The UDP-Glc:Glycoprotein Glucosyltransferase Is Essential for *Schizosaccharomyces pombe* Viability under Conditions of Extreme Endoplasmic Reticulum Stress

Sandra Fanchiotti, Fabiana Fernández, Cecilia D’Alessio, and Armando J. Parodi
Instituto de Investigaciones Bioquímicas Fundación Campomar, 1405 Buenos Aires, Argentina

**Abstract.** Interaction of monoglucosylated oligosaccharides with ER lectins (calnexin and/or calreticulin) facilitates glycoprotein folding but this interaction is not essential for cell viability under normal conditions. We obtained two distinct single *Schizosaccharomyces pombe* mutants deficient in either one of the two pathways leading to the formation of monoglucosylated oligosaccharides. The *alg6* mutant does not glucosylate lipid-linked oligosaccharides and transfers Man$_g$GlcNAc$_g$ to nascent polypeptide chains and the *gpt1* mutant lacks UDP-Glc:glycoprotein glucosyltransferase (GT). Both single mutants grew normally at 28°C. On the other hand, *gpt1/alg6* double-mutant cells grew very slowly and with a rounded morphology at 28°C and did not grow at 37°C. The wild-type phenotype was restored by transfection of the double mutant with a GT-encoding expression vector or by addition of 1 M sorbitol to the medium, indicating that the double mutant is affected in cell wall formation. It is suggested that facilitation of glycoprotein folding mediated by the interaction of monoglucosylated oligosaccharides with calnexin is essential for cell viability under conditions of extreme ER stress such as underglycosylation of proteins caused by the *alg6* mutation and high temperature. In contrast, *gls2/alg6* double-mutant cells that transfer Man$_g$GlcNAc$_g$ and that are unable to remove the glucose units added by GT as they lack glucosidase II (GII), grew at 37°C and had, when grown at 28°C, a phenotype of growth and morphology almost identical to that of wild-type cells. These results indicate that facilitation of glycoprotein folding mediated by the interaction of calnexin and monoglucosylated oligosaccharides does not necessarily require cycles of reglucosylation–deglucosylation catalyzed by GT and GII.

**Key words:** glycoprotein folding • *Schizosaccharomyces pombe* • glucosylation • glucosyltransferase • endoplasmic reticulum

**M**embrane-bound calnexin and its soluble homologue calreticulin behave in mammalian cells as authentic chaperones of the ER. They bind monoglucosylated high mannose-type oligosaccharides present in both misfolded and properly folded glycoproteins (Rodan et al., 1996; Zapun et al., 1997). Monoglucosylated structures may be formed by partial deglucosylation of the oligosaccharide transferred to nascent glucosylated structures by partial deglucosylation catalyzed by GT and GII. The yeast *S. pombe* calnexin is a transmembrane type I protein with a cytosolic tail and three highly conserved and one less conserved Ca$^{2+}$-binding motifs, as in mammalian calnexin.
Calnexin is essential for *S. pombe* viability and no calreticulin homologue was detected in this yeast genome (Jannatipour and Rokeach, 1995; Parlati et al., 1995a). Moreover, it has been shown that conditions that lead to the accumulation of misfolded glycoproteins in the ER induce calnexin-encoding mRNA synthesis (Jannatipour and Rokeach, 1995; Parlati et al., 1995a).

The oligosaccharide transferred to protein in *S. pombe* is Glc$_3$Man$_9$GlcNAc$_2$, as in most eukaryotic cells. It is processed in the ER to Man$_9$GlcNAc$_2$, thus indicating the presence of both GI and GII activities although the genes encoding these glucosidases have not yet been identified (Ziegler et al., 1994).

We have previously characterized the *S. pombe* GT. The enzyme was purified to homogeneity and found to be even more stringent in its differential glucosylation of denatured and native glycoproteins than its rat liver counterpart (Fernández et al., 1994). We showed that synthesis of *S. pombe* GT-encoding mRNA was induced under stress conditions that promoted accumulation of misfolded glycoproteins in the ER lumen (Fernández et al., 1996). However, disruption of the GT-encoding gene showed that the enzyme is not essential for cell viability and no discernible phenotype was observed (Fernández et al., 1996). On the other hand, the yeast *Sacharomyces cerevisiae* lacks GT, a key element in the quality control of glycoprotein folding, and calreticulin and its calnexin-like molecule presents significant structural variations with respect to the mammalian protein (Fernández et al., 1994; Parlati et al., 1995b; Jakob et al., 1998).

Facilitation of glycoprotein folding mediated by the interaction of calnexin/calreticulin with monoglucosylated oligosaccharides is dispensable for cell growth as shown by the yeast *S. pombe* (Jan-teraction of calnexin/calreticulin with monoglucosylated Jakob et al., 1998). ER lectins was therefore considered to be a back up system for other amino acid residues that are apparently required for the key element in the quality control of glycoprotein folding, and calnexin is essential for the same enzyme (Reitman et al., 1982). No monoglucosylated coproteins in the ER lumen (Fernández et al., 1996). How-ever, the yeast *Sacharomyces cerevisiae* is Glc$_3$Norte of *S. cerevisiae* ALG6 and putative Caenorhabditis elegans ALG6 gene products, respectively. An 876-bp fragment was obtained when primers Alg6s and Alg4 were used. As expected, it contained the 812-bp fragment. The 812-bp fragment in pGEMT-Easy vector (Promega Corp., Madison, WI) was treated with BglII and NheI enzymes. A 102-bp segment was thus eliminated and replaced by the ura4$^+$ gene (these sequence data are available from GenBank/EMBL/DDBJ under accession Nos. Z54342 and Z54342) were used as primers in PCR reactions: 5'-TAT-GTGGGNTGTYATATCNC-3' (Alg1, sense), 5'-DATNGTYYT-TYCTRGNACNYGRA-3' (Alg4, antisense), and 5'-CAYTGGATG-GARATHAC-3' (Alg6s, sense). The template was genomic *S. pombe* DNA. PCR reactions using primers Alg1s and Alg4 yielded an 812-bp fragment that was sequenced. The deduced amino acid sequence of this product was 82% and 80% homologous to the respective portions of *S. cerevisiae* ALG6 and putative Caenorhabditis elegans ALG6 gene products, respectively. To confirm the identity of the bands, the expected 1,684 bp (from 612 to 990) were used. As expected, it contained the 812-bp fragment. The 812-bp fragment in pGEMT-Easy vector (Promega Corp., Madison, WI) was treated with BglII and NheI enzymes. A 102-bp segment was thus eliminated and replaced by the ura4$^+$ gene (these sequence data are available from GenBank/EMBL/DDBJ under accession No. M36504: 1.8 kb) in an inverted position. The construct was liberated from the vector and used for transforming Sp95 cells. Ur4$^+$ cells (Alg6s; Sp95A strain) were selected. Colony PCR were performed using Alg6s (sense) and uraSC (sense, 5'-TGCTTCIIACAACATTACC-3'; this primer is complementary to the 3′ end of the ura4$^+$ gene). Selected colonies gave the expected 432-bp fragment. DNAs from wild-type (Sp95) and alg6 mutant cells were digested with BglII and NdeI (that do not cut the ura4$^+$ gene) and analyzed by Southern blotting. A 603-bp fragment obtained by digestion of the 812-bp portion of the alg6$^+$ gene with NheI and Spel was used as probe. Mutant and wild-type DNAs yielded 4.6-kb and 2.8-kb positive fragments, respectively. On the other hand, the mutant DNA 4.6-kb fragment gave a positive signal when an ura4$^-$/derived probe was used.

**Construction of the gpt1 Mutant.**

Construction of the gpt1 Mutant. The procedure used was similar to that previously described but Sp61 cells were used (Fernández et al., 1996). The resulting strain was named Sp61G1.

Excision of the ura4$^+$ Gene. The pBluescript plasmid containing the entire gpt1$^+$ gene (4,952 bp) (Fernández et al., 1996) was digested with BglII, thus eliminating 1,684 bp. The digestion product, containing the plasmid plus 612 bp of the S′ end and 2,656 bp of the S′ end of GT, was ligated. A 3,111-bp linear fragment obtained upon cutting the plasmid with PsiI and MluI was used to transform gpt1 mutant cells. Colonies resistant to fluoro-rotic acid (1 mg/ml) were analyzed by Southern blotting (see below). The strain was named Sp61G1.

Disruption of the alg6$^+$ Gene. The alg6$^+$ gene was disrupted in the gpt1-D1684 mutant (Sp61G4 strain), as described above for wild-type cells. The resulting strain was named Sp61G4A.

**Characterization of gpt1 and gpt1/alg6 Mutants**

DNAs from wild-type cells (Sp61), and from the gpt1::ura4$^+$ (Sp61G1), the gpt1-D1684 (Sp61G4) and the gpt1/alg6 (Sp61G4A) mutant cells were cut with BglII and analyzed in Southern blots with a 1,200-bp probe corresponding to the S′ end of the gpt1$^+$ gene. Wild-type DNA yielded two positive bands as BglII cuts at 612, 1,750, and 2,290 bp from the S′ end of the gpt1$^+$ gene whereas gpt1::ura4$^+$, gpt1-D1684, and gpt1/alg6 DNAs only showed a positive signal with the smaller band as 1,684 bp (from 612 to 2,296) from the gpt1$^+$ gene had been deleted for inserting the ura4$^+$ gene (gpt1 mutant) or for constructing the gpt1-D1684 mutant. Wild-type DNA gave a 2.8-kb positive signal, the same as gpt1::ura4$^+$ and gpt1-D1684 DNAs when the above mentioned 603-bp alg6$^+$ gene probe was used. On the other hand, gpt1/alg6 DNA gave the expected 4.6-kb band.

**Construction of the gsp2/alg6 Double Mutant**

Disruption of the GII-encoding gene (gsp2$^+$). Primers (sense 5′-TGGA-YGATAGAAYGARCC-3′, and antisense 5′-GCRGTGNGCICKR-AARRA-3′) were designed according to peptides conserved in three known GII sequences from other species (these sequence data are available from GenBank/EMBL/DDBJ under accession Nos. Z36098, D42041, and Z700753). A 414-bp fragment of the homologue *S. pombe* gene (here designated gsp2$^+$) was obtained by PCR using genomic wild-type DNA as

**Materials and Methods**

**Strains and Media**

The wild-type *S. pombe* strains used were Sp95 (h′, ura4-D18, ade6-M210, ade1, leu1-32), YE and minimal media were prepared as described (Moreno et al., 1991). The wild-type *S. pombe* strains used were Sp95 (h′, ura4-D18, ade6-M210, ade1, leu1-32), YE and minimal media were prepared as described (Moreno et al., 1991).
DNA and RNA procedures

Standard DNA manipulations were carried out as described (Sambrook et al., 1989). S. pombe genomic DNA was prepared as previously described (Moreno et al., 1989). Hybridization conditions were as previously described (Sambrook et al., 1989). RNA was extracted from cells grown under normal conditions (YE medium at 28°C) in the exponential growth phase at the same OD and submitted to Northern blotting as previously performed (Fernández et al., 1996). The probe used was a 1,004-bp DNA fragment of the BiP-encoding gene synthesized by PCR using genomic DNA as template.

Materials

[14C]Glucose (250 Ci/mol) and EasyTag Express protein labeling mix (>1,000 Ci/mmol) were from New England Nuclear (Boston, MA). Jack bean α-mannosidase and EndoH were from Sigma (St. Louis, MO). UDP-[14C]Glc (250 Ci/mol) were prepared as described by Wright and Robbins (1965). Polyclonal antiserum to recombinant S. pombe calnexin was a generous gift from Dr. L. Rokeach (Département de Biochimie, Université de Montréal, Montréal, Canada).

Substrates and Standards

[glucose-4,14C]Glc, Man, GlcNAc, [glucose-4,14C]Glc, Man, GlcNAc, [glucose-1,4C]Glc, Man, GlcNAc, [glucose-1,4C]Glc, Man, GlcNAc, [14C]Glc, Man, GlcNAc, [14C]Man, GlcNAc, and [14C]Man, GlcNAc were prepared as described previously (Fernández et al., 1994).

In Vivo Labeling of S. pombe Cells with [14C]glucose

Pulse labeling of S. pombe wild type, alg6, gpt1/alg6, and gpl2/alg6 cells with [14C]glucose was performed as described before for S. cerevisiae but 150 μCi of [14C]glucose was used (Fernández et al., 1994). Preincubations and incubations with the labeled precursor lasted for 15 min. Where indicated 5 mM 1-deoxynojirimycin (DNJ) was added. Isolation of Endo H–sensitive oligosaccharides was performed as already described (Fernández et al., 1994).

In Vivo Labeling of S. pombe Cells with [35S]Methionine and [35S]Cysteine

Wild type (Sp95), gls2, and alg6 mutant cells grown in YE medium were harvested, washed, and then suspended (2 × 10^8 cells) in 1.5 ml of 1% Yeast Nitrogen Base without amino acids (Difco Laboratories Inc., Deer- troit, MI) supplemented with adenine, leucine, and uracil. Samples were preincubated for 2 h at 28°C and incubated with 450 μCi of [35S]methionine plus [35S]cysteine (EasyTag Express protein labeling mix) for 5 min at the same temperature. Reactions were stopped by the addition of 10 mM sodium azide plus 10 mM iodoacetamide. Samples were then processed and calnexin was immunoprecipitated as described previously (Tabuchi et al., 1997). Samples were run on 9% SDS-PAGE and submitted to autoradiography.

Methods

Strong acid hydrolysis and treatment of oligosaccharides with Jack bean α-mannosidase were as described before (Fernández et al., 1994). Whatman 1 paper was used for chromatography. Solvents used were: (a) 1-propanol/nitromethane/water (5:2:4); (b) 1-butanol/pyridine/water (10:3:3); or (c) 1-butanol/pyridine/water (4:3:4). GII activity was assayed as described before using [glucose-4,14C]Glc, Man, GlcNAc as substrate (Ugalde et al., 1979). GT was assayed using yeast cell microsomes as an enzyme source and 8 M urea-denatured thyroglobulin as acceptor, as described previously (Trombetta et al., 1989). S. pombe and rat liver microsomes were prepared as already described (Trombetta et al., 1992; Fernández et al., 1994). Endomannosidase was assayed in S. pombe microsomes with [glucose-4,14C]Glc, Man, GlcNAc as substrate as already described (Gahn et al., 1991). Rat liver microsomes with or without S. pombe microsomes were used as positive controls.

Results

Glycosylation and Oligosaccharide-processing Pathways

The glycosylation and oligosaccharide processing path-

Fanchiotti et al. Protein Glucosylation in Schizosaccharomyces pombe 627
nose and very small amounts of glucose and galactose (Fig. 3). Strong acid hydrolysis of compounds in this figure yielded man-
P-Glc to Man9GlcNAc2-P-P-dolichol, respectively. Mu-

traceur that transfers the first glucose unit from dolichol-

The Journal of Cell Biology, Volume 143, 1998 628

Figure 1. Glycosylation and oligosaccharide processing pathways occurring in the ER of wild-type and mutant cells used in work reported here. D, dolichol; Pr, protein.

ways occurring in the ER of wild type and mutant cells used in the work reported here are depicted in Fig. 1. Gpt1+, gls2+, and alg6+ code for GT, for GII, and for the enzyme that transfers the first glucose unit from dolichol-

P-Glc to Man9GlcNAc2-P-P-dolichol, respectively. Mutants were obtained and genetically characterized by Southern blotting and PCR analysis as described under Materials and Methods (Table I).

Biochemical Characterization of Mutants

The alg6 Mutation. The gpt1+ gene encoding S. pombe GT has already been identified but as neither alg6+ nor gls2+ genes have yet been identified in S. pombe, it was necessary to characterize the alg6, gpt1/alg6, and gls2/alg6 mutants biochemically. For this purpose, the structure of protein-bound oligosaccharides synthesized in vivo by the wild-type and mutant cells in the presence or absence of the GI and GII inhibitor DNJ were compared. Incubation of wild-type cells with [14C]glucose for 15 min in the presence 5 mM DNJ yielded endo-β-N-acetylgalcosaminidase H (Endo H)-sensitive, protein-linked oligosaccharides that migrated on paper chromatography as Glc3Man9GlcNAc, and Glc1Man9GlcNAc standards (Fig. 2 A). Strong acid hydrolysis of compounds that migrated as either one of the last two standards produced labeled glucose and mannose units (Fig. 3 A). Omission of DNJ mainly produced compounds that migrated as Man9GlcNAc and Man8GlcNAc standards but a trailing in the position of Glc1Man9GlcNAc could be observed (Fig. 2 B). Strong acid hydrolysis of compounds in this figure yielded mannose and very small amounts of glucose and galactose (Fig.

3 B). From these results it is concluded that DNJ only par-

Table I. S. pombe Strains Used in the Present Study

| Strain   | Genotype |
|----------|----------|
| Sp95 (wt) | h0, ura4-D18, ade6-M216, leu1-32 |
| Sp61 (wt) | h-, ura4-D18, ade6-M210, ade1, leu1-32 |
| Sp95A (alg6) | h0, ura4-D18, ade6-M216, leu1-32, alg6::ura4+ |
| Sp61G (gpt1) | h-, ura4-D18, ade6-M210, ade1, leu1-32, gpt1::ura4+ |
| Sp61G4 | h-, ura4-D18, ade6-M210, ade1, leu1-32, gpt1::URA4 |
| Sp61G4 (gpt1/alg6) | h-, ura4-D18, ade6-M210, ade1, leu1-32, gpt1::URA4 |
| Sp95Ina (gls2) | h0, ura4-D18, ade6-M216, leu1-32, gls2::ura4+ |
| Sp95Ina2 | h0, ura4-D18, ade6-M216, leu1-32, gls2::ura4 |
| Sp95Ina2A (gls2/alg6) | h0, ura4-D18, ade6-M216, leu1-32, gls2::ura4, alg6::ura4+ |

Except for the first wild-type strains, all mutants were generated in the present study.

Figure 2. Characterization of alg6 mutant. Patterns of protein-linked oligosaccharides. Oligosaccharides synthesized in wild-

type (Sp95) and alg6 mutant cells incubated for 15 min with [14C]glucose in the presence or absence of DNJ were obtained by Endo H treatment and subjected to paper chromatography with solvent A. Standards: 1, Glc-Man,GlcNAc; 2, Glc-Man,GlcNAc; 3, Glc-Man,GlcNAc; 4, Man,Man,GlcNAc; and 5, Man,Man,GlcNAc.
hydrolysis of those compounds only produced mannose units (Fig. 3 E).

As only partial inhibition of GII by DNJ was attained, there are two interpretations to results shown above: in alg6 mutants, Man₉GlcNAc₂ was transferred to proteins and this compound was either demannosylated to Man₈GlcNAc₂ or glucosylated by GT to Glc₁Man₉GlcNAc₂ or alternatively, the disrupted gene (alg6¹) corresponds to the enzyme that transfers not the first but the second glucosyl residue to Man₉GlcNAc₂-P-dolichol. According to this interpretation, Glc₁Man₉GlcNAc₂ was transferred in the mutant obtained and this compound was deglucosylated and demannosylated to Man₉GlcNAc₂ and Man₈GlcNAc₂. Characterization of the gpt1/alg6 double mutant discarded this last possibility.

The gpt1/alg6 Double Mutation. Incubation of gpt1/alg6 mutant cells with [¹⁴C]glucose yielded protein-linked Man₉GlcNAc and Man₈GlcNAc both in the presence and absence of DNJ (Fig. 4, A and B). Strong acid hydrolysis of compounds obtained under both conditions only produced labeled mannose residues (Fig. 4, C and D). Furthermore, microsomes obtained from the double mutant were completely devoid of GT activity (not shown; the assay and results were as described before for the gpt1 mutant; Fernández et al., 1996). These results indicate that in gpt1/alg6 double-mutant cells Man₉GlcNAc₂ was transferred to nascent polypeptide chains and that this compound was not glucosylated. They further indicate that the same compound was transferred in the single alg6 mutant.

The gls2/alg6 Double Mutation. Incubation of gls2/alg6 mutant cells with [¹⁴C]glucose in the absence of DNJ yielded a mixture of Glc₁Man₉GlcNAc₂, Man₉GlcNAc₂, and Man₉GlcNAc₂ (Fig. 5 A). The same result was obtained in the presence of the GII inhibitor (not shown). Strong acid degradation of above mentioned compounds yielded glucose and mannose (Fig. 5 B). No GII activity was detected in gls2/alg6 microsomes when assayed with [glucose-¹⁴C]Glc₁Man₉GlcNAc as substrate.

Presence of the Unfolded Protein Response in alg6, gpt1/alg6, and gls2/alg6 Mutants

Accumulation of misfolded proteins in the ER leads to the so-called unfolded protein response, that is, to the induction of chaperones and other proteins that facilitate protein folding (BiP, calnexin, GT, etc.) (Pidoix and Armstrong, 1992; Jannatipour and Rokeach, 1995; Parlati et al., 1994).
1995a; Fernández et al., 1996). Detection of this response is rather simple in *S. pombe* as under normal conditions a single BiP-encoding mRNA is synthesized. Accumulation of misfolded proteins leads to the induction of not only this mRNA species but also of a slightly smaller mRNA (Pidoux and Amstrong, 1992). Synthesis of both BiP-encoding mRNAs was detected in *alg6*, *gpt1/alg6*, and *gls2/alg6* mutants but not in wild-type cells grown under normal conditions (Fig. 6 A). The most plausible explanation for induction of BiP-encoding mRNA in cells carrying the *alg6* mutation might be that underglycosylation of glycoproteins occurs in them as has been described for *S. cerevisiae* mutants transferring Man₉GlcNAc₂ to proteins (Balou et al., 1986). Cell-free assays have shown that in most eukaryotic cells Glc₃Man₉GlcNAc₂ is transferred ~10–25-fold more efficiently than Man₉GlcNAc₂ (Bosch et al., 1988). It has been reported that underglycosylation of glycoproteins triggers misfolding of glycoproteins (Marquardt and Helenius, 1992; Imperiali and Rickert, 1995). To confirm that underglucosylation of glycoproteins occurred in *S. pombe* mutants carrying the *alg6* mutation, wild-type, *gls2*, and *alg6* cells were incubated for 5 min with a mixture of [35S]methionine and [35S]cysteine, lysed in the presence of iodoacetamide and material immunoprecipitated by a calnexin antiserum run on SDS-PAGE under reducing conditions. As depicted in Fig. 6 B, whereas a single positive band appeared in wild-type cells, two positive signals were detected in the *alg6* mutant. Their difference in size was of ~2 kD, a value consistent with the presence of a single oligosaccharide in *S. pombe* calnexin (Janntipour and Rokeach, 1995; Parlati et al., 1995a). The *gls2* mutant yielded a single positive signal that migrated slightly above that of wild-type cells. This result is consistent with the fact that in this mutant the oligosaccharide retains two glucose units.

**Growth and Morphology of Wild Type, *alg6*, *gpt1*, and *gpt1/alg6* cells at 28°C and 37°C**

Alg6 and gpt1 cells grew at about the same rate as wild-type cells at 28°C (Fig. 7 A). Moreover, their short rod shapes were similar to those of wild-type cells (Fig. 8, A–C). On the other hand, gpt1/alg6 cells grew much more slowly than wild-type cells and growth stopped at a lower OD (Fig. 7 A). Gpt1/alg6 cells tended to clump and had a rounded shape with clearly visible inclusions (Fig. 8 D). All mutant cells grew at 37°C except for gpt1/alg6 that did not grow at this temperature (Fig. 9). Growth of gpt1 mutant cells at the high temperature was similar to that of wild-type cells, whereas *alg6* cells grew somewhat less efficiently (Fig. 9). It is worth mentioning in experiments shown in Fig. 9 all strains were allowed to grow for 5 d except for *gls2/alg6* and *gpt1/alg6* that were incubated for 7 and 10 d, respectively. Both *alg6* and *gls2/alg6* mutants showed the so-called thermosensitive morphology at 37°C, characterized by swollen cells, displaying pear, lemon, and round shapes (Ishiguro et al., 1997).

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**Figure 5.** Characterization of *gls2/alg6* mutant. Patterns of oligosaccharides and monosaccharide composition. (A) Oligosaccharides synthesized by *gls2/alg6* mutant cells upon incubation with [14C]glucose in the absence of DNJ were run on paper chromatography with solvent A. (B) Compounds synthesized were submitted to strong acid hydrolysis and resulting products run on paper chromatography with solvent B. Standards: 1, Glc₃Man₉GlcNAc₂; 2, Man₉GlcNAc₂; 3, Man₅GlcNAc₂; 4, galactose; 5, glucose; and 6, mannose.

**Figure 6.** The unfolded protein response and underglycosylation of glycoproteins in *S. pombe* mutant cells. (A) Wild-type, *alg6*, *gpt1/alg6*, and *gls2/alg6* cells were grown in YE medium at 28°C, mRNAs were extracted and submitted to Northern blotting. A fragment of BiP-encoding DNA was used as probe. (B) Wild-type, *gls2*, and *alg6* mutant cells were incubated for 5 min with [35S]methionine and [35S]cysteine, and lysed in the presence of iodoacetamide. Material immunoprecipitated by antisera to *S. pombe* calnexin was run on SDS-PAGE under reducing conditions and submitted to autoradiography.

**Figure 7.** Growth of wild-type and mutant cells at 28°C. Minimal medium plus adenine was used. The medium was supplemented with leucine and uracil (wild type) or with leucine (gpt1, *alg6*, and gpt1/*alg6*). Sp95 or Sp61 wild-type cells yielded similar growth curves.
Rescue of the Wild-Type Phenotype in gpt1/alg6 Mutants

The rounded shape of gpt1/alg6 cells suggested that synthesis of one of the components of the cell wall was affected. A classical way to test this hypothesis is to grow cells in a hyperosmotic medium. As shown in Fig. 10 A, gpt1/alg6 cells recovered the wild-type morphology when grown in 1 M sorbitol. Double-mutant cells grew at 28°C at the same rate as wild-type ones in the presence of the above mentioned compound (not shown). To confirm that the phenotype observed in those double mutants was caused by the absence of GT, cells were transfected with two expression vectors, pREP1-gpt1+ and pREP41-gpt1+ both coding for GT. They respectively had strong and medium promoters and when transfected into gpt1 mutants they yielded cells displaying 4–10- and 1–2-fold the GT activity of wild-type cells. Gpt1/alg6 cells recovered the wild-type morphology (Fig. 10 B) and growth rate at 28°C (Fig. 7 B), and the ability to grow at 37°C (Fig. 9) when transfected with any of these expression vectors. The wild-type phenotype was not rescued by the vectors lacking the gpt1+ gene (Fig. 10 C).

It was reported that under physiological conditions of pH and salt concentration, GT interacted with hydrophobic peptides and that this interaction was blocked by denatured glycoproteins. It was postulated that this interaction could be one of the recognition elements that determine the exclusive glucosylation of misfolded species (Sousa and Parodi, 1995). The in vivo interaction of GT with certain misfolded ER proteins under conditions of Ca2+ depletions has also been reported (Choudhury et al., 1997). To check if the rescue of the wild-type phenotype by the GT-expressing vectors was caused by the catalytic activity of the enzyme or by its capacity of binding hydrophobic domains, two point mutations were introduced at the COOH-terminal domain of GT. This domain is believed to harbor the catalytic activity as it is 68–70% identical among the four known GT sequences (Drosophila melanogaster, S. pombe, C. elegans, and rat liver) (Parker et al., 1995; Fernández et al., 1996; these sequence data are available from GenBank/EMBL/DDBJ under accession No. U28735; our unpublished results) and also has a limited but significant homology with bacterial glycosyltransferases that use UDP-Glc or UDP-Gal as sugar donor (Parker et al., 1995). The changes introduced were Y1312A and D1352A. Both amino acids are not only conserved in the above mentioned GTs but also in at least nine bacterial glycosyltransferases. The mutated GT in pREP1 (pREP1-gpt1YD-AA) was completely inactive when expressed in gpt1/alg6 cells (Table II). The double-mutant cells expressed similar amounts of GT when transfected with pREP1-gpt1+ or with pREP1-gpt1YD-AA as judged by Coomassie
brilliant blue staining of SDS-PAGE of the soluble content of microsomes. The high molecular weight of the enzyme and its absence in non-transformed cells eased its identification. The mutated enzyme did not revert the gpt1/alg6 phenotype (Fig. 10 D) and did not allow growth of the double mutant at 37°C (not shown). This result confirmed that the enzymatic activity of GT was responsible for the correction of the phenotype and strongly suggested that the correction observed was mediated by the interaction of monoglucosylated species with calnexin.

Growth and Morphology of gls2/alg6 Cells

Gls2/alg6 cells grew at 28°C at almost the same rate as wild-type or alg6 cells (not shown). Moreover, the double-mutant cells displayed the same morphology as wild-type or alg6 cells when grown at 28°C (Fig. 11, A and B) and were able to grow at 37°C although not as efficiently as alg6 mutants (Fig. 9). As mentioned above, both alg6 and gls2/alg6 mutant cells showed the so-called thermosensitive morphology at 37°C, characterized by swollen cells, displaying pear, lemon, and round shapes (Ishiguro et al., 1997). The fact that gls2/alg6 cells exhibited a phenotype of growth and morphology clearly different from that of gpt1/alg6 cells indicates that facilitation of glycoprotein folding mediated by the interaction of monoglucosylated oligosaccharides with calnexin does not necessarily require cycles of reglucosylation–deglycosylation catalyzed by the opposing activities of GT and GII.

Discussion

It has been reported that in wild-type S. pombe cells the Glc3Man9GlcNAc2 transferred is deglucosylated to Man9GlcNAc in the ER, and then elongated by the addition of mannose and galactose residues in the Golgi (Ziegler et al., 1994). However, we have always obtained small amounts of Man9GlcNAc2. This compound was presumably not produced in the Golgi but in the ER as it was detected after very short incubations with the labeled precursor (5 min) or after long incubations (90 min) under conditions that prevented almost completely (addition of 5 mM DTT to the incubation medium) or completely (incubation at 39°C) exit of glycoproteins from the ER. Man9GlcNAc2 was not formed by an endomannosidase activity similar to that described previously in other cell types (Lubas and Spiro, 1987), as we have been unable to detect such activity in S. pombe microsomal membranes (see Materials and Methods). A phylogenetic survey has indicated a late evolutionary appearance of the enzyme (Dairaku and Spiro, 1997). Accordingly, no endomannosidase activity has been detected so far in unicellular organisms including protozoa and yeasts (Gañán et al., 1991; Gotz et al., 1991; Dairaku and Spiro, 1997).

Labeling of intact mutant cells with [14C]glucose for 15 min provided further evidence against the possibility that Man9GlcNAc2 could be formed by an endomannosidase activity. As depicted in Fig. 4, Man9GlcNAc2 was produced in gpt1/alg6 mutant cells, in which no glucosylated oligosaccharides (the substrate for the endomannosidase) were synthesized. In addition, incubation of gls2 mutant cells (Sp951a strain; see Table I) with [14C]glucose led to the formation of protein-bound Glc2Man9GlcNAc2 and Glc2Man8GlcNAc2. No glucose-free compounds were detected, thus indicating that removal of the glucose units was not required for formation of oligosaccharides containing eight mannose residues.

In contrast to what was observed in mammalian, plant and protozoan cells incubated with [14C]glucose (see Parodi, 1993 for references), Glc1Man8GlcNAc2 was not detected in any of the S. pombe wild-type or mutant cells used in this study (Glc3Man9GlcNAc2 would have migrated on paper chromatography between Man9GlcNAc and

| Table II. Activity of Wild Type and Mutant GT Expressed in gpt1/alg6 Cells |
|-----------------------------|-----------------|-----------------|-----------------|
| Addition                   | pREP1-gpt1     | pREP1-gpt1YD-AA | pREP1          |
| Thyroglobulin              | 13,840          | 240             | 330             |
|                            | 98,550          | 1,080           | 900             |

Values expressed are cpm/mg of microsomal protein precipitated by 10% trichloroacetic acid.
Man₆GlcNAc). The absence of Glc₃Man₆GlcNAc₂ formation suggests that the mannosidase responsible for Man₆GlcNAc₂ formation and GT might be located in different ER subcompartments. An alternative possibility is that the Man₆GlcNAc₂ isomer formed may lack the manno unit to which the glucose residue is added by GT.

Glycoproteins synthesized in *S. pombe* mutants that transfer Man₆GlcNAc₂ to proteins (alg6 mutants) were found to be underglycosylated. It is known that in cell-free assays the oligosaccharyltransferase transfers Glc₃Man₆GlcNAc₂ about 10–25-fold faster than Man₆GlcNAc₂ (Bosch et al., 1988). Glycoproteins synthesized in *S. cerevisiae* mutants that transfer the last compound were also found to be underglycosylated (Ballou et al., 1986). It is not surprising, therefore, that induction of BiP-encoding mRNA was detected in all *S. pombe* strains having the alg6 mutation. The extreme plasticity of the ER folding machinery compensated folding problems caused by glycoprotein underglycosylation by increased synthesis of chaperones and other folding-assisting proteins (BiP, GT, calnexin, etc.) and no discernable phenotype could be observed in those mutants at 28°C.

Facilitation of glycoprotein folding mediated by the interaction between monoglycosylated oligosaccharides and calnexin prevented appearance of a discernable phenotype in alg6 mutants because gpt1/alg6 double-mutant cells, in which no monoglycosylated oligosaccharides were formed, grew very slowly and with an altered morphology at 28°C and were unable to grow at 37°C. A wild-type phenotype was restored upon transfection with a GT-encoding plasmid. Two amino acid changes in the proposed catalytic domain of GT that abolished its glucosylating activity resulted in the non restoration of the wild-type phenotype. This fact, as well as the already known induction of BiP-encoding mRNA in cells submitted to conditions that promote accumulation of misfolded proteins in the ER (Fernández et al., 1996), confirmed the involvement of monoglycosylated oligosaccharides created by GT in facilitation of glycoprotein folding. Moreover, the fact that Glc₃Man₆GlcNAc₂ could only be detected in alg6 mutants when DNJ had been added to the medium or when the gls2² gene had been disrupted confirmed that *S. pombe* GT is involved, together with GII, in glucosylation–deglucosylation cycles, like its mammalian counterpart, and not in the synthesis of cellular polysaccharides. Single gpt1 mutants did not show a discernable phenotype as in them no underglycosylation of glycoproteins is expected to occur and probably increased synthesis of BiP and other folding-assisting proteins compensate for the absence of glycoprotein reglucosylation.

As mentioned above, many glycoproteins are expected to have folding problems in gpt1/alg6 mutant cells. However, not all glycoproteins are affected in the same manner as the requirement for the presence of all oligosaccharides for correct folding varies among different glycoproteins (see Helenius, 1994, and references therein). The one (or ones) that is responsible for the phenotype observed is probably somehow involved in cell wall formation as normal growth and morphology could be restored in a hyperosmotic medium (addition of 1 M sorbitol). It is worth mentioning that *S. pombe* mutant cells affected in the galactomannan content displayed a rounded morphology similar to that of gpt1/alg6 cells (Ribas et al., 1991). Moreover, the morphological alteration in those mutants was corrected when grown in 1 M sorbitol the same as with the double mutants described in the present report. Synthesis of galactomannan is initiated in the ER upon transfer of Glc₃Man₆GlcNAc₂ to a protein core.

This is the first report showing that protein reglucosylation by GT is required for cell viability under conditions of extreme ER stress such as glycoprotein underglycosylation and high temperature.

It has been reported that proper folding was arrested upon preventing the dissociation of influenza hemagglutinin (a glycoprotein having seven N-linked oligosaccharides) with calnexin/calreticulin by the addition of glucosidase inhibitors to an in vitro translation system (Hebert et al., 1996). It was concluded that on-and-off cycles of interaction between the glycoprotein and the lectins catalyzed by GT and GII were required for folding facilitation. This would be analogous to what happens with chaperones of the Hsp70 family. Mutations in the NH₂-terminal portion that abolish the ATPase activity and thus delay dissociation of the chaperones and misfolded proteins, prevent proper folding (Hendrick and Hartl, 1993). Apparently cycles of reglucosylation–deglucosylation are not essential for facilitation of proper folding of the glycoprotein(s) responsible for the observed phenotype in gpt1/alg6 cells as gls2/alg6 double-mutant cells that transferred Man₆GlcNAc₂ and were unable to remove the glucose units added by GT as they lacked GII, had a phenotype of
growth and morphology very similar to that of wild-type cells at 28°C and were able to grow at 37°C. It has been reported that at least two oligosacchrides in RNase B were required to immunoprecipitate the glycoprotein with anti-calnexin antibodies (Rodan et al., 1996). This indicates that the affinity of calnexin for monoglcosylated oligosacchrides is not as high as those of classical plant lectins. This was confirmed by the fact that isolated monoglcosylated oligosacchrides could be washed out with buffer from an immobilized calnexin column (Ware et al., 1995). It may be speculated, therefore, that even in the absence of GII association–dissociation cycles of binding of calnexin with the glycoprotein(s) responsible for the altered phenotype of gpt1Δalg6 cells could occur if the glycoprotein had a single (or few) oligosaccharide.

As mentioned above, calnexin is essential for S. pombe viability (Jannatipour and Rokeach, 1995; Parlati et al., 1995b). The fact that gpt1Δalg6 mutant cells grew, albeit at a slower rate, at 28°C indicates that calnexin has another role, besides that of binding monoglcosylated oligosacchrides in this yeast. The calnexin homologue in S. cerevisiae is not essential for survival, and although mammalian cells devoid of calnexin are viable it is unknown whether cells lacking both calnexin and calreticulin can survive (Parlati et al., 1995b; Scott and Dawson, 1995).

It is worth mentioning that S. cerevisiae alg5 or alg6 mutants are similar to the gpt1Δalg6 double mutant in S. pombe as Man3GlcNAc2 is transferred to proteins in them and there is no GT in S. cerevisiae. Nevertheless, no thermosensitive phenotype has been observed in S. cerevisiae alg5 or alg6 mutants (Jakob et al., 1998).

Trypanosomatid protozoa are similar to alg6 mutants in S. pombe as in them unglcosylated oligosacchrides are transferred to proteins and they have GT (Parodi, 1993). However, in this case no underglycosylation of glycoproteins is expected to occur as the protozoan oligosaccharyltransferase transfers, in cell-free assays, Glc3Man9GlcNAc2 and Man3GlcNAc2, while the same reaction is catalyzed by a protein that distinguishes between denatured and native proteins. However, it may be speculated that the specificity of the oligosaccharyltransferase in these protozoa is an evolutionary consequence of the loss of the ability to form Glc3Man3GlcNAc2 to prevent underglycosylation of proteins and thus a potential source of stress.

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