated that UDP-galactose was produced by sequential reaction of galactokinase and Gal7 without UDP-glucose/galactose epimerase (Figs 6 and 7b). These results also suggest that Sch. pombe is able to transport galactose from the medium into the cytosol by unidentified transporters. In Sac. cerevisiae, Gal2p (Tschopp et al., 1986), which is an integral plasma membrane protein predicted to contain 12 transmembrane domains (Nehlin et al., 1989) is able to transport galactose. GAL2 is also regulated at the level of transcription in response to galactose by Gal4p (De Robichon-Szulmajster, 1958; Lohr et al., 1995; Platt & Reece, 1998), and loss of Gal2p’s permease activity renders cells unable to utilize galactose as a sole carbon source (Douglas & Condie, 1954). In contrast, a GAL2 homologue was not found in the Sch. pombe genome. Multiple genes that encode hexose transporters (Ght1 to Ght6) have been identified in Sch. pombe (Heiland et al., 2000). These transporters are involved in glucose, fructose and gluconate transport from the extracellular milieu into the cytosol (Heiland et al., 2000). However, a galactose transporter has not yet been identified (Fig. 7b). Using the uge1Δgal10Δ strain should help reveal not only the function of galactokinase and Gal7p, but also the galactose transport machinery in Sch. pombe. Schizosaccharomyces japonicus and Sch. octosporus also lack genes involved in regulation of the Leloir pathway, suggesting that the features of galactose metabolism identified in Sch. pombe may also apply in other Schizosaccharomyces species.

We were not able to analyse the mutarotase domain of Gal10p in this study. Gal10p was efficiently expressed under low (0.1%) glucose conditions, suggesting that mutarotase activity might be elevated under these conditions. Further studies will be necessary to determine how galactokinase and Gal7p are regulated under various growth conditions in Sch. pombe.

ACKNOWLEDGEMENTS

We thank Drs Taro Nakamura and Yoko Giga-Hama for providing Sch. pombe plasmids and strains. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES


Edited by: M. Schweizer