Glycoprotein Biosynthesis in the \textit{alg3} \textit{Saccharomyces cerevisiae} Mutant

I. ROLE OF GLUCOSE IN THE INITIAL GLYCOSYLATION OF INVERTASE IN THE ENDOPLASMIC RETICULUM*

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Oligosaccharides on invertase restricted to the endoplasmic reticulum (ER) in \textit{alg3}, \textit{sec18} yeast at 37 °C were found to be 20% wild type Man$_{n}$GlcNAc and 80% Man$_{a-2}$Man$_{a-2}$Man$_{a-3}$(Man$_{a-6}$)Man$_{b-4}$GlcNAc$_{2}$ (Verostek, M. F., Atkinson, P. H., and Trimble, R. B. (1991) \textit{J. Biol. Chem.} 266, 5547–5551). These results suggested that \textit{alg3} was slightly leaky, but did not address whether the oligosaccharide-lipid Man$_{a}$GlcNAc$_{2}$ and Man$_{b}$GlcNAc$_{3}$ precursors were glucosylated in \textit{alg3} yeast. Therefore, an \textit{alg3}, \textit{sec18}, \textit{glsl} strain was constructed to delete the \textit{GLsl} gene product. Thus, the vast majority of the \textit{N}-linked glycosylation in the ER of \textit{alg3} yeast (>75%) occurs by transfer of Man$_{b}$GlcNAc$_{2}$ without prior addition of the 3 glucose normally found on the lipid-linked precursor.

In both mammalian and yeast cells, Glc$_{2}$Man$_{a}$GlcNAc$_{2}$ is built up sugar by sugar on dolichol pyrophosphate and then transferred as a unit to specific asparagine residues in nascent proteins in the lumen of the ER (1). After transfer, the oligosaccharide is modified by specific trimming and elongation reactions, which in yeast lead to the formation of Man$_{b}$GlcNAc$_{2}$ by the stepwise removal of the 3 glucose and 1 mannose (1). Previous work by Huffaker and Robbins (2) and by Orlean (3) utilizing the \textit{alg3} and \textit{dpm1} strains of yeast, respectively, depict only Man$_{a}$GlcNAc$_{2}$ in profiles of lipid-linked oligosaccharides released by mild acid hydrolysis. Recent studies from our laboratory have identified the precursor for elongation of endo H-resistant mannan in \textit{alg3}, \textit{sec18} (37 °C) yeast to be Man$_{a-2}$Man$_{a-2}$Man$_{a-3}$(Man$_{a-6}$)Man$_{b-4}$GlcNAc$_{2}$ (4).

Although Glc$_{2}$Man$_{a}$GlcNAc$_{2}$ was isolated from class E Thy-1" murine cells (5), it is not currently known whether a glucosylated Man$_{a}$GlcNAc$_{2}$ oligosaccharide was initially transferred during protein glycosylation by \textit{alg3} yeast. Precursor glycosylation appears necessary in mammalian cells (6, 7), but this is not the case in yeast, where failure to add glucose to oligosaccharide-lipid (\textit{alg5}-1 and \textit{alg6}-1 mutations) reduces but does not prevent glycoprotein biosynthesis (8). Recent studies with a conditional ts mutation in the yeast Man-P-Dol synthase (\textit{DPM1}) gene have not resolved this question, as Man$_{a}$GlcNAc$_{2}$-PP-Dol accumulated on shift to the nonpermissive temperature (3).

The purpose of this study was to determine whether glucose was involved in the transfer of lipid-linked oligosaccharide to protein in \textit{alg3} yeast. GLSL codes for glucosidase I (9), which is responsible for trimming the terminal \textit{a1,2}-linked glucose from newly transferred Glc$_{2}$Man$_{a}$GlcNAc$_{2}$ in the ER. An

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‡The abbreviations used are: ER, endoplasmic reticulum; endo H, endo-\textit{a1,2-N}-acetylglucosaminidase H (EC 3.2.1.96); PNGase F, peptide-\textit{N}-acetyl-\textit{a2,3}-glucosaminyl asparagine amidase (EC 3.5.1.52); dan- syl, 5-(dimethylamino)-1-naphthylsulfonyl; PAGE, polyacrylamide gel electrophoresis; Dol, dolichol; G or Glc, glucose; M or Man, mannose; N, GlcNAc, N-acetylgalactosamine; alg, asparagine-linked glycosylation; sec, secretion defective.
**Experimental Procedures**

**Materials**

Saccharomyces cerevisiae haploid strain X79-1A(a) (alg3 - sec18 - gls1) provided by M. Carlson (2); haploid strain SF782-8A(a) (sec18-l, gls1, pep4) was provided by R. Schekman (9). These two strains were mated, zygotes isolated and sporulated, the tetrads dissected, and the four spores grown out. Temperature-sensitive isolates requiring uracil and adenine were screened at 26°C for growth on YEPD plates containing 2.4 mM sodium citrate, pH 5.5, containing 5 mM sodium azide, and 1 mg of external invertase from 5 mg of cells. The major loss in activity seen in steps 3 and 4 of Table 1 is the removal of the nonglycosylated internal form of invertase, which represents about 50% of the total activity and as shown in Figs. 1 and 2 migrates at 59 kDa on SDS-PAGE (15).

**Oligosaccharide Release and Purification—N-Linked oligosaccharides were hydrolyzed from invertase sequentially by treatment with endo H (purified from transformed Escherichia coli) (16) followed by PNGase F according to standard deglycosylation protocols (17); endo H will cleave N-linked chains only if they are substituted with a mannose residue linked α1,3 to the upper arm α1,6-linked mannose of the core (18). Invertase (100 mg) was denatured in a boiling water bath for 5 min in 0.05 M sodium citrate buffer, pH 5.5, containing 0.1 M β-mercaptoethanol and 120 mg of SDS (1.2-fold weight excess). After cooling, endo H was added to ~100 milliunits/ml and the sample was incubated for 17 h at 30°C, after which the presence of endo H activity was confirmed by assay with pH-dansyl-AsnGlcNAcMan3 as substrate (17).

**Methods**

**Cell Growth—** S. cerevisiae strains were grown in media consisting of the following in grams/liter of distilled water adjusted to pH 6.5 with NaOH: Bacto-yeast nitrogen base with amino acids, 6.7; Bacto-peptone, 8.0; adenine sulfate, 0.2; succinic acid, 2.4. A 100-ml starter culture grown overnight at 26°C was used to inoculate a 2.5-liter Bio-Flow I11 benchtop fermentor (New Brunswick Scientific Co., Inc.) containing 2 liters of medium and 600 ml of 0.06% glucose to the 100 ml of culture remaining in the fermentor.

**Materials**

Saccharomyces cerevisiae haploid strain X79-1A(a) (alg3 - sec18 - 1ade1 - 1ade2 - ura3 - 598) was provided by T. C. Huffer and P. W. Robbins (2); haploid strain SF782-8A(a) (sec18-l, gls1, pep4) was provided by R. Schekman (9). These two strains were mated, zygotes isolated and sporulated, the tetrads dissected, and the four spores grown out. Temperature-sensitive isolates requiring uracil and adenine were screened for retention of glucose on oligosaccharides as an internal marker by SDS-PAGE. A representative of each phenotype was transformed using the lithium acetate procedure (10) with a multicopy plasmid (pRB58) obtained from M. Carlson (11), which carries the external invertase (SUC2) gene. Bio-Gel P-4 and SDS-PAGE gels were from Bio-Rad and Immobilon membranes from Millipore. Sepharose S-500 Superfine was a product of Pharmacia LKB Biotechnology Inc. and DE52-cellulose was obtained from Whatman. Anti-rabbit IgG alkaline phosphatase immunoblot kit was obtained from Promega Biotech. Sigma was the source for all chemicals.

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et al. (19). The sweep width in the 11.7 tesla field was 900–1000 Hz using 512 increments in $t_2$. There were 1024 data points in $t_2$ and an acquisition time of 0.4 s with a recycle time of 1.1 s. Both $t_1$ and $t_2$ were zero-filled to 2048 points. A total of 64 transients were run for each free-induction decay. Data were analyzed on a Varian VX4000 work station and on a Silicon Graphics Iris using Hare Research, Inc., software. Resonance intensities were integrated by cutting out and weighing peaks from expansions of the anomeric and C2-H regions of one-dimensional spectra. The anomeric proton of the core $\beta$1,4-linked mannose (residue 3) was obscured by the residual HOH peak. However, residue 3’s C2-H resonance is found at 4.233 ppm in Man$_3$GlcNAc$_2$ (4) and upfield at 4.158 ppm when the core $\alpha$1,3-linked lower-arm mannose is 6-O-substituted (20).

General Methods—Neutral hexose was determined by a scaled-down version (21) of the phenol-sulfuric acid assay (22) with mannose as standard. Protein was determined either according to Bearden (23) at 595 nm, using bovine serum albumin as a standard, or by absorbance at 280 nm (14). Invertase assay was assayed colorimetrically by a modification (24) of the two-step method described by Goldstein and Lampen (25). Radioactivity was measured in “Ready Protein” (Beckman) with a Beckman LS-3801 scintillation spectrometer. Protein was determined either according to Bearden or Lampen. Radioactivity was measured in “Ready Protein” (Beckman) with a Beckman LS-3801 scintillation spectrometer. Invertase antibodies were raised in rabbits (26) using carbohydrate-free internal invertase as the immunogen (15). SDS-PAGE was performed on 8% acrylamide slab gels (0.5 mm) (27), which were either stained with 0.5% Coomassie Blue R-250 or transferred to Immobilon membranes and Western blotted using either rabbit anti-invertase and alkaline phosphatase-conjugated anti-rabbit IgG (Promega Bio- tech) with visualization as described (20) or digoxigenin-labeled ConA and alkaline phosphatase-conjugated anti-digoxigenin (Boehringer Mannheim) with visualization as described (28).

RESULTS

Cell Growth and Invertase Derepression

To determine the role of glucose in protein glycosylation in the alg3 mutant background, it was necessary to restrict the secreted glycoprotein form of invertase to the ER under conditions where any glucose present on oligosaccharides initially transferred from oligosaccharide-lipid to the protein would not be removed. Thus, a strain was constructed, alg3,sec18,glsl, which included the sec18 mutation to prevent trafficking of invertase from the ER at 37 °C (29) and the glsl mutation to prevent normal glucose trimming in the ER (9). When grown in the 2.5-liter benchtop fermentor this strain typically yielded 0.5 g of (wet weight) cells/g of glucose, or 40 g of (wet weight) cells/liter of media containing 10% (w/v) glucose. This result agrees with data previously obtained by Wang et al. (30) who demonstrated a similar ratio of glucose consumed to wet weight of cell yield.

Aliquots of alg3,sec18,glsl cells growing at 26 °C taken at 3 h before and at selected times after the derepression of invertase synthesis on temperature shift to 37 °C were analyzed for both residual glucose and invertase activity with the results shown in Fig. 1, panel B. The invertase activity measured represents both the internal nonglycosylated form that migrates on SDS-PAGE as a sharp band at 59 kDa and an increasing proportion with time at 37 °C of the larger glyco- sylated form restricted to the ER by the sec18 mutation (Fig. 1, panel A). Although external invertase was synthesized maximally when the concentration of glucose in the medium dropped below 0.5%, even at high glucose concentrations (>5%), a small background of external invertase routinely was present in cells as observed by Western blot analysis (Fig. 1, -3 h). This pre-existing glycosylated invertase appears to be the source for the small fraction of Man$_3$GlcNAc$_2$ oligosaccharide which, as will be shown in the accompanying study (31), has the same structure as the major Man$_3$GlcNAc$_2$ isomer found on invertase secreted by alg3 yeast at 26 °C.

FIG. 1. Relationship between glucose consumption and the derepression of invertase synthesis at 37 °C by alg3,sec18,glsl yeast. Panel A shows invertase Western blots at the times indicated relative to the temperature shift to 37 °C at 0 h. Note the size of the purified glycosylated form of sec18,glsl (37 °C) invertase in the left margin which is included as a marker (M) for size comparison. A total of 50 ng of invertase, which consisted of both the nonglycosylated internal and glycosylated external forms was electrophoresed in each lane for blotting. Panel B shows the actual level of residual glucose and total invertase activity in cells Western blotted in panel A at the same time points relative to the temperature shift to 37 °C at 0 h.

### TABLE I

| Step          | Volume | Total activity$^a$ | Total Specific activity | Recovery  |
|---------------|--------|-------------------|------------------------|----------|
| 1. Crude cell extract | 2120  | 16.1   | 1183       | 35       | 100 |
| 2. Ammonium sulfate | 2125  | 9.8    | 1124       | 54       | 95  |
| 3. Hollow fiber | 720   | 3.3    | 944        | 397      | 80  |
| 4. DE52-cellulose | 512   | 0.7    | 636        | 1853     | 54  |
| 5. Sephacyr S-300 | 35    | 3.6    | 518        | 4163     | 44  |

$^a$ Protein was determined according to Bearden (23) using bovine serum albumin as a standard for steps 1–3. Protein concentration for steps 4 and 5 was determined from the absorbance at 280 nm (ε$_{280}^\text{nm}$ = 22.5; Ref. 14).

### Purification of the Glycosylated ER Form of Invertase and Oligosaccharide Isolation

Glycosylated invertase of high specific activity (4163 units/mg protein) was purified with an overall 44% recovery from a crude cell extract by precipitation with ammonium sulfate and acetic acid, and chromatography on DE52-cellulose and Sephacryl S-300 columns (Table I). However, approximately 50% of the initial invertase activity present was the intracellular (internal) nonglycosylated form, which was selectively removed in steps 3 and 4 of the purification scheme. Thus, from the recovery of phenol sulfurlc acid-positive material
and the low level of residual internal form in the purified preparation used as a source of alg3 oligosaccharides (Fig. 2), recovery of nearly 90% of the secreted (external) glycosylated form is estimated. Maximum recovery of the glycosylated external invertase was achieved only when ammonium sulfate fractionation preceded the pH 4 precipitation with glacial acetic acid.

Fig. 2 shows that the oligosaccharides on purified alg3,sec18,glsl invertase were largely endo H-resistant, although some increase in mobility was observed on digestion of the 37 °C ER form (compare lane 1 with lanes 2, 5, with 6, and 8 with 9 in Fig. 2). PNGase F-treatment markedly increased invertase’s mobility, but not to the extent of the recovery of nearly 90% of the secreted (external) glycosylated form is estimated. Maximum recovery of the glycosylated external invertase was achieved only when ammonium sulfate fractionation preceded the pH 4 precipitation with glacial acetic acid.

Structural Assignments by NMR

The one-dimensional NMR spectra for the Glc3Man3GlcNAc and Hex6GlcNAc2 pools are shown in Fig. 3. The carbohydrate structures were determined by methylation and both 1D and 2D 'H NMR analyses. The near identical elution positions of Glc3Man3GlcNAc and Hex6GlcNAc2 is due to the larger hydrodynamic volume of GlcNAc compared to Man.

Fig. 3 shows the Bio-Gel P-4 profiles, which reveal on the basis of Phenol sulfuric acid assay, that endo H released 17% of the oligosaccharides as Glc3Man3GlcNAc (panel A) while PNGase F released the remainder as Hex5GlcNAc2 and Man6GlcNAc2 (panel B). Leaking of approximately 17% of the Man6GlcNAc2-PP-Dol past the alg3 block leads to normal lipid-linked oligosaccharide synthesis of endo H-sensitive mannan, consistent with previous results obtained in studies at 37 °C using the alg3,sec18 mutant (4). The endo H- and PNGase F-released oligosaccharides were pooled separately and exchanged with H2O for 1D and 2D phase-sensitive DQF-COSY 1H NMR analyses (see “Experimental Procedures”).

Chemical shifts (6, ppm) for C1- H protons are summarized in Table II. The Man6GlcNAc2 chemical shifts assigned in this study with resonance identification numbers above each residue for cross-referencing in Figs. 4 and 5, Table II, and the text. Numbers next to the residue identification numbers are relative molar values of signal intensities integrated under the resonance peaks.

Chemical shifts (6, ppm) for C1- H protons are summarized in Table II. The Man6GlcNAc2 chemical shifts assigned in the previous study (4) for residues 1a/b, 2, 3, 4, 5, 8, and 11 have been included in Table II for comparison with the additional oligosaccharides isolated from alg3,sec18,glsl (37 °C) invertase. Linkage assignments for the mannose and N-acetylglucosamine residues (Fig. 4 and Table II) were based on the existing library of chemical shifts for model compounds and oligosaccharides of similar configuration (12, 21, 31-36). Those for the glucose residues were based on assignments reported previously for Glc3Man3GlcNAc isolated from the yeast mutant mnn2,sec18,glsl (37), for the model tetrasaccha-
Fig. 4. One-dimensional $^1$H NMR spectra of alg3,sec18,glu1 (37°C) invertase oligosaccharides at 500 MHz and 23°C. Panel A shows the GlcManGlcNAc released by endo H (from Fig. 3, panel A), while panel B shows the Hex$_6$GlcNAc$_5$ pool released by PNGase F (from Fig. 3, panel B). Numerals beside the peaks refer to the residue identification numbers in the accompanying structures, text, and Table II and the values in parentheses are the relative molar peak area integrations. Chemical shifts ($\delta$, ppm) in the assigned isomers are summarized in Table II. The sample concentrations in the NMR tubes were: 1.7 mM for GlcManGlcNAc and 1 nM for Hex$_6$GlcNAc$_5$. 
the new terminal α,α-linked glucose (G3). Since the sum of ppm, the reporter resonance for the α,3-linked glucose (G1)

H-H NMR spin decoupling analysis to establish the C2-H assignments for the glucotriose constituents. The C2-H’s can be seen to reside at chemical shifts expected for the glucose axial protons, not the region expected for mannosyl equatorial protons.

HexGlcNAc5—Inspection and integration of the Hexa GlcNAc2 spectrum (Fig. 4, panel B) revealed the presence of two species which could be assigned as Glc3Man6GlcNAc and a unique isomer of Man6GlcNAc2 (Fig. 4, panel B, and Table II). The integrated sum of resonance intensities for residues 5, 8, 4, 2 and 3 (see below) was 5 mol indicating that these residues are common to all structures in the pool (see Fig. 4, panel B). The glucose resonance intensities, however, were not 1 mol, suggesting that an endo H-resistant mannosyl compound of the same size as Glc3Man6GlcNAc had copurified in the Hexa pool. Integration of the 1D spectrum (Fig. 4, panel B) provided values for α,3-linked glucose residues G1 and G3 of 0.6 mol each, and since G6 is only found at 5.535 ppm when substituted by an α,1,2-linked glucose (G3) (37), this provides evidence that G1-3 are all present at the same amount. Note that the anomeric proton for G6 overlaps the α-anomer of the reducing-end GlcNAc (1α) at 5.190 ppm. The 0.6 mol value for G6 deduced above can be calculated directly by subtracting from the 1.1 mol of signal integrated at 5.190 ppm the value for 1α, 0.5 mol, which itself is derived by subtracting the 0.5-mol value for the β-anomer integrated at 4.694 ppm (1b) from the 1-mol value for the reducing-end. The presence of a unique resonance cross-peak at 5.032(C1-H)/4.224(C2-H) ppm (Fig. 5, panel B) verifies the 3-O-substitution of residue 11 (32). Thus, based on the glucose content, 60% of the HexaGlcNAc2 species are Glc3Man6GlcNAc, in which lower arm residue 11 of the core Man6GlcNAc2 (4) bears the glucotriose unit. Note that the J1,3 cross-peaks for the monosaccharide constituents of the Glc3 extension are identical to those seen in Glc3Man6GlcNAc (compare Fig. 5, panels A and B).

Subtraction of the anomeric proton values (0.6 mol) for the monosaccharide constituents of Glc3Man6GlcNAc leaves 0.4 mol of intensity for residues 5, 8, 4, and 2 in the Man6 core structure. Also present were 0.8 mol at 5.138 ppm and 1.4 mol at 5.04 ppm (Fig. 4, panel B). The former chemical shift is where α,6-linked 2-O-substituted (6i) and/or α,3-linked terminal (3t) mannose reside, while the latter is where α,1,2-linked terminal (2t) and/or 3-0-substituted α,1,2-linked (2′) mannose reside. The sum of C2-H proton intensities for residues 3 and 11 at 4.229-4.224 ppm plus that at 4.160 ppm was 2 mol. Since core residue 3 is present in all species, by difference 1 mol of intensity at 4.224 ppm belongs to residue 11, signifying that all of this residue is 3-O-substituted. As 60% of this substitution is due to G6 in the Glc3Man6GlcNAc2 isomer derived above, 40% must be substituted with mannosone. This accounts for 0.4 mol of intensity at 5.138 ppm, the chemical shift of α,3-linked terminal mannose (32).

The shift in 0.4 mol of residue 3’s C2-H from 4.235 to 4.160 ppm is indicative of an α,1,6-linked substitution of core-linked residue 5 by residue 12 (20). Thus, 40% of the species contain residue 12, which when 2-O-substituted resides at 5.144 ppm and accounts for the remaining 0.4 mol of resonance intensity at this chemical shift. Residue 11’s anomeric proton is found at about 5.04 ppm and accounts for 1 of the 1.4 mol of signal integrated at this chemical shift. The 0.4 mol difference at 5.04 ppm corresponds to residue 13, the α,1,2-linked mannose that terminaly substitutes residue 12 (Fig. 4, panel B).
Glucosylation of alg3 Oligosaccharides

TABLE II
Assignment of $^1$H chemical shifts to individual oligosaccharide isomers released by endo H and PNGase F from invertase secreted by alg3,sec18,glsl yeast at 37 °C

| Chemical Shifts (δ, ppm) for Monosaccharide Constituents in Isomers† | Hex$_2$GlcNAc | Man$_2$GlcNAc$_2$ | Hex$_3$GlcNAc$_2$ |
|---------------------------------------------------------------|----------------|----------------|----------------|
| C1-H’s                                                        |               |                |                |
| G$_3$ -2G1α-                                                  | 5.535         | 5.535          | 5.535          |
| 5 3'B                                    | 5.345         | 5.340          | 5.345          |
| 8 2'B                                    | 5.304         | 5.301          | 5.302          |
| G$_1$ -3G1α-                                                  | 5.273         | 5.270          | 5.270          |
| 2a αGn                                               | 5.250         |                |                |
| 1a αGn                                               | 5.184         | 5.190          | 5.190          |
| G$_3$ G1α-                                                  | 5.185         | 5.180          |                |
| 6 6'B                                    | 5.150         |                |                |
| 12 6'B                                    | 5.138         |                |                |
| 14 3′-0-2i                               | 5.138         |                |                |
| 7α/β 3′c                                           | 5.101/5.074   | 5.048          |                |
| 13β/β 2′-0-6i                                     | 5.040         | 5.040          | 5.032          |
| 11 2′                                            | 5.040         | 5.032          | 5.032          |
| 9 2′                                            | 5.040         |                |                |
| 4 6′                                            | 4.863         | 4.914          | 4.920          |
| 3 6′                                            | 4.770         | 4.769          | 4.772          |
| 1b αGn                                               | 4.685         | 4.694          | 4.694          |
| 2b αGn                                               | 4.694         | 4.611          | 4.610          |
| 2 core Gn                                           | 4.611         | 4.610          |                |
| C2-H’s                                                        |               |                |                |
| 3 8′i                                            | 4.235         | 4.233          | 4.229          |
| 11 2′i                                            | 4.226         | 4.224          | 4.224          |
| 3 8′i                                            |                | 4.160          |                |

| % Abundance = | 17 | 73 | 6 | 4 |

† Linkages: , α1,6; ∆, α1,3; →, α1,2; ←, B1,4. Symbols: O, Man; ■, GlcNAc; ●, Glc.

‡ Residue names are based on the linkage of a particular residue, how it is substituted, and its relative position in the molecule. For example, 6t refers to a terminal α1,6-linked mannose, 3′t to a 2-O-substituted internal α1,3-linked mannose, 3′-0-2 to an α1,3-linked terminal mannose on an α1,2-linked mannose, 3′t to a terminal α1,3-linked mannose on the upper (α6 branch) arm, and 2i to an internal α1,2-linked mannose.

The assignment of α1,2-linked residue 13 is verified by the J$_{1,3}$ cross-peak at 5.046(C1-H)/4.067(C2-H) ppm (Fig. 5, panel B). The assignment of the two isomers in the Hex$_3$ pool satisfies all integrations in the 1D spectrum (Fig. 4, panel B). The unique isomer of Man$_3$GlcNAc$_2$, representing 40% of the Hex$_3$ pool but only 4% of the total oligosaccharides present, has been derived from the Man$_3$GlcNAc$_2$ precursor by addition of a terminal α1,3-linked mannose to residue 11 and α1,6-linked mannose residue 12 to the lower arm core residue 5, which itself is 2-O-substituted by terminal residue 13. This Man$_3$GlcNAc$_2$ isomer, present on external invertase in the cells prior to derepression at 37 °C (Fig. 1), is the major Man$_3$ core structure found on invertase secreted by alg3 sec18 glsl yeast at 26 °C in the accompanying paper (31). Of the oligosaccharides that were endo H-resistant on alg3 invertase (83% of the total in Table II), 88% were Man$_3$GlcNAc$_2$ while only 7% became glucosylated to yield Glc$_2$Man$_3$GlcNAc$_2$. The novel Man$_3$GlcNAc$_2$ isomer was only 5% of the endo H-resistant oligosaccharides recovered.

DISCUSSION

The current study sought to determine whether glucose was involved in the transfer of the glycan from oligosaccharide-lipid to protein in yeast. Structural studies were performed on the oligosaccharides released enzymatically from the alg3 sec18 glsl triple-mutant invertase, which had accumulated in the ER during incubation at 37 °C when ER→Golgi
traffic was disrupted by the sec18 ts defect. This mutant provided preparative amounts of oligosaccharide, which were subjected to analyses by one- and two-dimensional $^3$H NMR spectroscopy. Integration of the resonance intensities from the 1D expansions revealed that GlcMan$_3$GlcNAc was the only oligosaccharide released by endo H (Fig. 4, panel A, and Table II), and that PNGase F-released oligosaccharides corresponded in size to Man$_2$GlcNAc$_2$ and Hex$_3$GlcNAc, which were assigned as GlcMan$_3$GlcNAc$_2$ and a novel isomer of Man$_2$GlcNAc$_2$ (Fig. 4, panel B, and Table II).

The pathway for the synthesis of N-linked glycans in both mammalian and yeast cells starts with the transfer of GlcMan$_3$GlcNAc$_2$ from dolichyl pyrophosphate to protein, and continues with the specific trimming to Man$_2$GlcNAc$_2$ by the stepwise removal of the 3 glucose residues followed by the middle arm $\alpha$1,2-linked terminal mannose residue always found in cells (Fig. 1). Glc3Man$_6$GlcNAc has been isolated from class E Thy-1- cells responding in size to Man$_6$GlcNAc and HexsGlcNAc$_2$, which were assigned as Glc3Man$_5$GlcNAc and a novel isomer of Man$_2$GlcNAc$_2$ (Fig. 3, panel A) served as a control showing the absence of glucosidase I, for if alg3,secl8,glsl glucosylation of the Man$_6$ structure is very slow relative to the transfer of glucosidase I (42). By contrast, Glc3Man$_6$GlcNAc$_2$ has been isolated from class E Thy-1- cells (5) and Chinese hamster ovary cells (43), which argues for the need for precursor glucosylation in mammalian cells.

$^3$H NMR spectroscopy of the endo H-sensitive oligosaccharide confirmed it to be Glc3Man$_6$GlcNAc$_2$ (Fig. 4, panel A, and Table II). This agrees with earlier work showing that N-linked oligosaccharides accumulated with a size corresponding to GlcMan$_3$GlcNAc$_2$ in the sec18,glsl mutant at 37 °C (9). The formation of only Glc3Man$_6$GlcNAc in the endo H-sensitive oligosaccharide pool (Fig. 3, panel A) was unprecedented.

Evidence that in mammalian cells only glucosylated lipid-linked glycans are transferred to nascent proteins (7), while yeast are capable of transferring truncated lipid-linked oligosaccharides (40). Nonglucosylated oligosaccharides are transferred to protein by the alg5, alg6, and dse1 mutants, which accumulate Man$_2$GlcNAc$_2$-PP-Dol (8, 41), and an underglycosylated donor is accumulated by the alg6, mutant, which transfers GlcMan$_3$GlcNAc$_2$-PP-Dol (42).

The formation of only Glc3MansGlcNAc in the endo H-released oligosaccharides (Table I) is consistent with results obtained using a GlcMan$_3$GlcNAc$_2$-PP-Dol substrate in wild type yeast cells (9). This agreement with earlier work showing that alg3,secl8,glsl mutants which lack vacuolar $\alpha$-mannosidase, Ref. (42) suggests that the four additional mannoses added from Man-P-Dol to form the $\alpha$1,6-linked branch of the precursor Man$_3$GlcNAc$_2$ significantly enhance both the rate of oligosaccharide-lipid glucosylation and the subsequent ER glucosidase trimming reactions. The latter has been observed in vitro using a GlcMan$_3$GlcNAc$_2$ derived from GlcMan$_3$GlcNAc by jack bean $\alpha$-mannosidase treatment (47).

In summary, we have shown that in alg3 yeast, the failure to add glucose to lipid-linked oligosaccharides may reduce but does not serve to limit glycoprotein biosynthesis under the laboratory growth conditions employed. Any reduction in the efficiency of invertebrate glucosylation due to the lack of glucose on oligosaccharide-lipid was minor, as quantitative carbohydrate recoveries in the current work indicate that invertebrate subunits were associated with an average of two endo H-sensitive and seven endo H-resistant chains, which is very close to the average of 10 occupied sites per subunit on wild type invertase (45). It is not clear whether alg3’s vigor is due to leakage of about 20% of the oligosaccharides past the alg3 block to become wild type GlcMan$_3$GlcNAc$_2$, followed by processing to normal mannan, or to the relaxed specificity of yeast oligosaccharide transferase in glucosylating nascent peptides with truncated nonglucosylated oligosaccharides.

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