A misfolded protein conformation is not a sufficient condition for *in vivo* glucosylation by the UDP-Glc:glycoprotein glucosyltransferase

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A key element in the quality control of glycoprotein folding is the UDP-Glc:glycoprotein glucosyltransferase (GT), which in cell-free assays exclusively glucosylates misfolded glycoproteins. In order to test if such a protein conformation is a sufficient condition for *in vivo* glucosylation of all *N*-linked oligosaccharides by GT, a *Schizosaccharomyces pombe* double mutant (*glc2/alg6*) was constructed. With this mutant, Man₉GlcNAc₂ is transferred to proteins and no removal of glucose units added by GT occurs as it lacks glucosidase II. The same proportion of glucosylated (Glc₁Man₉GlcNAc₂) and unglucosylated (Man₉GlcNAc₂ and Man₈GlcNAc₂) endoplasmic reticulum (ER)-specific compounds was produced when cells were pre-incubated for 10, 20 or 30 min and further incubated with [¹⁴C]glucose for 10 min at 28°C with or without 5 mM dithiothreitol (DTT), thus indicating not only that DTT did not affect protein glucosylation but also that no increased glucosylation of glycoproteins occurred in the presence of the drug. Monitoring Golgi-specific modifications of oligosaccharides after pulse-chase experiments performed in the presence or absence of 5 mM DTT showed that exit of the bulk of glycoproteins synthesized from the ER and thence their proper folding had been prevented by the drug. Cells pulse-chase labeled at 37°C in the absence of DTT also yielded glucosylated and unglucosylated protein-linked oligosaccharides without Golgi-specific modifications. It was concluded that a misfolded protein conformation is not a sufficient condition for *in vivo* glucosylation of all *N*-linked oligosaccharides by GT.

Keywords: glucosidase II/glucosyltransferase/glycoprotein folding/*Schizosaccharomyces pombe*

Introduction

The model currently proposed for the quality control of glycoprotein folding in the endoplasmic reticulum (ER) of mammalian cells involves three components; lectins which recognize monoglucosylated high mannose-type oligosaccharides (membrane-bound calnexin and its soluble homolog, calreticulin), glucosidase II (GII), which removes the glucoses from di- and monoglucosylated derivatives and the UDP-Glc:glycoprotein glucosyltransferase (GT). In cell-free assays this enzyme transfers

a single glucose unit to glucose-free oligosaccharides only when the protein moieties of glycoproteins do not have their proper tertiary structure (Sousa *et al.*, 1992; Helenius *et al.*, 1997). It is assumed that upon partial deglucosylation of the transferred oligosaccharide by the successive action of glucosidase I (GI) and GII, the monoglucosylated compounds thus formed are recognized by the above mentioned lectins. Further deglucosylation of the oligosaccharides by GII would liberate the glycoproteins from their lectin anchors. The oligosaccharides would then be reglucosylated by GT, and thus recognized again by the lectins, only if the glycoproteins have not yet adopted their native structures. The deglucosylation-reglucosylation cycle would continue until proper glycoprotein folding is achieved. Correctly folded glycoproteins would then be allowed to follow their normal transit through the secretory pathway whereas misfolded species would be retained and eventually degraded in the proteasomes (Zhang *et al.*, 1997). The lectin-oligosaccharide interaction would not only retain misfolded species but also facilitate their proper folding by preventing aggregation (Hebert *et al.*, 1995).

The yeast *Schizosaccharomyces pombe* has the three basic elements of the quality control of glycoprotein folding: two independent groups have reported the existence in this fission yeast of a calnexin-like molecule which has a 40% amino acid identity with the mammalian lectin (Jannatipour and Rokeach, 1995; Parlati *et al.*, 1995a). As with mammalian calnexin, the *S.pombe* form is a transmembrane type I protein with a cytosolic tail and three highly conserved and one less conserved Ca²⁺-binding motifs. Both groups also reported the induction of calnexin-encoding mRNA by conditions that lead to the accumulation of misfolded glycoproteins in the ER, thus strongly suggesting a role for fungal calnexin in cellular stress conditions (Jannatipour and Rokeach, 1995; Parlati *et al.*, 1995a). Calnexin is essential for *S.pombe* viability and no other homolog (for instance a calreticulin-like molecule which in mammalian cells is ~35% identical with calnexin) was detected in the *S.pombe* genome (Jannatipour and Rokeach, 1995; Parlati *et al.*, 1995a).

Concerning the glucosidases, it has been reported that the oligosaccharide transferred in *S.pombe* is Glc₃Man₉GlcNAc₂, the same as in most eukaryotic cells and that it is processed in the ER to Man₉GlcNAc₂, thus indicating the presence of both GI and GII, although the genes encoding them 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). As with calnexin, the synthesis of *S.pombe* GT-encoding mRNA was found

to be induced under stress conditions which promoted the accumulation of misfolded glycoproteins in the ER lumen (Fernández *et al.*, 1996). Disruption of the GT-encoding gene showed that the enzyme was not essential for cell viability and no discernible phenotype could be observed (Fernández *et al.*, 1996). On the other hand, the yeast *Saccharomyces cerevisiae* lacks GT and calnexin and its calnexin-like molecule presents significant structural variations with respect to the mammalian protein (Jakob *et al.*, 1998; Fernández *et al.*, 1994; Parlati *et al.*, 1995b).

GTs purified from very different sources as mammalian, yeast and insects cells (Trombetta and Parodi, 1992; Fernández *et al.*, 1994; Parker *et al.*, 1995) were found to share the property of exclusively glucosylating denatured glycoproteins in cell-free assays. A misfolded conformation of the acceptor substrate was found to be a necessary and sufficient condition for glucosylation. Work reported here was performed in order to test whether such conformation is a sufficient condition for *in vivo* glucosylation of all *N*-linked oligosaccharides by GT. In order to ensure that only GT glucosylation was assayed and that no glucose residues were removed by GII, an *S.pombe* double mutant (*gls2/alg6*) that transfers Man₉GlcNAc₂ to nascent polypeptide chains and is devoid of GII activity was constructed. Results presented indicate that a misfolded conformation is not a sufficient condition for *in vivo* glucosylation of all *N*-linked oligosaccharides by GT.

Results

Genetic characterization of the double mutant strain

The procedure used for obtaining the *gls2/alg6* double mutant strain is outlined in Materials and methods. The GII-encoding gene (*gls2*⁺) was identified and disrupted by inserting the *ura4*⁺ gene in an inverted position. The *gls2::ura4*⁺ mutant was thus generated. Portions of *gls2*⁺ and *ura4*⁺ were then excised thus generating the *gls2::ura4* mutant. This mutant was then transformed with a portion of the *alg6*⁺ gene having the *ura4*⁺ gene inserted in an inverted position. The *alg6*⁺ gene (also identified here) encodes the dolichol-P-Glc-dependent glucosyltransferase which transfers the first glucose unit to Man₉GlcNAc₂-P-P-dolichol. Consequently the *gls2/alg6* (*gls2::ura4/alg6::ura4*⁺) double mutant was generated. When primers GIIBs and GIIBa (see Materials and methods for identity of the primers), which are complementary to portions of the *gls2*⁺ gene were used in PCR reactions, the expected band of 542 bp was obtained with wild-type DNA as a template and bands of the same length (1108 bp) were obtained with both *gls2::ura4* and *gls2::ura4/alg6::ura4*⁺ mutant DNAs (Figure 1A, lanes 2, 4 and 6). On the other hand primers Alg6s (not present in the construct used for disrupting the *alg6*⁺ gene but complementary to a portion of the gene located at the 5' end) and *uraSC* (complementary to *ura4*⁺) did not yield detectable bands in PCR reactions when wild-type or *gls2::ura4/alg6*⁺ DNAs were used as templates (Figure 1A, lanes 1 and 3). The expected 423 bp fragment was synthesized with *gls2/alg6* (*gls2::ura4/alg6::ura4*⁺) DNA (Figure 1A, lane 5).

The *gls2/alg6* mutant cells grown in the absence of exogenous stress showed an induction of the binding protein (BiP)-encoding mRNA. Accumulation of mis-

Fig. 1. Genetic characterization of *gls2/alg6* mutant cells. (A) PCRs using *alg6*⁺- and *ura4*⁺- or only *gls2*⁺-specific primers were performed using DNA from wild-type as a template (lanes 1 and 2, respectively), *gls2* (*gls2::ura4*) (lanes 3 and 4, respectively) or *gls2/alg6* (*gls2::ura4/alg6::ura4*⁺) (lanes 5 and 6, respectively) cells. (B) BiP- and actin-encoding mRNA species were detected by Northern blotting in wild-type (lane 1) and *gls2/alg6* (*gls2::ura4/alg6::ura4*⁺) (lane 2) cells. Upper bands correspond to BiP mRNA whereas the three lower ones correspond to actin mRNA.

folded proteins in the *S.pombe* ER leads to the so-called unfolded protein response, i.e. to the induction of mRNAs coding for chaperones and other proteins that facilitate proper folding of recently synthesized species (BiP, calnexin, GT etc.) (Pidoux and Armstrong, 1992; Jannatipour and Rokeach, 1995; Parlati *et al.*, 1995a; Fernández *et al.*, 1996). We determined by Northern analysis the mRNA species coding for BiP, the more abundant ER chaperone, in wild-type and *gls2/alg6* mutant cells grown in the absence of exogenous stress. It has been reported that in *S.pombe*, accumulation of misfolded species in the ER triggers synthesis of two BiP mRNAs, one of them having the same size as the mRNA present in wild-type cells grown under normal conditions and the other having a slightly smaller size (Pidoux and Armstrong, 1992). This was precisely the result observed with double mutant cells as two bands appeared when the BiP probe was used (Figure 1B). Results were scanned and intensities obtained for BiP mRNAs were normalized with those obtained for actin mRNA, a constitutively expressed species. Taking the ratio obtained for wild-type cells as 1, that of *gls2/alg6* cells was 2.2.

The most plausible explanation for this result is that as Man₉GlcNAc₂ and not Glc₃Man₉GlcNAc₂ is transferred in *gls2/alg6* cells, an underglycosylation of glycoproteins is expected to occur, as has been observed in *S.cerevisiae* mutants which transfer the former oligosaccharide (Ballou *et al.*, 1986). It is known that the triglycosylated compound is transferred in cell-free assays 10- to 25-fold faster than that lacking the glucose units (Bosch *et al.*, 1988). Moreover, underglycosylation is one of the factors triggering accumulation of misfolded glycoproteins in the ER (Marquardt and Helenius, 1992; Imperiali and Rickert, 1995). As with BiP, it may be expected that *gls2/alg6* cells show an increased synthesis of chaperones and other folding facilitating proteins (GT, calnexin etc.).

Biochemical characterization of the double mutant strain

It has been reported that in wild-type *S.pombe* cells the transferred oligosaccharide (Glc₃Man₉GlcNAc₂) is deglycosylated to Man₉GlcNAc₂ in the ER and that further processing in the Golgi involves addition of mannose and

Fig. 2. Biochemical characterization of *gls2/alg6* mutant cells. Wild-type (WT) (A and B) or *gls2/alg6* mutant (MUT) (D) cells were pre-incubated for 15 min and incubated with [¹⁴C]glucose for the same period. DNJ (5 mM) was added where indicated during pre-incubation and incubation. Endo H-sensitive oligosaccharides were run on paper chromatography with solvent A. Standards: 1, Glc₁Man₉GlcNAc₂; 2, Glc₂Man₉GlcNAc₂; 3, Glc₃Man₉GlcNAc₂; 4, Man₉GlcNAc₂ and 5, Man₈GlcNAc₂. In (C) GII activity was assayed in wild-type and *gls2/alg6* mutant cell microsomes using [glucose-¹⁴C]Glc₁Man₉GlcNAc₂ as substrate. The percentage of label released as glucose is represented in ordinates.

galactose units (Ziegler *et al.*, 1994). Incubation of wild-type *S.pombe* cells with [¹⁴C]glucose for 15 min in the presence of 5 mM 1-deoxynojirimycin (DNJ; a GI and GII inhibitor) led to the synthesis of protein-linked Glc₂Man₉GlcNAc₂, Glc₁Man₉GlcNAc₂, Man₉GlcNAc₂ and Man₈GlcNAc₂ (Figure 2A). Contrary to what has been reported, we have observed formation of small amounts of Man₈GlcNAc₂ in many short-term incubations of intact cells with the labeled precursor. Omission of the glucosidase inhibitor produced Man₉GlcNAc₂ and Man₈GlcNAc₂ (Figure 2B). This showed that deglucosylation of the transferred oligosaccharide (Glc₃Man₉GlcNAc₂) was extremely rapid in *S.pombe* and that *in vivo* DNJ did not affect GI and only partially inhibited GII. As a similar result was obtained with *N*-methyl-DNJ it was decided to disrupt the gene encoding GII in order to completely prevent removal of glucose units added by GT. In addition, to ensure that all protein-linked monoglucosylated oligosaccharides had been produced by GT and not by deglucosylation of the compound transferred in wild-type cells (Glc₃Man₉GlcNAc₂), a mutant that transferred Man₉GlcNAc₂ was required.

Disruption of the GII-encoding gene (denominated *gls2*⁺) produced mutant cells (*gls2*) which upon incubation with [¹⁴C]glucose for 15 min in the absence of DNJ led to the production of Glc₂Man₉GlcNAc₂ and a smaller amount of Glc₂Man₈GlcNAc₂ (data not shown). No compounds containing three, one or no glucose units were produced. Moreover, *in vitro* assays of GI and GII using microsomes as enzyme source and [glucose-¹⁴C]Glc₃Man₉GlcNAc₂ or [glucose-¹⁴C]Glc₁Man₉GlcNAc₂ as substrates showed that the *gls2* mutant had normal amounts of GI and completely lacked GII activity. On the other hand, disruption of only the gene (denominated *alg6*⁺)

Fig. 3. The effect of DTT on protein glucosylation. Double mutant (*gls2/alg6*) cells were pre-incubated for 30 min at 28°C and further incubated for 10 min with [¹⁴C]glucose in the absence (A) or presence (B) of 5 mM DTT. Endo H-released oligosaccharides were run on paper chromatography with solvent A. Substances in (A) and (B) were eluted, submitted to strong acid hydrolysis and run on paper chromatography with solvent B (C and D, respectively). Standards: 1, Glc₁Man₉GlcNAc₂; 2, Man₉GlcNAc₂; 3, Man₈GlcNAc₂; 4, mannose; 5, glucose and 6, galactose.

encoding the dolichol-P-Glc-dependent glucosyltransferase which transfers the first glucose unit to Man₉GlcNAc₂-P-P-dolichol yielded mutants (*alg6*) that, upon incubation with [¹⁴C]glucose for 15 min, formed Man₉GlcNAc₂ and Man₈GlcNAc₂ in the absence of DNJ, and a mixture of Glc₁Man₉GlcNAc₂, Man₉GlcNAc₂ and Man₈GlcNAc₂ in its presence (data not shown).

The *gls2/alg6* double mutant was completely devoid of GII activity as judged by incubating microsomes with [glucose-¹⁴C]Glc₁Man₉GlcNAc₂ as substrate (Figure 2C). An incubation of intact *gls2/alg6* cells with [¹⁴C]glucose for 15 min in the absence of DNJ led to the production of protein-linked Glc₁Man₉GlcNAc₂, Man₉GlcNAc₂ and Man₈GlcNAc₂ (Figure 2D). The same pattern was obtained when the incubation was performed in the presence of 5 mM DNJ (data not shown). Strong acid hydrolysis of compounds shown in Figure 2D yielded labeled glucose and mannose units (data not shown; a similar result is depicted in Figure 3C and D). It is worth mentioning that although all labeling procedures were performed with mutant cells in the exponential phase of growth, the apparent proportion of glucosylated and unglucosylated compounds synthesized varied in samples withdrawn in the early, medium or late stages. This was due, at least partially, to different relative specific activities of the glucose and mannose residues in the protein-linked oligosaccharides. Apparently the endogenous content of unlabeled sugar nucleotides depended on the stage of the culture. It is for this reason that results shown in Table I, experiments 2 and 3 and in Figures 3 and 5 were performed in each case with samples withdrawn from the same cultures.

Table I. The effects of DTT and inhibition of glycosylation on glycoprotein glucosylation

Experiment ^a	Additions	Pre-incubation	Incubation	Result ^b
1	–	10 min	30 min	319–277
	thyroglobulin	10 min	30 min	3526–3519
	thyroglobulin + 5 mM DTT	10 min	30 min	3520–3452
2	–	10 min	10 min	0.18
	5 mM DTT	10 min	10 min	0.16
	–	20 min	10 min	0.18
	5 mM DTT	20 min	10 min	0.17
	–	30 min	10 min	0.18
	5 mM DTT	30 min	10 min	0.16
3	5 mM DTT	10 min	10 min	0.18
	5 mM DTT + cycloheximide	10 min	25 min	0.16

^aExperiment 1 corresponds to a GT cell-free assay. Experiments 2 and 3 were performed with intact cells.

^bResults in experiment 1 represent c.p.m. in 10% trichloroacetic acid insoluble material. In experiments 2 and 3 they represent the ratio of c.p.m. incorporated as glucose over mannose in *N*-linked oligosaccharides. For further explanation see text.

The *gls2/alg6* cells grew in Yeast Extract (YE) medium at 28°C at the same rate as wild-type and both cell types had the same morphology under the light microscope. On the other hand double mutant cells had the so-called thermosensitive morphology when grown at 37°C as they swelled up, displaying pear, lemon and round shapes (Ishiguro *et al.*, 1997).

The effect of dithiothreitol (DTT) on protein glucosylation

Several procedures have been employed for promoting misfolding of glycoproteins in the ER of intact cells. The most widely used are inhibition of protein glucosylation, addition of a calcium ionophore or of a reducing agent to the medium and a heat shock. The first two were discarded for being impractical as glycoprotein glucosylation is precisely the object of the study and GT requires Ca²⁺ for activity. Therefore, glucosylation of glycoproteins was studied under misfolding conditions promoted by addition of a reducing agent or by a heat shock. Concerning the first condition, it has been reported that addition of DTT at millimolar concentrations to mammalian and yeast cells delays folding (and thence secretion) of disulfide-containing proteins in the ER by modifying the redox potential in the lumen of the organelle. Removal of the drug (or addition of oxidized glutathione) elicited proper folding and secretion of the retained species thus indicating that misfolded proteins were not in irreversible aggregates (Jamsa *et al.*, 1994; Simons *et al.*, 1995). On the other hand, folding and secretion of disulfide-free proteins was not affected by DTT. *S.cerevisiae* cells not only remained viable but were able to divide at higher DTT concentrations and for much longer periods than those employed in the present report (Jamsa *et al.*, 1994; Simons *et al.*, 1995). In addition, we have often employed reduction with DTT for converting native glycoproteins into substrates for GT in cell-free assays. DTT at a 5 mM concentration did not affect *S.pombe* GT activity as judged by a cell-free assay performed using denatured thyroglobulin as acceptor (Table I, experiment 1).

In order to determine whether addition of DTT to intact cells promoted an increased glucosylation of glycoproteins, *gls2/alg6* cells were pre-incubated for 10, 20 and 30 min and further incubated with [¹⁴C]glucose for 10 min, both in the presence or absence of 5 mM DTT. Total cell proteins were degraded with a non-specific protease and endo-β-*N*-acetylglucosaminidase H (Endo H)-sensitive oligosaccharides were liberated from the resulting glycopeptides. The chromatographic patterns of the oligosaccharides obtained were similar (if not identical) in all samples and revealed, as expected, the presence of Glc₁Man₉GlcNAc₂, Man₉GlcNAc₂ and Man₈GlcNAc₂. Strong acid hydrolysis of the saccharides showed the presence of labeled glucose and mannose units. The patterns of oligosaccharides of the 30 min pre-incubation–10 min incubation samples, obtained with and without DTT in the medium, as well as their respective monosaccharide compositions are depicted in Figure 3A–D. It may be observed that the patterns of oligosaccharides (Figure 3A and B) and that of monosaccharides (Figure 3C and D) are practically superimposable.

It is worth stressing that due to the short incubation period with the label, no Golgi-specific modification of oligosaccharides (addition of mannose and galactose units to Man₉GlcNAc₂) had occurred in samples with or without DTT. Therefore, label in mannose residues in oligosaccharides is an indication of protein glucosylation whereas label in glucose units indicates protein glucosylation. In order to compare protein glucosylation under the different experimental conditions employed and to compensate for differential losses in the manipulation of samples, protein glucosylation was expressed as the ratios of label in the glucose over that in the mannose units. Results obtained are depicted in Table I, experiment 2. As similar values were obtained in all cases it may be concluded that no increased glucosylation occurred in the presence of the drug. In addition results obtained show that not only GT activity but also other steps involved in protein glucosylation (synthesis of UDP-Glc, transport of the sugar nucleotide through the ER membrane etc.) were not affected by the drug. The insensitivity of the glucosylating machinery to DTT agrees with previous results of Hebert *et al.* (1995): they showed that influenza virus hemagglutinin (a glycoprotein having seven *N*-linked oligosaccharides) translated in the presence of 4 mM DTT in an *in vitro* system using dog pancreas microsomes was in a misfolded conformation and only partially glucosylated. Addition of castanospermine (a GII inhibitor) promoted further glucosylation of hemagglutinin molecules, thus indicating that in the absence of castanospermine misfolded hemagglutinin was continuously glucosylated by GT and deglycosylated by GII.

Several explanations may be envisaged for the absence of an increased glucosylation in the presence of DTT: (i) contrary to what happens in mammalian and *S.cerevisiae* cells, DTT does not promote glycoprotein misfolding in *S.pombe*; (ii) *gls2/alg6* cells have low amounts of GT so they are unable to glucosylate all the oligosaccharides that are continuously transferred; (iii) only an extremely minor proportion of glycoproteins synthesized have disulfide bridges and thence practically no misfolded species are generated by DTT, or alternatively, (iv) a

Fig. 4. DTT-induced synthesis of misfolded glycoproteins. Wild-type *S.pombe* cells were pre-incubated (10 min) and further incubated with [^{35} S]Met and [^{35} S]Cys (10 min), both in the presence and absence of 5 mM DTT. Samples were processed as described under Materials and methods, incubated with anti *S.pombe* calnexin polyclonal antiserum and protein A-Sepharose and run on SDS-PAGE under non-reducing (NR) or reducing (R) conditions.

misfolded conformation is not a sufficient condition for glucosylation of all *N*-linked oligosaccharides.

In order to rule out the first possibility wild-type cells were pre-incubated with or without 5 mM DTT for 10 min and further incubated with [^{35} S]Met and [^{35} S]Cys for another 10 min. Cells were then disrupted in the presence of *N*-ethylmaleimide and labeled proteins that reacted with anti *S.pombe* calnexin polyclonal antiserum were run on SDS-PAGE under reducing and non-reducing conditions. As shown in Figure 4, identical migration of the lectin synthesized in the presence or absence of DTT was observed under the first condition. Under non-reducing conditions the lectin synthesized in the presence of DTT had the same migration as fully reduced calnexin whereas that synthesized in the absence of the drug migrated somewhat faster. Results obtained showed, therefore, that DTT had effectively inhibited proper folding of an ER glycoprotein synthesized during a period similar to that employed for labeling oligosaccharides with [^{14} C]glucose. A similar effect of DTT on mammalian calnexin migration under reducing and non-reducing conditions in SDS-PAGE has been observed previously (Hebert *et al.*, 1995). There are four Cys residues that are conserved in calnexins from several origins including those from mammals and *S.pombe* (Jannatipour and Rokeach, 1995). At least two of those residues form a disulfide bridge (Hebert *et al.*, 1995).

To rule out the second possibility, i.e. that the amount of GT in *gls2/alg6* cells might be not enough for glucosylating all the oligosaccharides which are continuously transferred, double mutant cells were pre-incubated for 10 min in the presence of 5 mM DTT and further incubated with [^{14} C]glucose for 10 min. Cycloheximide (50 μ g/ml) was then added and the sample was incubated for 15 additional minutes. Cycloheximide inhibits protein synthesis and thence glycosylation but not glucosylation. The patterns of protein-linked oligosaccharides and of monosaccharide constituents of aliquots taken before and after addition of the protein synthesis inhibitor were similar and resembled those shown in Figure 3. The ratios of label in the glucose over that in mannose units are shown in Table I, experiment 3. As similar values were obtained for both samples, it may be concluded that no additional glucosylation occurred in the absence of glycosylation.

Protein-linked oligosaccharides formed by the *gls2/alg6* double mutant cells under normal conditions

In order to discern between the last two possibilities mentioned above for non-induction of glycoprotein

Fig. 5. *N*-linked oligosaccharides formed by *gls2/alg6* mutant cells in long-term incubations. Cells (*gls2/alg6*) were labeled as described under Materials and methods in the absence (A) or presence (B) of 5 mM DTT. Endo H-released oligosaccharides were run on paper chromatography with solvent A. Standards: 1, Glc₁Man₉GlcNAc and 2, Man₉GlcNAc. For further details see Materials and methods.

glucosylation by DTT (low amount of disulfide containing glycoproteins, that is low amount of acceptor substrates generated by DTT or alternatively, that a misfolded conformation might not be a sufficient condition for glucosylation of all *N*-linked oligosaccharides) double mutant cells were pre-incubated for 15 min, then incubated for 60 min with [^{14} C]glucose and further incubated for 30 min with an excess of unlabeled glucose, both in the presence or absence of 5 mM DTT. As mentioned above, it has been described that in mammalian and *S.cerevisiae* cells DTT affects folding and thence secretion of disulfide-containing proteins but does not affect secretion of disulfide-free species. It was speculated, therefore, that if the drug had not generated a significant amount of misfolded glycoproteins, Golgi-specific processing of oligosaccharides (addition of mannose and galactose units to Man₉GlcNAc₂) would be similar in both samples. On the contrary, if DTT affected folding and secretion of the majority of glycoprotein molecules formed, addition of galactose and mannose residues to transferred oligosaccharides would be mainly observed in cells incubated without DTT.

Double mutant cells pulse-chased as described above (15 min pre-incubation–60 min incubation with [^{14} C]glucose–30 min chase with unlabeled glucose, in the absence of DTT) yielded a rather complex pattern of protein-linked oligosaccharides (Figure 5A). The main peak migrated as a Glc₁Man₉GlcNAc standard but a trailing toward Man₉GlcNAc was observed. In addition, several larger compounds, migrating between 7 and 23 cm in Figure 5A, were obtained.

Compounds migrating between 26 and 32 cm in Figure 5A (i.e. as Glc₁Man₉GlcNAc and Man₉GlcNAc standards) were treated with Jack bean α -mannosidase. Compounds obtained migrated as Glc₁Man₅GlcNAc, Glc₁Man₄GlcNAc and mannose standards (Figure 6A). In addition, a very small amount of a compound migrating

Fig. 6. Structural characterization of oligosaccharides. Compounds migrating between 26 and 32 cm in Figure 5A (A), between 7 and 22 cm in Figure 5A (B) and between 26 and 34 cm in Figure 5B (C) were submitted to α -mannosidase degradation. Reaction products were run on paper chromatography with solvent C. Standards: 1, mannose; 2, ManGlcNAc; 3, Glc₁Man₄GlcNAc and 4, Glc₁Man₅GlcNAc.

as a ManGlcNAc standard was observed (Figure 6A, inset). This confirms the presence of Man₉GlcNAc in the sample as degradation of this compound with α -mannosidase produces mannose and ManGlcNAc. The monosaccharides in this disaccharide are linked by a bond in a β configuration. Strong acid hydrolysis of compounds migrating either as Glc₁Man₅GlcNAc or Glc₁Man₄GlcNAc standards in Figure 5A produced labeled galactose, glucose and mannose units (Figure 7A). It may be concluded, therefore, that compounds migrating between 26 and 32 cm in Figure 5A were Glc₁Man₉GlcNAc, Gal₁Man₉GlcNAc and a small amount of Man₉GlcNAc.

Degradation of compounds migrating between 7 and 23 cm in Figure 5A produced mannose and α -mannosidase resistant compounds that were larger than the Glc₁Man₅GlcNAc standard (Figure 6B). Strong acid hydrolysis of the α -mannosidase-resistant cores yielded galactose, glucose and mannose units (Figure 7B).

We conclude, therefore, that upon transfer of Man₉GlcNAc₂ to protein, part of the protein-linked compound was glucosylated by GT to form Glc₁Man₉GlcNAc₂, the other part was galactosylated to form Gal₁Man₉GlcNAc₂, whereas further addition of mannose and galactose units to compounds mentioned above produced the larger saccharides that migrated between 7 and 23 cm in Figure 5A. Consequently, the double mutant cells yielded compounds being structurally similar to those described to occur in wild-type cells, except for the retention of glucose units caused by the *glc2* mutation. As previously mentioned, addition of glucose to Man₉GlcNAc₂ occurs in the ER whereas mannose and galactose units are added in the Golgi.

Fig. 7. Structural characterization of oligosaccharides. Compounds migrating as standard Glc₁Man₅GlcNAc in Figure 6A (A), between 0 and 5 cm in Figure 6B (B) or as standard Glc₁Man₅GlcNAc in Figure 6C (C) were submitted to strong acid hydrolysis and run on paper chromatography with solvent B. Standards: 1, mannose; 2, glucose and 3, galactose.

Protein-linked oligosaccharides formed by the *glc2/alg6* double mutant cells under conditions that delay ER protein folding

The same labeling protocol described in the previous section was repeated but with 5 mM DTT in the medium. Total label incorporation (mainly in polysaccharides and in amino acids in proteins; label in Endo H-released compounds was always <0.5% of the total) was almost identical under normal and protein misfolding-promoting conditions. This indicated that those conditions did not significantly affect the overall cellular energy production.

The pattern of protein-linked oligosaccharides obtained is shown in Figure 5B. The main peak migrated as a Glc₁Man₉GlcNAc standard and a clearly noticeable shoulder in the position of Man₉GlcNAc appeared. Treatment of the sample with α -mannosidase yielded compounds migrating as Glc₁Man₅GlcNAc, Glc₁Man₄GlcNAc, ManGlcNAc and mannose standards (Figure 6C). Strong acid hydrolysis of compounds migrating as either one of the two first standards yielded glucose and mannose residues (Figure 7C). It may be concluded that the compounds present in Figure 5B were Glc₁Man₉GlcNAc and Man₉GlcNAc. Except for a very small peak migrating between 18 and 20 cm in Figure 5B, corresponding to material larger than the Glc₁Man₉GlcNAc₂ standard, no Golgi modification appeared, thus indicating that the bulk of glycoproteins synthesized in *S.pombe* cells had disulfide bridges as they had been retained in the ER. The scant amount of label in the substance migrating between 18 and 20 cm precluded its structural identification but it presumably corresponds to Golgi-processed compounds.

The relative proportion of glucosylated and unglucosylated oligosaccharides in Figure 5B was calculated as previously described (Gañán *et al.*, 1991). Briefly, the label in mannose in ManGlcNAc divided by the sum of the label in mannose in the same compound plus that in

Fig. 8. *N*-linked oligosaccharides formed by wild-type and *gls2/alg6* mutant cells under heat shock. Wild-type (A) or double mutant (*gls2/alg6*) (B) cells were labeled as described under Materials and methods (15 min pre-incubation, 60 min incubation with the label and 30 min chase) at 38 or 37°C, respectively. Endo H-released oligosaccharides were run on paper chromatography with solvent A. Standards: 1, Glc₁Man₉GlcNAc; 9, Man₉GlcNAc and 8, Man₈GlcNAc.

mannose in Glc₁Man₄GlcNAc divided by four, plus that in Glc₁Man₅GlcNAc divided by five gave the relative proportion of unglucosylated compounds. No labeled glucosamine appeared in the experiment shown in Figure 7C, thus indicating that those residues were not significantly labeled during the 60 min incubation with [¹⁴C]glucose. This fact facilitated quantitation of mannose label in ManGlcNAc. The relative proportion of unglucosylated compounds obtained was ~45%. Glc₁Man₉GlcNAc is slightly over-represented in Figure 5B because, as may be calculated from Figure 7C, the specific activity of glucose was about 2-fold higher than that of mannose.

Protein-linked oligosaccharides formed under a heat shock

It has been reported that a heat shock promotes misfolding of ER proteins in *S.pombe* as this treatment triggers a strong induction of BiP-encoding mRNA synthesis, which is the same as in other species (Pidoux and Armstrong, 1992). In order to confirm this finding and to evaluate the amount of glycoproteins synthesized under a heat shock which were unable to properly fold, wild-type *S.pombe* cells grown at 28°C were pulse-chased with [¹⁴C]glucose-unlabeled glucose as described above, but at 38°C. As depicted in Figure 8A, only protein-linked Man₉GlcNAc₂ and Man₈GlcNAc₂ were detected. As the secretion mechanism is not expected to be thermosensitive at 38°C in wild-type cells (these cells grow normally at 39°C), the absence of Golgi-specific oligosaccharide elongation indicated that all glycoproteins synthesized under the heat shock were unable to properly fold as they had been retained in the ER.

Folding of glycoproteins in the *gls2/alg6* mutant cells is expected to be especially sensitive to heat shock because, as mentioned above, the mutant glycoproteins are thought to be underglycosylated and cells display the so-called thermosensitive morphology at 37°C. It was tested, therefore, if unglucosylated oligosaccharides were present in glycoproteins that misfolded due to a heat shock. The pattern of protein-linked oligosaccharides synthesized by

double mutant cells submitted to the same pulse-chase protocol but at 37°C (Figure 8B) was very similar to that formed in the presence of DTT but at 28°C (Figure 5B). Oligosaccharides migrated as Glc₁Man₉GlcNAc, Man₉GlcNAc and Man₈GlcNAc standards, that is, no Golgi-specific processing was detected in the heat-shock sample and not all protein-linked oligosaccharides were glucosylated. This result showed that, the same as found upon DTT addition, a misfolded conformation generated by a heat shock did not lead to glucosylation of all *N*-linked compounds.

Discussion

Gls2/alg6 double mutant cells were used in work presented here to ensure that all the glucose units present in protein-linked oligosaccharides had been added by GT and that no removal of those units had occurred.

We have found that induction of glycoprotein misfolding upon addition of millimolar DTT concentrations to the medium did not promote an increase in GT-mediated glycoprotein glucosylation. It was proved that this result could not be due to: (i) failure of DTT to induce glycoprotein misfolding; (ii) a deleterious effect of DTT on cellular glucosylating machinery; (iii) low amounts of the glucosylating enzyme and (iv) low amounts of disulfide-containing glycoproteins synthesized in *S.pombe* ER (i.e. a low amount of misfolded species created upon DTT addition). It was concluded that, contrary to what happens in cell-free assays, a misfolded conformation is not a sufficient condition for *in vivo* GT-mediated glycoprotein glucosylation. Addition of millimolar concentrations of DTT to the incubation medium has been widely used before in yeast cells. The drug prevents folding and secretion of disulfide-containing species whereas it does not affect proteins devoid of those bonds (Jamsa *et al.*, 1994; Simons *et al.*, 1995).

The other procedure employed for promoting glycoprotein misfolding (heat shock) was found to prevent proper folding of all glycoproteins synthesized in wild-type cells as only ER-specific processing oligosaccharides was detected. A significant proportion of unglucosylated, ER-retained, oligosaccharides was found when the same experiment was performed with *gls2/alg6* mutant cells, thus confirming that a misfolded conformation is not a sufficient condition for *in vivo* GT-mediated glucosylation of all *N*-linked oligosaccharides.

It is unknown whether all *N*-linked oligosaccharides present in denatured glycoproteins having several of them may be glucosylated by GT in cell-free assays. Nevertheless, the fact that all natural denatured glycoproteins having a single oligosaccharide tested so far (for instance ribonuclease B, soybean agglutinin, phytohemagglutinin etc., see Sousa *et al.*, 1992; Trombetta and Parodi, 1992) were found to be glucosylated strongly suggests that denaturation of the protein moiety is a necessary and sufficient condition for cell-free glucosylation. Chemically synthesized glycoproteins (neoglycoproteins) also support the idea that glycoprotein denaturation is a necessary and sufficient condition for cell-free glucosylation by GT: a single glycopeptide was chemically linked to either one of two cysteine units located at different positions in the primary sequence of a staphylococcus nuclease. Both

neoglycoproteins were equally glucosylated by GT when in a misfolded conformation (Sousa and Parodi, 1995).

The property of exclusively glucosylating misfolded glycoproteins in cell-free assays was shared by GTs purified from mammalian, insect and yeast cells (Trombetta and Parodi, 1992; Fernández *et al.*, 1994; Parker *et al.*, 1995). It was also reported that GT glucosylation was dependent on the enzyme recognition of protein domains exposed in denatured but not in native conformations as neither glycopeptides or native glycoproteins were glucosylated by the enzyme (Sousa *et al.*, 1992). It was suggested that those protein domains could be clusters of hydrophobic amino acids as it was found that the enzyme was retained by hydrophobic but not by hydrophilic peptides linked to Sepharose 4B at physiological conditions of pH and salt concentration. Denatured but not native glycoproteins prevented binding (Sousa and Parodi, 1995). Exposure of hydrophobic amino acid side chains is the only feature common to all protein moieties of denatured glycoproteins.

Exposed protein domains were apparently not the only recognition elements required for glucosylation as the enzymatic reaction was not inhibited by denatured non-glucosylated proteins. It was found that a very effective inhibition occurred, nevertheless, when the glucosylation reaction was challenged with denatured glycoproteins to which the oligosaccharides had been removed with Endo H (Sousa and Parodi, 1995). Denatured non-glucosylated proteins having identical or very similar amino acid primary sequences to Endo H-treated species did not inhibit the glucosylation reaction. It was concluded, therefore, that GT also required accessibility to the innermost *N*-acetylglucosamine units of the oligosaccharide in order to catalyze glucosylation (Sousa and Parodi, 1995). Structural studies have shown that there is an interaction of the *N*-acetylglucosamine unit with neighboring amino acids in native conformations (Dessen *et al.*, 1995). Moreover, evidence was presented indicating that both recognition elements (the exposed protein domains and the innermost *N*-acetylglucosamine units) had to be covalently linked as no glucosylation occurred in incubation mixtures containing glycopeptides and denatured non-glucosylated proteins. A chemical cross-linking of both element yielded, however, highly efficiently glucosylated substrates (Sousa and Parodi, 1995).

Glucosylation-deglucosylation cycles catalyzed by GT and GII have been described to occur in glycoproteins that misfolded due to amino acid mutations or to the addition of DTT to the medium (Suh *et al.*, 1989; Hebert *et al.*, 1995). In order to explain the fact that not all *N*-linked oligosaccharides were glucosylated *in vivo*, it may be speculated that some of the above mentioned GT recognition elements are hindered as soon as they enter the ER lumen. For instance, immediate proper folding not of an entire glycoprotein but only of domains where the oligosaccharides are attached could prevent recognition of the innermost *N*-acetylglucosamine unit. Alternatively, the possibility exists that certain protein domains whose recognition by GT is required for glucosylation could be occluded as soon as they enter the ER lumen by chaperones such as BiP bind hydrophobic amino acids or by other proteins that recognize those same elements. Hsp70 chaperones such as BiP show a certain specificity toward

the hydrophobic sequences they bind (Rüdiger *et al.*, 1997). It should be expected, therefore, that not all the hydrophobic domains would be blocked by the chaperone. The interaction of glycoproteins synthesized by *S.cerevisiae* in the presence of 4 mM DTT with BiP has been described already (Simons *et al.*, 1995). As proper folding was artificially prevented in work reported here by the addition of DTT or by raising the temperature, a permanent blocking of certain GT recognition elements by chaperones is expected to occur. In any case, results presented here clearly show that unlike what happens in cell-free assays, glycoprotein misfolding is not a sufficient condition for *in vivo* GT-mediated glucosylation.

This result is relevant to the quality control of glycoprotein folding. As mentioned above, it is assumed that species not properly folded oscillate between non-glucosylated and monoglucosylated states. The latter are recognized by calnexin/calreticulin and this interaction facilitates folding of bound species and prevents their exit from the ER. What the present report implies is that certain *N*-linked oligosaccharides (and probably also certain glycoprotein species containing one or only few *N*-linked compounds) never participate in an interaction with calnexin/calreticulin mediated by GT. *N*-linked oligosaccharides not recognized by GT would only interact with the lectins through the monoglucosylated oligosaccharides generated by deglucosylation of the transferred compound (Glc₃Man₉GlcNAc₂). Nevertheless, as deglucosylation of this compound is an extremely rapid process (see for instance Figure 2B), a severe limitation in the interaction of oligosaccharides not recognized by GT with calnexin/calreticulin is expected to occur.

It is unknown if specific oligosaccharides in individual misfolded glycoproteins are not glucosylated *in vivo* or if, alternatively, the same oligosaccharide may be glucosylated in some molecules and not in others. It is worth mentioning that it was reported that only ~50–60% of *N*-linked oligosaccharides in properly folded, mature glycoproteins had been glucosylated by GT in *Trypanosoma cruzi* (Gañán *et al.*, 1991). When individual glycoproteins were studied, it was found that the same oligosaccharide had been glucosylated in some molecules but not in others (Labriola *et al.*, 1995). It was suggested that glucosylation was restricted to molecules that had had folding problems, probably because they had not been recognized by the proper chaperones at the proper place and at the proper time. It is possible, therefore, that the same as that found in properly folded glycoproteins, also in individual permanently misfolded ones *in vivo* glucosylation of the same oligosaccharide could occur in some molecules but not in others. A study of the structure of the oligosaccharides of individual glycoproteins synthesized by *gls2/alg6* cells in the presence of DTT will be required for discerning between both possibilities.

Materials and methods

Strains and media

Escherichia coli DH5a was used for cloning procedures. The *S.pombe* strain used was h⁹⁰, *ura4-D18*, *ade6-M216*, *leu1-32*. The YE medium contained 3% glucose, 0.5% Yeast Extract (Difco) and was supplemented with adenine.

Disruption of the *GII*-encoding gene

Known *S.cerevisiae* (DDBJ/EMBL/GenBank accession No. Z36098), human (DDBJ/EMBL/GenBank accession No. D42041) and putative *Caenorhabditis elegans* (DDBJ/EMBL/GenBank accession No. Z700753) *GII* amino acid sequences were aligned and primers (sense 5'-TGGAAAYGAYATGAAYGARCC-3' and antisense 5'-IGCRTGNGC-ICKRAARAA-3') were designed according to peptides WNDMNEP and FFRAHA which are conserved in the three sequences mentioned above (amino acids 535–541 and 668–673 from *S.cerevisiae* *GII*). A 414 bp fragment of the homolog *S.pombe* gene (here designated *gls2*⁺) was obtained by PCR using genomic wild-type DNA as template. The fragment was sequenced and an antisense primer (5'-CCACCG-AACAAACAATTTCAGC-3') was designed according to the sequence of positions 355–375 of the 414 bp fragment. This primer and a sense one derived from the conserved sequence NAAETW (5'-AAYGCNGCNG-ANACNTGG-3', positions 330–335 of *S.cerevisiae* *GII*) were used to synthesize a 998 bp fragment by PCR, using DNA as a template. The 998 bp fragment was cloned in a pGEMT-Easy vector (Promega) and sequenced. The conceptual translation of this fragment showed homologies of 49, 43 and 47% with the corresponding respective fragments of human, *S.cerevisiae* and putative *C.elegans* *GII* sequences. The *ura4*⁺ marker gene (DDBJ/EMBL/GenBank accession No. M36504) was introduced in reverse orientation in position 569 (*Hind*III site) of the 998 bp fragment. Wild-type cells (see above) were transformed with a linear 2766 bp *NotI* restriction DNA fragment containing the *ura4*⁺ gene flanked by 672 bp (5') and 330 bp (3') of *gls2*⁺ DNA. A standard lithium acetate protocol was used. The resulting *GII* minus mutant (*gls2*) was then h⁹⁰, *ura4*-D18, *ade6*-M216, *leu1*-32, *gls2::ura4*⁺.

Excision of the *ura4*⁺ gene

The vector containing the 998 bp fragment of *gls2*⁺ gene having *ura4*⁺ at position 569 (*Hind*III site) in an inverted position was treated with *Bsu*36I. An 1198 bp fragment was liberated that had 210 bp of the *gls2*⁺ plus 988 bp of the 3' end of *ura4*⁺. The digestion products were run on an agarose gel and a 4565 bp band containing the vector with 359 bp of the 5' end of the *gls2*⁺, 776 bp of the 5' end of the *ura4*⁺ and 430 bp of the 3' end of the *gls2*⁺ genes was extracted and ligated. *E.coli* cells were transformed, the plasmids isolated and a fragment of 1565 bp liberated from them with *NotI* which cuts in the polylinker region of the vector. The fragment had the portions of *gls2*⁺ and *ura4*⁺ genes mentioned above. The *gls2::ura4*⁺ mutant was then transformed with the 1565 bp fragment as above but after transformation cells were grown overnight on YE medium to dilute the *ura4*⁺ gene product and then plated on minimal medium, plus adenine, leucine, uracil and fluororotic acid (1 mg/ml). After 7 days, a colony PCR was performed of fluororotic acid-resistant cells (i.e. *gls2::ura4*). For colony PCR the oligonucleotide primers were 5'-GTCGTCCGCTTCTCCCTC-3' (*GIIBs*) and 5'-CCCC-ATAATGAATAGCAT-3' (*GIIBa*). The primers are complementary to portions of the *gls2*⁺ gene fragment and generate a 542 bp fragment in wild-type cells, a 2306 bp fragment in *gls2::ura4*⁺ cells and a 1108 bp fragment in *gls2::ura4* cells. All clones yielded the expected 1108 bp fragment.

Construction of the *gls2/alg6* double mutant

Degenerate oligonucleotide primers which code for three peptides that are conserved in the *ALG6* *S.cerevisiae* (DDBJ/EMBL/GenBank accession No. Z74910) and the possible *ALG6* *C.elegans* (DDBJ/EMBL/GenBank accession No. Z54342) sequences were used as primers in PCR reactions. Genomic *S.pombe* DNA was used as a template. The peptides were YWGLDYPP, FQ/HVHEKTIL and HWMEIT, and the respective primers were 5'-TAYTGGGGNTYNGAYTATCCNCC-3' (*Alg1*⁺, sense), 5'-DATNGTYTTYTCRTGNACNYGRA-3' (*Alg4*⁺, antisense) and 5'-CAYTGGATGGARATHAC-3' (*Alg6s*, sense). PCRs using primers *Alg1*⁺ and *Alg4*⁺ yielded an 812 bp fragment (annealing temperature 52°C) that was sequenced. Conceptual translations of this product gave homologies of 82 and 80% with the respective portions of *S.cerevisiae* *ALG6* and putative *C.elegans* *ALG6* genes, respectively. An 876 bp fragment was obtained when primers *Alg6s* and *Alg4*⁺ were used. The fragment was sequenced and as expected it was found that it contained the 812 bp fragment. The 812 bp fragment in pGEMT-Easy vector (Promega) was treated with *Bgl*II and *Nhe*I enzymes. A 102 bp segment was thus eliminated from the *alg6*⁺ gene fragment. The *ura4*⁺ gene in an inverted position replaced the segment. The construct had at its 5' and 3' ends 148 bp and 562 bp fragments of the *alg6*⁺ gene. The construct was liberated from the vector with *Kpn*I and *Xba*I that cut it in the polylinker region. The *gls2::ura4* cells were then transformed with this linear fragment having portions of the *alg6*⁺ gene interrupted

with *ura4*⁺. Transformants were grown in minimal medium supplemented with adenine and leucine and *ura4*⁺ cells were selected. Colony PCRs were performed using primers *GIIBs* and *GIIBa* (see above) or *Alg6s* (sense) and *uraSC* (sense, 5'-TGCTCCTACAACATTACC-3'). This primer is complementary to a portion of the 3' end of the *ura4*⁺ gene. Selected colonies (*gls2/alg6*) gave the expected fragments of 1108 bp with *GIIBs* and *GIIBa* primers and of 423 bp with the *Alg6s* and *uraSC* ones. Resulting cells were then h⁹⁰, *ura4*-D18, *ade6*-M216, *leu1*-32, *gls2::ura4*, *alg6::ura4*⁺.

RNA procedures

Cells were grown under normal conditions (YE medium at 28°C). RNA was extracted from cells in exponential phase of growth at the same OD and submitted to Northern blotting as performed previously (Jannatipour and Rokeach, 1995). Probes used were DNA fragments synthesized by PCR using genomic DNA as template and had 1004 and 973 bp from *BiP*- and actin-encoding genes, respectively.

Materials

[¹⁴C]glucose (250 Ci/mol) and EasyTag Express protein labeling mix (>1000 Ci/mmol) were from New England Nuclear. Jack bean α-mannosidase and Endo H were from Sigma. UDP-[¹⁴C]Glc (250 Ci/mol) was prepared as described by Wright and Robbins (1965). Anti recombinant *S.pombe* calnexin polyclonal antiserum was a generous gift from Dr Luis Rokeach, Département de Biochimie, Université de Montréal, Montréal, Canada.

Substrates and standards

[glucose-¹⁴C]Glc₁Man₉GlcNAc, [glucose-¹⁴C]Glc₃Man₉GlcNAc, [glucose-¹⁴C]Glc₂Man₉GlcNAc, [glucose-¹⁴C]Glc₁Man₄GlcNAc, [glucose-¹⁴C]Glc₁Man₅GlcNAc, [¹⁴C]ManGlcNAc, [¹⁴C]Man₉GlcNAc and [¹⁴C]Man₉GlcNAc were prepared as described previously (Fernández *et al.*, 1994).

In vivo labeling of *S.pombe* cells with [¹⁴C]glucose

Short-term labeling of *S.pombe* wild-type and *gls2/alg6* cells with [¹⁴C]glucose (Figures 2 and 3) was performed as described before for *S.cerevisiae* but 150 µCi of [¹⁴C]glucose was used (Fernández *et al.*, 1994). Incubation with the label lasted for 15 min (Figure 2) or 10 min (Figure 3). Where indicated 5 mM DNJ was added during the 15 min pre-incubation and 15 min incubation (Figure 2), or 5 mM DTT was added during the different pre-incubation periods and 10 min incubation with the label (Figure 3). Long-term labeling of *gls2/alg6* mutant cells (Figure 5) was performed as follows: cells (0.5 g) in the early exponential phase of growth were harvested, washed three times with 1% Yeast Nitrogen Base (Difco) and resuspended in 1.8 ml of the same solution. Two aliquots of 1 ml each were withdrawn. One of them received DTT (5 mM final concentration). Both aliquots were incubated for 15 min at 28°C after which 200 µCi of [¹⁴C]glucose was added. Unlabeled glucose (0.1 M final concentration) was added after 60 min and incubation prolonged for 30 additional min. Isolation of Endo H-sensitive oligosaccharides was performed as already described (Fernández *et al.*, 1994). Where indicated pre-incubation, incubation and chase were performed at 38°C with wild-type (Figure 8A) or at 37°C with double mutant (*gls2/alg6*) cells (Figure 8B), in the absence of DTT.

In vivo labeling of *S.pombe* cells with [³⁵S]Met and [³⁵S]Cys

S.pombe wild-type cells (2×10⁸) were pre-incubated at 28°C for 10 min either in the presence or absence of 5 mM DTT in 0.2 ml of Yeast Nitrogen Base without amino acids (Difco), supplemented with adenine, uracil and leucine after which 120 µCi of [³⁵S]Met plus [³⁵S]Cys were added. Incubations were stopped after 10 min at the same temperature upon addition of 1 mM cycloheximide plus 20 mM *N*-ethylmaleimide. One volume of 40 mM sodium azide plus 2 M sorbitol and 50 mM Tris-HCl buffer pH 7.5 was then added and mixtures left on ice for 5 min. Samples were then processed and immunoprecipitated as described by Tabuchi *et al.* (1997). Anti *S.pombe* calnexin antiserum was used. Both samples were then divided in halves. Non-reduced and DTT-reduced immunoprecipitates were run on 12% SDS-PAGE and submitted to autoradiography.

Methods

Strong acid hydrolysis and treatment of oligosaccharides with Jack bean α-mannosidase were as described previously (Fernández *et al.*, 1994). Whatman 1 papers were used for chromatography. Solvents employed 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). *GI* and *GII*

activities were assayed as described before using [glucose-¹⁴C]Glc₃Man₉-GlcNAc₂ and [glucose-¹⁴C]Glc₁Man₇₋₉GlcNAc as substrates, respectively (Ugalde *et al.*, 1979). GT was assayed using wild-type *S.pombe* cell microsomes as enzyme source and 8 M urea-denatured thyroglobulin as acceptor as described by Trombetta *et al.* (1989).

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References

- Ballou, L., Gopal, P., Krummel, B., Tammi, M. and Ballou, C. (1986) A mutation that prevents glucosylation of the lipid-linked oligosaccharide precursor leads to underglycosylation of secreted yeast invertase. *Proc. Natl Acad. Sci. USA*, **83**, 3081–3085.
- Bosch, M., Trombetta, S., Engstrom, U. and Parodi, A.J. (1988) Characterization of dolichol diphosphate oligosaccharide:protein oligosaccharyltransferase and of glycoprotein processing glucosidases occurring in trypanosomatids. *J. Biol. Chem.*, **263**, 17360–17365.
- Dessen, A., Gupta, D., Sabewsan, S., Brewer, C.F. and Sacchettini, J.C. (1995) X-ray crystal structure of the Soybean agglutinin cross-linked with a biantennary analog of the blood group I carbohydrate antigen. *Biochemistry*, **34**, 4933–4942.
- Fernández, F., Trombetta, S.E., Hellman, U. and Parodi, A.J. (1994) Purification to homogeneity of UDP-Glc:glycoprotein glucosyltransferase from *Schizosaccharomyces pombe* and apparent absence of the enzyme from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **269**, 30701–30706.
- Fernández, F., Jannatipour, M., Hellman, U., Rokeach, L. and Parodi, A.J. (1996) A new stress protein: synthesis of *Schizosaccharomyces pombe* UDP-Glc:glycoprotein glucosyltransferase mRNA is induced under stress conditions but the enzyme is not essential for cell viability. *EMBO J.*, **15**, 705–713.
- Gañán, S., Cazzulo, J.J. and Parodi, A.J. (1991) A major proportion of N-glycoproteins is transiently glucosylated in the endoplasmic reticulum. *Biochemistry*, **30**, 3098–3104.
- Hebert, D.N., Foellmer, B. and Helenius, A. (1995) Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell*, **81**, 425–433.
- Helenius, A., Trombetta, E.S., Hebert, D.N. and Simons, J.F. (1997) Calnexin, calreticulin and the folding of glycoproteins. *Trends Cell Biol.*, **7**, 193–200.
- Imperiali, B. and Rickert, K.W. (1995) Conformational implications of asparagine-linked glycosylation. *Proc. Natl Acad. Sci. USA*, **80**, 7466–7470.
- Ishiguro, J., Saitou, A., Durán, A. and Ribas, J.C. (1997) *cpsI*⁺, a *Schizosaccharomyces pombe* gene homologue of *Saccharomyces cerevisiae* FKS genes whose mutation confers hypersensitivity to cyclosporin A and papulacandin B. *J. Bacteriol.*, **179**, 7653–7662.
- Jakob, C.A., Burda, P., te Heesen, S., Aebi, M. and Roth, J. (1998) Genetic tailoring of N-linked oligosaccharides: the role of glucose residues in glycoprotein processing in *Saccharomyces cerevisiae* in vivo. *Glycobiology*, **8**, 155–164.
- Jamsa, E., Simonen, M. and Makarow, M. (1994) Selective retention of secretory proteins in the yeast endoplasmic reticulum by treatment of cells with a reducing agent. *Yeast*, **10**, 355–370.
- Jannatipour, M. and Rokeach, L.A. (1995) The *Schizosaccharomyces pombe* homologue of the chaperone calnexin is essential for viability. *J. Biol. Chem.*, **270**, 4845–4853.
- Labriola, C., Cazzulo, J.J. and Parodi, A.J. (1995) Retention of glucose residues added by the UDP-Glc:glycoprotein glucosyltransferase delays exit of glycoproteins from the endoplasmic reticulum. *J. Cell Biol.*, **130**, 771–779.
- Marquardt, T. and Helenius, A. (1992) Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. *J. Cell Biol.*, **117**, 505–513.
- Parker, C.G., Fessler, L.I., Nelson, R.E. and Fessler, J.H. (1995) *Drosophila* UDP-Glc:glycoprotein glucosyltransferase: sequence and characterization of an enzyme that distinguishes between denatured and native proteins. *EMBO J.*, **14**, 1294–1303.
- Parlati, F., Dignard, D., Bergeron, J.J. and Thomas, D.Y. (1995a) The calnexin homologue *cnx1*⁺ in *Schizosaccharomyces pombe*, is an essential gene which can be complemented by its soluble ER domain. *EMBO J.*, **14**, 3064–3072.
- Parlati, F., Dominguez, M., Bergeron, J.J. and Thomas, D.Y. (1995b) *Saccharomyces cerevisiae* CNE1 encodes an endoplasmic reticulum (ER) membrane protein with sequence similarity to calnexin and calreticulin and functions as a constituent of the ER quality control apparatus. *J. Biol. Chem.*, **270**, 244–253.
- Pidoux, A.L. and Armstrong, J. (1992) Analysis of the BiP gene and identification of an ER retention signal in *Schizosaccharomyces pombe*. *EMBO J.*, **11**, 1583–1591.
- Rüdiger, S., Germeroth, L., Schneider-Mergener, J. and Bukau, B. (1997) Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *EMBO J.*, **16**, 1501–1507.
- Simons, J.F., Ferro-Novick, S., Rose, M.D. and Helenius, A. (1995) BiP/Kar2p serves as a molecular chaperone during carboxypeptidase Y folding in yeast. *J. Cell Biol.*, **130**, 41–49.
- Sousa, M. and Parodi, A.J. (1995) The molecular basis for the recognition of misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. *EMBO J.*, **14**, 4196–4203.
- Sousa, M., Ferrero-García, M. and Parodi, A.J. (1992) Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. *Biochemistry*, **31**, 97–105.
- Suh, P., Bergmann, J.E. and Gabel, C.A. (1989) Selective retention of monoglucosylated high mannose oligosaccharides by a class of mutant vesicular stomatitis virus G protein. *J. Cell Biol.*, **108**, 811–819.
- Tabuchi, M., Iwaihara, O., Ohtani, Y., Ohuchi, N., Sakurai, J.-I., Morita, T., Iwahara, S. and Takegawa, K. (1997) Vacuolar protein transport sorting in fission yeast: cloning, biosynthesis, transport and processing of carboxypeptidase Y from *Schizosaccharomyces pombe*. *J. Bacteriol.*, **179**, 4179–4189.
- Trombetta, S.E. and Parodi, A.J. (1992) Purification to apparent homogeneity and partial characterization of rat liver UDP-glucose:glycoprotein glucosyltransferase. *J. Biol. Chem.*, **267**, 9236–9240.
- Trombetta, S., Bosch, M. and Parodi, A.J. (1989) Glucosylation of glycoproteins by mammalian, plant, fungal and trypanosomatid protozoa microsomal proteins. *Biochemistry*, **28**, 8108–8116.
- Ugalde, R.A., Staneloni, R.J. and Leloir, L.F. (1979) Microsomal glucosidases acting on the saccharide moiety of the glucose-containing dolichyl diphosphate oligosaccharide. *Biochem. Biophys. Res. Commun.*, **91**, 1174–1181.
- Wright, A. and Robbins, P.W. (1965) The enzymatic synthesis of uridine diphosphate [¹⁴C]glucose. *Biochim. Biophys. Acta*, **104**, 594–595.
- Zhang, J.-X., Braakman, I., Matlack, K.E.S. and Helenius, A. (1997) Quality control in the secretory pathway: the role of calreticulin, calnexin and BiP in the retention of glycoproteins with C-terminal truncations. *Mol. Biol. Cell*, **8**, 1943–1954.
- Ziegler, F.D., Gemmil, T.R. and Trimble, R.B. (1994) Glycoprotein synthesis in yeast. Early events in N-linked oligosaccharide processing in *Schizosaccharomyces pombe*. *J. Biol. Chem.*, **268**, 12527–12535.

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