N-Glycans in nearly all eukaryotes are derived by transfer of a precursor GlcManNac2 from dolichol (Dol) to consensus Asn residues in nascent proteins in the endoplasmic reticulum. The Saccharomyces cerevisiae alg (asparagine-linked glycosylation) mutants fail to synthesize oligosaccharide-lipid properly, and the alg9 mutant, accumulates Man_Nac2-P-P-Dol. High-field 1H NMR and methylation analyses of Man_Nac2 released with peptide-N-glycosidase F from invertase secreted by Δalg9 yeast showed its structure to be Manα1,2Manα1,2Manα1,3Manα1,3Manα1,6-Manβ1,4GalNacβ1,4GalNacαβ, confirming the addition of the α1,3-linked Man to Man_Nac2-P-P-Dol prior to the addition of the final upper-arm α1,6-linked Man. This Man_Nac2 is the endoglycosidase H-sensitive product of the Alg3p step. The Δalg9 Hexα1,6→GlcNac elongation intermediates were released from invertase and similarly analyzed. When compared with alg3 sec18 and wild-type core mannans, Δalg9 N-glycans reveal a regulatory role for the Alg3p-dependent α1,3-linked Man in subsequent oligosaccharide-lipid and glycoprotein glycan maturation. The presence of this Man appears to provide structural information potentiating the downstream action of the endoplasmic reticulum glucosyltransferases Alg6p, Alg8p and Alg10p, glucosidases Gls1p and Gls2p, and the Golgi Och1p outerchain α1,6-Man branch-initiating mannosyltransferase.

With the exception of some protists, all eukaryotes co-trans- ferred Man_Nac2 and GlcNac2 from that found in higher eukaryotes, this yeast provides a good model system to investigate the structural information contained in the GlcMan_Nac2-P-P-Dol precursor transferred to protein, since nearly the entire phosphorodolichol and much of the Golgi processing pathways have been dissected by the generation of the alg and mnn mutants (7–10). Genes for a number of steps in OSL biosynthesis were not readily identified in yeast, because their absence produced no apparent phenotype in the presence of wild-type OST. However, in recent work, Aebi’s group has isolated ALG3, ALG6, ALG8, ALG9, and ALG10 loci by rescuing through complementation of the respective synthetically lethal phenotypes occurring in conjunction with mutant subunits of OST (1, 11–13). Subsequent to their isolation, strains for each deletion have been generated in a wild-type OST background. These mutants provide an advantage for investigating Golgi-processed N-glycan structures, because gene ablation prevents N-linked glycan structures associated with leaaky alleles, for example alg3–1 (14).

The alg9 mutants accumulate endo H-sensitive Man_Nac2-P-P-Dol, the glycan of which is shown in this work to be Manα1,2Manα1,2Manα1,3Manα1,3Manα1,6-Manβ1,4GalNacβ1,4GalNacαβ, differing from Man_Nac2-P-P-Dol of alg3 mutants only by the addition of the central arm-initiating α1,3-linked Man (see residue 7 in Scheme I). In addition, we define downstream processing of Δalg9 glycans found on secreted invertase as was done previously on wild-type and alg3 sec18 invertase as a model secreted glycoprotein.
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(15, 16). This study reveals the strong regulatory influence addition of this central arm α1,3-linked Man plays in subsequent ER and Golgi processing events. Findings here provide implications regarding the in vivo substrate specificities of the ER glucosyltransferases and glucosidases and of the Golgi Och1p α1,6-Man branch-initiating enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—S. cerevisiae haploid strain yg414 (Mata ade2-101 ura3-52 his3-200 lys2-801 alg9Δ::G418) was supplied by S. te Heesen (ETH, Zurich, Switzerland). The Δalg9 strain was transformed with the pRS315 multityp gene invertase plasmid for overexpression using the lithium acetate procedure (17). Bio-Gel P-4 was from Bio-Rad. Sephacryl S-300 Superfine was a product of Amersham Pharma Biotech, and DE32-cellulose was obtained from Whatman. Cellulose TLC plates were purchased from EM Separations Technology. The [2-3H]Man was obtained from American Radiolabeled Chemicals, Inc. Ecolume scintillation mixture was purchased from ICN Pharmaceuticals, Inc. Sigma was the source of 99.8% and 99.96% D2O, while 99.996% D2O was from Cambridge Isotopes Laboratories.

**Golgi Glycosylation**—Small pellets were resuspended in 50 mM sodium phosphate buffer, pH 8.5, containing 500 μM CuCl2, 10 mM MgCl2, and 1 mM 1,000.55 mm. The yeast suspension was beaten for 60 s and cooled on ice for 5 min. This was repeated six times or until the yeast cells were more than 90% disrupted as determined in the light microscope (magnification, ×400). The slurry was removed from the blender, rinsed with buffer, and centrifuged at 2,000 × g for 10 min at 4 °C in 40-ml polycarbonate tubes to remove the nuclei and cell walls. The supernatant was transferred into a 40-ml polycarbonate tube and centrifuged at 40,000 × g for 60 min at 4 °C. The supernatant was discarded, and the microsomal pellet without the brownish mitochondrial upper layer was gently resuspended in buffer and dispersed in a small Dounce homogenizer with a Teflon pestle. The final protein concentration was determined by the method of Bearden (21) using bovine serum albumin (Sigma) as a standard. Microsomes (30–40 mg of protein) were from 30-ml beads in liquid N2 and stored at −80 °C.

**Glycolipid Biosynthesis in Vitro**—Glycolipids were synthesized as described previously (22). Incorporation of [3H]Man from GDP-[3H]Man or [14C]Man from GDP-[14C]Man into endogenous acceptors was performed in a final reaction volume of 110 μl. For synthesis of preparative amounts of Δalg9 OSL, the reactions were incubated for 20 min at 27 °C. All reactions were terminated by addition of 2 ml of CHCl3:CH3OH (2:1 v/v). The glycolipids were extracted by a modified procedure of Waechter et al. (20). The CHCl3/CH3OH (2:1) and CHCl3/CH3OH (1:0.3) extracts contained Man-P-Dol and/or OSL standards, respectively, on their behavior on silica-gel and cellulose TLC. For preparation of large amounts of OSL, reactions were scaled up 2-fold. To reduce OSL glucosylation, some reactions contained 10 μM UDP. The resulting OSL was purified on cellulose TLC plates using Man-P-Dol and/or OSL standards. Purified OSL was scraped from TLC plates, resuspended in 4 ml of CHCl3/CH3OH/Δalg9-S (1:1:0.3), the matrix removed by centrifugation, the supernatant concentrated under reduced pressure at 28 °C, and the residue resuspended in a small volume of fresh CHCl3/CH3OH/Δalg9-S (1:1:0.3).

**Glycosylase Digestions**—To isolate transferred oligosaccharides, microsomal pellets were solubilized in 1% SDS in 50 mM sodium phosphate buffer (pH 8.5) with heating. For endo H digestion, the pH was adjusted to 5.5 with 1.0M phosphoric acid and endo H added at 50 units/ml. The reactions were incubated at 37 °C for 16 h, and endo H activity was verified by hydrolysis of ManGlcNAc-Asn-dansyl, followed by paper chromatography of the released GlcNAc-Asn-dansyl moiety (23). For PNGase F digestions, SDS was removed from the solubilized pellet protein by extraction with 50% acetonitrile. The resulting pellets were resuspended in 50 mM sodium phosphate buffer, pH 8.5, boiled to resolubilize them, and PNGase F was added to 100 units/millil. The reaction was incubated at 30 °C for 16 h, and activity was verified by hydrolysis of [7H]dansyl-fetuin-pentaglycopeptide, followed by paper chromatography (24).

**Purification of External Invertase and Oligosaccharide Isolation**—Secreted invertase was purified from crude cell extracts by 35% ammonium sulfate and pH 4 precipitations, followed by DE-52 and Sephacryl S-300 chromatography as described previously (15). A typical purification from 225 g of cells yielded 47 mg of external invertase. The N-linked oligosaccharides were hydrolyzed from invertase by treatment with PNGase F as described above, isolated by solvent precipitation as described previously (25), then chromatographed on a calibrated column of Bio-Gel P-4 (95 cm × 16 mm) with 0.1 M acetic acid, 1% 1-butanol as the eluant at 8.8 ml/h and room temperature. Fractions of 0.75 ml were collected, and aliquots were assayed for total hexose and radioactivity, which included an internal marker of ManGlcNAc-[3H]Holo.

**Mass Spectrometry**—MALDI-TOF mass spectrometry was performed on a Bruker Reflex instrument. Samples of 25–50 pmol were prepared with 2.5-dihydrobenzoic acid as matrix. Data accumulated for 10–50 3-ns pulses of the 337 nm laser were averaged for each sample. Analyses were performed in linear and reflective mode.

**Invertase Assay**—External invertase activity was followed through the purification scheme using a modification of the method of Goldstein and Lampen (26).

**Methylation Linkage Analysis**—Samples were analyzed by methylation as described (27) using the NaOH/MelSO method. Briefly, the free hydroxyls of the oligosaccharides were deprotonated with NaOH/MeSO. Then CH3I was added to replace the free hydroxyls with methoxy groups. The methoxylated oligosaccharide was hydrolyzed in strong acid, evaporated under low pressure, and applied to Whatman silica-gel 60A TLC plates. The plates were developed twice with CH3CN-CHCl3-CH3OH, 3:9:1 (v/v/v), thoroughly dried between each ascent, and rapidly dipped into a solution containing 3 g of N-trifluoroacetylated mannosylithioated and 50 ml of concentrated H2SO4 in 1 liter of CH3OH. The plates were resolved, and the bands were scraped in 99.996% D2O at 120 °C. All saccharide standards were purchased from Sigma.

**1H NMR Spectroscopy**—Oligosaccharides (0.2–0.5 mg) were prepared in 99.996% D2O and twice from 99.96% D2O, followed by lyophilization. Lyophilized samples were dried over P2O5 in vacuo for a day or more, then reconstituted in 0.5 ml of 99.996% D2O to a final concentration of 0.25–0.70 mM. Samples were transferred into 5-mm tubes (Wilmad Co., no. 535pp, previously washed and exchanged with 99.8% D2O), flame-sealed, and examined at 160 °C for 1D and 2D DQF-CPMG-1H NMR spectroscopy at 500 MHz as described previously (28–30). Spectral width in the 11.7-tesla field was 1502 Hz for all experiments. For acquisition of 1D data, 1024 scans were collected over 4096 data points. The limit of resolution was 0.0045 ppm based on the ratio of the width of the widest peak at half-height (2.26 Hz) to the number of Hz per ppm (500.13 Hz/ppm). For homonuclear 2D DQF-COSY, 1.5 s of low power presaturation on residual DHO at 4.79 ppm was applied. Data collection for the 2D experiments was 4096 data points in t2 and 512 complex data points in the indirect t1 dimension.

**Glucosylation in Vivo**—Δalg9 S. cerevisiae cells were grown overnight to stationary phase and collected by centrifugation for 5 min at 3000 rpm and room temperature in a Sorvall TCS centrifuge equipped with an H400 rotor. The yeast were washed twice in glucose-free YP medium by centrifugation, and incubated in the presence or absence of 5 mM castanospermine (Sigma) for 1.5 h in YP + 1% glucose. The yeast were again washed twice in glucose-free YP medium by centrifugation, and cells (2 × 107) were resuspended in 400 μl of YP + 0.15% glucose containing 500 μCi of [2-3H]Man (20 Ci/mmol) (American Radiolabeled Chemicals, Inc.). After 2 min of labeling 2000-fold excess of unlabeled Man was added to reactions. Aliquots of 125 μl were taken at 0, 1, and 10 min, and reactions were terminated by rapid addition to 4 ml of CHCl3/CH3OH (2:1) while vortexing.

**General Methods**—Neutral hexose was determined by a scaled-down version of the phenol-sulfuric assay with mannose as the standard (31). Protein was determined using either the method of Bearden (21), with bovine serum albumin as standard, or in the case of purified invertase by absorbance at 280 nm (25). Radioactivity was measured with a Beckman LS-3801 scintillation spectrometer in Ecolome (ICN) scintillation fluid.
RESULTS

Characterization of Δalg9 Invertase N-Linked Glycans—Δalg9 yeast were transformed with pRB58 (see “Experimental Procedures”), and several transformants producing invertase activities of over 700 IU/g (wet weight) were accessioned. The best overproducer of invertase, JCY362, was chosen for external invertase purification from the Δalg9 background, which yielded high specific activity enzyme (79 mg, 3990 units/mg protein) with an overall recovery of 72% from 535 g of cells. Bio-Gel P-4 size exclusion chromatography of the PNGase F-released glycans from this invertase preparation provided four major pools, labeled A through D in Fig. 1, which eluted on the calibrated column in volumes consistent with Hex 6GlcNAc2 (fractions 130–136), Hex 7GlcNAc2 (fractions 124–129), Hex8.5GlcNAc2 (fractions 118–123), and Hex 10GlcNAc2 (fractions 113–117), respectively. Each pool was rechromatographed, and the central 85% of the resulting peaks were re-pooled for further analysis (data not shown).

MALDI-TOF MS Analysis of Bio-Gel P-4 Pools A–D—Fig. 2A shows the MS spectrum of pool A, revealing one size isomer with masses of 1420 and 1436 Da for the sodium- and potassium-adducted species, respectively, the expected Mr for a Hex6GlcNAc2 oligosaccharide. The HPAEC data (Fig. 3) confirm that pool A consisted of a single Hex6GlcNAc2 isomer. The inset in Fig. 2A shows that endo H at 50 milliunits/ml for 16 h at 37 °C, conditions that will not hydrolyze the reducing-end GlcNAc from the alg3 Man5GlcNAc2 precursor (25), removed a mass equivalent to the reducing-end GlcNAc (204 Da). This result is consistent with the notion that the Hex6GlcNAc2 is the first endo H-sensitive structure in the N-linked glycosylation pathway, the expected product of the Alg3p step shown in Scheme I (32).

Pool B contained two glycan sizes; major ions of Hex7GlcNAc2 appeared at 1581 and 1597 Da for the sodium- and potassium-adducted species, respectively, the expected Mr for a Hex7GlcNAc2 oligosaccharide. Although MS is not wholly quantitative, signal intensities from the size neighbors in a homologous structural series should be relatively proportional. On this basis, Hex6GlcNAc2 represented approximately 90% of pool B while Hex7GlcNAc2 represented approximately 10%. As Fig. 2C shows, pool C contained both Hex6GlcNAc2 and Hex7GlcNAc2, the former comprising approximately 20% of the pool while the latter made up the remaining 80%. Pool D contained a single glycan size consistent with Hex10GlcNAc2 sodium and potassium forms at 2067/2083 Da, respectively.

HPAEC Analysis—In order to estimate the number of branch isomers present in the four oligosaccharide pools, each was analyzed by HPAEC using an analytical Dionex CarboPak PA-100 column (4 × 250 mm). As indicated above, pool A gave a single peak (Fig. 3) and co-eluted with the smallest Δalg9 glycan from OSL (data not shown), consistent with the hypothesis that it is the core alg9 isomer unmodified by further processing after transfer to protein and transport through the secretory pathway. Quantitation of pool A from the Bio-Gel P-4 column indicated this to be the most abundant isomer among the Hex6–10GlcNAc2 structures, representing 24% of the N-
glycan chains found on secreted invertase.

Fig. 3 shows that pool B yielded five peaks corresponding to the presence of a minimum of five branch isomers, with the most abundant glycan species representing 53% of the profile area. The remaining four minor peaks contained 24%, 16%, 4%, and 2% of the total profile area, respectively.

Pool C provided six peaks, where the major species represented approximately 68% of the total area of the analytes (Fig. 3). The other peaks were 10%, 10%, 6%, 4%, and 2% of the profile area, respectively.

Pool C provided six peaks, where the major species represented approximately 68% of the total area of the analytes (Fig. 3). The other peaks were 10%, 10%, 6%, 4%, and 2% of the profile area, respectively.

Pool A: Hex 6GlcNAc2—The smallest oligosaccharide found in pool A is Hex 6GlcNAc2. This structure is shown in Scheme I A, which represents the Glc3Man9GlcNAc2 transferred to protein in wild-type cells (boxed area indicates the residues conserved in the core alg9 glycan). In addition, this structure is shown in Scheme I B, which represents the alg3 Man5GlcNAc2 core. Finally, in pool D five glycan peaks were separated. The main species represented 61% of the chromatogram area. Other isomers represented 24%, 7%, 4%, and 4% of the total peak area present in the HPAEC profile.

NMR Analysis of DeltaAlg9 Core Oligosaccharides—In order to determine what structures were present in each of the Bio-Gel P-4 pools A-D, 1D 1H NMR spectra were collected for each. These are shown as a montage in Fig. 4, and integrations of proton intensities for established reporter groups present in expansions of the spectra are summarized in Table I. The Man added by Och1p is boxed (36).

Within each pool, the number and amount of each isomer assigned agreed closely with the number and area of HPAEC peaks present (Fig. 3) and size proportions estimated by MALDI-TOF MS (Fig. 2). The subsequent paragraphs explain how each assignment was made. Some assignments required DQF-COSY experiments for validation, which are presented in Figs. 5 and 6.

Pool A: Hex6GlcNAc2—The smallest oligosaccharide found
on secreted invertase in \( \Delta \text{alg9} \) yeast was \( \text{Hex}_6 \text{GlcNAc}_2 \), which was completely endo H-sensitive to removal of the reducing end GlcNAc (Fig. 2A, inset). Given the substrate specificity of endo H, the \( \Delta \text{alg9} \) \( \text{Hex}_6 \text{GlcNAc}_2 \) structure should be the Alg3p product, the cytosolic \( \text{Man}_5 \text{GlcNAc}_2 \) precursor with the added middle arm \( \alpha \)1,3-linked Man residue 7 (Scheme I, B and C, respectively). The determination of the glycan structures in a homologous series by high field proton NMR is quite straightforward, because the addition of subsequent glycosidic linkages results in characteristic chemical shift resonances that can be compared with large libraries of related glycan proton chemical shifts available in the literature and data bases. Importantly, the 1D spectra can be accurately integrated to assign the components of complex mixtures (33).

Integration of signals in Fig. 4A (Table I, pool A) between 4.70 and 5.40 ppm provides 6.00 mol of hexose protons, including 1 mol for residue 3 obscured by residual HDO signal centered at 4.78 ppm. Note the 1-mol resonance intensity integrated at 5.11 ppm, which is indicative of terminal \( \alpha \)1,3-linked Man residue 7 (33, 34). This resonance is the only detectable difference on comparing the \( \Delta \text{alg9} \) \( \text{Man}_6 \text{GlcNAc}_2 \) NMR spectrum with that of \( \text{alg3-1} \text{Man}_6 \text{GlcNAc}_2 \) (14) described previously and included in Scheme I. A 2D DQF-COSY experiment (data not shown) showed \( J_{1,2} \) cross-peak for residue 7 at 5.11(C1-H)/4.05(C2-H) ppm, confirming this assignment (34). In addition, residue 4's C2-H shifts downfield on 3-O substitution from 3.97 to 4.12 ppm, verifying addition of residue 7 to residue 4 (Scheme IC). As will be seen, this resonance is present in all glycans \( \text{Hex}_6 \text{GlcNAc}_2 \) and larger, which means the \( \Delta \text{alg9} \) \( \text{Man}_5 \text{GlcNAc}_2 \) is an invariant core component of all the \( \text{Man}_{\alpha5-14} \text{GlcNAc}_2 \) structures elucidated.

In one preparation of \( \Delta \text{alg9} \) \( \text{Hex}_7 \text{GlcNAc}_2 \), \( \text{Glc}_1 \text{Man}_5 \text{GlcNAc}_2 \) was found to be a minor species (8%). This assignment was made on the basis of the total anomeric proton integration and presence of \(-0.08 \text{ mol of Glc anomeric resonance intensity at} \ 5.25 \text{ ppm} J_{1,2} \text{ coupled (3.5 Hz)} \text{ with its C2-H at} \ 3.54 \text{ ppm expected for an} \ \alpha \text{-anomeric proton with an axial C2-H as found in Glc (14–16, 25, 34, 35). This species was present in the shoulder of the P-4 Bio-Gel fractions most distant from the} \ V_o \text{ and is not considered further when assigning} \ \text{Hex}_{\alpha7-10} \text{GlcNAc}_2 \text{ structures.}

**Pool B: Hex}_{\alpha7-8} \text{GlcNAc}_2 — Pool B integrated to a total of 7.10 mol of anomeric protons (Table I), assuming 1 mol of intensity for residue 3 obscured by the HDO signal. This indicates that 90% of the pool is \( \text{Hex}_7 \text{GlcNAc}_2 \) and 10% is \( \text{Hex}_8 \text{GlcNAc}_2 \), in good agreement with MALDI-TOF MS distribution observed in Fig. 2B. C1-H resonance intensity in excess of the core \( \text{Man}_5 \text{GlcNAc}_2 \) structure was present at chemical shifts 4.89, 5.03, 5.12, and 5.25 ppm (Table I). At 4.89 ppm, 1.20 mol of resonance intensity was present. One mol was from residue 4 of the core structure (Scheme IB), and 0.20 mol was assigned to the branch-forming unsubstituted \( \alpha \)1,6-linked Man residue 12. The resonances for 4 and 12 correlate with their definitive C2-H resonances at 4.13 and 3.98 ppm, respectively (14–16, 25, 34). Thus, 20% of Pool B is isomer 7a (Scheme II), which is the expected product of the Och1p outer chain initiating enzyme (36).

At 5.03 ppm 1.06 mol of resonance intensity was integrated. One mol of this signal was assigned to residue 11 of the core structure and the additional 0.06 mol to residue 13, the \( \alpha \)1,2-Man cap on residue 12 (see Scheme ID). The predicted 2D DQF-COSY cross-peaks for these residues were observed at 5.03(C1-H)/4.08(C2-H) ppm (Fig. 5A) as expected for all terminal \( \alpha \)1,2-linked mannosides. Together with the size constraint placed on the pool by total integrated C1-H resonance intensity, mass spectrometry data, and the above resonance assignments, \(-6 \% \) of pool B contain residues 12 and 13 as their Golgi modification to the \( \Delta \text{alg9} \) \( \text{Man}_6 \text{GlcNAc}_2 \) core to yield isomer 8c (Scheme II).

The total resonance intensity between 5.11 and 5.15 ppm was 1.74 mol. One mol is from \( \alpha \)1,3-linked Man residue 7 in the core structure (Scheme IC), while the other 0.74 mol represents \( \alpha \)1,3-linked Man residue 14 and the \( \alpha \)2-O-substituted residue 12 in isomer 8c assigned above. Both of the 3-O-substituting Man’s C2-Hs were observed in the 2D DQF-COSY spectrum at 4.08 ppm (Fig. 5A) (15, 25, 37). Subtracting 0.06 mol of residue 12 present in 8c from the 0.74 mol integrated leaves 0.68 mol of \( \alpha \)1,3-linked Man C1-H resonance, which is seen as residue 14 on the basis of the intense 2D DQF-COSY cross-peak at 5.14(C1-H)/4.06(C2-H) ppm (Fig. 5A). This defines isomer 7b (Scheme II) as a major constituent of pool B. Furthermore,
residue 11 shows the expected $J_{1,2}$ cross-peak at 5.05(C1-H)/4.22(C2-H) ppm resulting from its 3-O substitution (Fig. 5A and Scheme ID) (34).

A very weak base-line 5.12(C1-H)/4.22(C2-H) ppm resonance was also observed, but is not apparent in the projection in Fig. 5A. This cross-peak is assigned to residue 16, which was previously shown to be due to a novel 3-O-substituting mannose in $\text{alg}3 \sec 18$ invertase oligosaccharides (15, 25). As will be shown below, components of the Hex$_3$GlcNAc$_2$ and Hex$_4$GlcNAc$_2$ pools also contain the Man$_{al}3\text{Man}_{al}1,3$-disaccharide. Furthermore, a minor component of pool B (2\%) eluted at 11 min on HPAEC with a component of the Hex$_3$GlcNAc$_2$ isomers in pool C (see Fig. 3), also predicted to contain residue 16 (see Table I, Scheme II, and Fig. 5B). These two are most probably the same isomer, and its presence in pool B may have resulted from combining overlapping fractions from multiple Bio-Gel P-4 runs. Taken together, these calculations are consistent with about 60\% of pool B being isomer 7b and about 4\% isomer 8d (Scheme II).

The resonance intensity at 5.25 ppm was 0.10 mol (Table I) and indicates the presence of residue $G_1$. This assignment is supported by the 2D DQF-COSY cross-peak at 5.25(C1-H)/3.54(C2-H) ppm (Fig. 5A), which is in the region expected for this axial ring proton (25, 35). The resonance peak indicates that 10\% of pool B is the core $\Delta\text{alg}9$ glycan, assigned isomer 7c, which retained the $\alpha$1,3-Glc on transport through the Golgi after leaving the ER (Scheme II).

For the oligosaccharide structures studied here, a C2-H resonance intensity can appear at 4.22 ppm from residue 3 in the absence of the through space effect caused by residue 12’s substitution of residue 5 (15, 37), the 3-O substitution of residue 11 by 14 or $G_1$ (25), or the 3-O substitution of residue 14 with 16 (see Scheme ID). In pool B, 1.52 mol of resonance intensity was present at 4.22 ppm (Table I), which, as noted above, reveals a principle $J_{1,2}$ cross-peak in 2D DQF-COSY spectrum with residue 11’s C1-H at 5.04 ppm, already assigned to the major constituent of this pool, isomer 7b. Isomers 7b and 8d have residue 11 by 14 or $G_1$, or the 3-O substitution with 16 by 11 (25), or the 3-O substitution of residue 14 with 16 (see Scheme ID). Subtracting 1 mol of intensity for the common core 1,6-linked Man residues 4 and 12 (see Table I, Scheme II, and Fig. 5B). These two are most probably the same isomer, and its presence in pool B may have resulted from combining overlapping fractions from multiple Bio-Gel P-4 runs. Taken together, these calculations are consistent with about 60\% of pool B being isomer 7b and about 4\% isomer 8d (Scheme II).

The total molar proton intensity in C1-H

| Residue no. | Linkage$^a$ | Nominal chemical shift (ppm) | $\text{alg}3\text{Man}_\alpha$ Pool A | $\text{alg}3\text{Man}_\beta$ Pool C |
|-------------|-------------|-----------------------------|--------------------------------------|----------------------------------|
| C1-H        |             |                             | Pool B | Pool D |
| C2-H        |             |                             | Pool C | Pool D |
|             |             |                             |        |       |

$^a$ All linkages are $\alpha$ unless otherwise indicated.

$^b$ Integrated intensities are taken from the $\text{alg}3 \text{Man}_\alpha$ $\text{GlcNAc}_2$ $^1$H NMR data in Ref. 14.

$^c$ Core residue 3 could not be integrated accurately due to water suppression, but must be present in all N-linked glycan cores.
Hex$_8$–9GlcNAc$_2$ size, all isomers with unsubstituted 12, or without 12 altogether, must contain a lower arm substitution on residue 11. These can be 3-O substitutions by G1 or 14 (15). Residue 14 can also be 3-O-substituted with 16. Representations of these structures are shown in Scheme II as isomers 8a, 8b, 8d, and 9a.

Between 5.03 and 5.05 ppm, 1.72 mol of a$_{1,2}$-linked terminal Man anomeric protons were integrated (Table I). One mol is from the core residue 11 (Scheme I C), and the other 0.72 mol results from residue 13, which 2-O-substitutes 12. Between 5.11 and 5.14 ppm, there was 2.82 mol of proton resonance intensity. One mol is from core residue 7 (Scheme ID). Residue 12's cross-peak, when substituted with 13, is found at 5.14(C1-H)/4.02(C2-H) ppm (Fig. 5 B), and indicates that 72% of the pool contains structures in which the a$_{1,6}$-Man outer chain branch initiation has an a$_{1,2}$-Man cap. Thus, residue 7 and 2-O-substituted 12 account for 1.72 of the 2.82 mol of resonance intensity between 5.11 and 5.14 ppm, which leaves 1.10 mol to assign.

Between 5.11 and 5.14 ppm also are found signals for terminal a$_{1,3}$-Man (residues 7, 14, 15, and 16) and 3-O-substituted a$_{1,3}$-linked Man (residues 14 or 15) (Scheme ID and Refs. 15, 16, and 34). The presence of a$_{1,3}$-linked Man (14, 15, and 16) is shown by the strong 2D DQF-COSY cross-peak at 5.14(C1-H)/4.06(C2-H) ppm (Fig. 5B). The 3-O substitution of an a$_{1,3}$-linked Man causes a diagnostic downfield shift in the substituted residue's C3-H from 3.95 to 4.02 ppm (15). The presence of 3-O-substituted 14 and/or 15 on alg9 glycans is shown by the presence of the cross-peak correlations at 5.12(C1-H)/4.22(C2-H) ppm (Fig. 5B) and 4.22(C2-H)/4.02(C3-H) ppm (Fig. 6B). Integrating the 3-O-substituted C2-H signal at 4.22 ppm provided 1.20 mol of proton intensity (Table I). A trace amount of this signal was due to residue 3's C2-H when residue 12 is absent, as discussed above for pool B isomers 7b, 7c, and 8d (Scheme I). Evidence for this assignment, despite the absence of 3's J$_{1,2}$ 2D DQF-COSY cross-peak due to HDO suppression (Fig. 5B), is a weak (~0.10 mol) J$_{2,3}$ 2D DQF-COSY cross-peak at 4.22/C2-H/3.68/C3-H ppm (data not shown). This corresponds to 10% of pool C being isomer 8d, which is likely to be the co-eluting 11-min HPAEC peak seen in pool B (Fig. 3 and Scheme II).

Subtracting isomer 8d's contribution (~0.10 mol) to the 1.20 mol of intensity at 4.22 ppm leaves 1.10 mol, which correlates exactly with the residual 1.10 mol of 5.14 ppm resonance intensity calculated above (2.82 mol total minus 1.72 mol for 7 and 2-O-substituted 12) and assignable to terminal a$_{1,3}$-linked

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**Scheme II. Interrelationship of alg9 Hex$_{6-10}$GlcNAc$_2$ species deduced in this study.** The identifiers for structures are those used in the text. The regions of the secretory pathway in which the reactions occur are indicated at the top as bracketed areas denoted ER and Golgi. Glycans of interest are indicated as: A, alg3-type Glc$_x$Man$_y$GlcNAc$_z$; B, the family of N-glycans exhibiting persistent Glc; C, mature wild-type core-filled isomers or substrates for elongation to "mannan"; D, pathways leading to structures containing 16 (see text); E, structures leading to both normal core-filling or isomers containing 16. The asterisk appears above the glucosylated structures deduced from *in vivo* [2-3H]Man labeling studies (see text). Glycans 8x and 9x were not seen but are implied by the presence of 10d. The Alg3p substrate Man$_x$GlcNAc$_y$ from which all structures in this study are derived is indicated by .
Man. These are assigned collectively to 3-O-substituted residues 14, and/or 15, whose C1-, C2-, and C3-H cross-peaks have been identified above. Structure 9c can be assigned as the major component (68%) of pool C on the basis of its Man9GlcNAc2 size, the large signal for 2-O-substituted 12 (Fig. 5B), and one α1,3-linked terminal Man distributed between residues 11 and 13. Accounting for all integrated protons, 2D DQF-COSY cross-peak intensities, and Hex8,9GlcNAc2 size distribution of the pool, the remaining isomers in pool C are estimated to be: 8a, 6%; 8b, 2%; 8d, 10%; 9a, 10%; and 9b, 4%, which are in very good agreement with the number of separated peaks and their areas seen in pool C by analytical HPAEC (Fig. 3).

Pool D: Hex10GlcNAc2 Pool—The total integrated Man and Glc anomeric proton intensity of this pool was 10.0 mol, which agrees with a single MALDI-TOF MS mass for a Hex10GlcNAc2 sized glycan (Fig. 2D). Increased C1-H resonance intensity above that provided by the core Hex6GlcNAc2 was observed at 5.25, 5.14, 5.04, and 4.89 ppm (Table I).

At 5.02–5.04 ppm 2 mol of resonance intensity for terminal α1,2-linked Man was integrated. Subtracting 1 mol for core residue 11 leaves 1 mol assigned to α1,2-linked Man residue 13. This assignment is supported by the strong 2D DQF-COSY cross-peak at 5.05(C1-H)/4.22(C2-H) ppm (Fig. 5C) and indicates the presence of residue 13 on all pool isomers (Scheme ID).

At 4.89–4.91 ppm 1.07 mol of resonance intensity for 2-O-unsubstituted α1,6-linked Man was integrated. Subtracting 1 mol for core residue 4 leaves 0.07 mol. Since 12 is fully substituted by 13, in order to satisfy the Hex10GlcNAc2 size constraint, the additional 0.07 mol of 4.89–4.91 ppm resonance is likely to be present as an additional α1,6-linked Man on the new lower arm α1,6-linked branch and is assigned as residue 17 substituting residue 12. This structure is supported by the presence of a 2D DQF-COSY cross-peak of low intensity at 4.91(C1-H)/4.04(C2-H) ppm (not apparent in Fig. 5C) and assigns 7% of pool D as isomer 10d (Scheme ID).

At 5.25 ppm 0.03 mol of resonance intensity was observed for G1, and the 2D DQF-COSY cross-peak at 5.25(C1-H)/3.54(C2-H) ppm confirms its presence. Thus, 3% of pool D is 10e, Glc1Man9GlcNAc2, which appears to retain an untrimmed glucose on exit from the ER to the Golgi (Table I).

At 5.11–5.15 ppm 3.90 mol of resonance intensity was integrated for core residue 7 and 1 mol from 2-O-substituted residue 12 documented above. This leaves
1.90 mol of resonance to be assigned to α1,3-linked residues 14, 15, and 16 (Scheme ID), and, indeed a strong cross-peak is seen in the 2D DQF COSY spectrum of Pool D (Fig. 5A–C) at 5.14(C1-H)/4.06(C2-H) ppm, which is characteristic of the terminal 3-O-linked residues 14, 15, and 16. At 5.12(C1-H)/4.22(C2-H) ppm, another strong cross-peak was observed, indicating that a considerable amount of 14 and/or 15 in pool D isomers is 3-O-subsituted with residue 16. This substitution has a unique J_3,2 signature cross-peak at 4.22(C2-H)/4.02(C3-H) ppm, which is more intense in pool D than seen in pool C (Fig. 6, compare panels B and C).

At 4.22 ppm 1.93 mol of C2-H proton resonance intensity was integrated. As already described for the other pools, this is due to the 3-O-substitution of 11 with G1, or 14, of 15, or of 14 and/or 15 with 16 (Scheme ID). As described above for pool C, cross-peaks for all of these residues were observed in pool D’s 2D DQF-COSY spectra (Figs. 5C and 6C, respectively). Distribution of the integrated protons, consistent with relative cross-peak resonance intensities, and the Hex10GlcNAc2 size constraint, allows assignment of the two major residues as 10a (61%) and 10b (24%) (Scheme II). These structures account for 1.80 of the 1.90 mol of resonance to be assigned to α1,3-linked residues 14, 15, and 16 at 4.22 ppm leaving 0.23 mol, and isomers 10d and 10e, assigned above, account for an additional 0.13 mol, leaving 0.10 mol to assign. Similarly, isomer 10a, 10b, 10d, and 10e account for 1.80 of the 1.90 mol of δ 5.13 resonance intensity calculated in the previous paragraph. Isomer 10e (5%) accounts for the remaining 0.10 mol of 3-O-subsituted Man at 4.22 ppm, the 0.10 mol of α1,3-Man protons at 5.13–5.15 ppm, and the 0.05 mol of unsubstituted residue 11 at 5.03 ppm. These assignments are in very good agreement with the pool’s HPAEC profile, both in the number of peaks found and their respective areas (Fig. 3).

In Vitro Characterization of alg9 N-Linked Oligosaccharides—Because Man5GlcNAc2 was the smallest glycan released from Δalg9 secreted invertase, either this or its glucosylated form would be expected to be present on the Δalg9 OSL precursor. Fig. 7 shows comparative permethylation analysis of in vitro and in vivo-synthesized alg9 Hex6GlcNAc2. The former was released by mild acid hydrolysis from OSL synthesized in vitro under limiting glucosylation conditions (see “Experimental Procedures”), while the latter was the pool A Man6GlcNAc2.

The data for both the 3H-labeled in vitro product and the unlabeled in vivo product overlap completely, indicating they are the same structure. The labeled and unlabeled glycans were also digested with α1,2-mannosidase, which caused the products of both to migrate two hexose units smaller on Bio-Gel P-4 columns confirming this assignment (data not shown). The permethylation profiles are most consistent with the presence of 2,3,4,6 tetra-O-methyl Man, 3,4,6 tri-O-methyl Man, 2,4,6 tri-O-methyl Man, and 2,4 di-O-methyl Man. This distribution, coupled with α1,2-mannosidase digestion data, and 1D 1H NMR spectrum of the pool A (Fig. 4A) indicate that both compounds are Man1,2Man1,2Man1,3Man1,6Manβ1,4GlcNAcβ1,4GlcNAc (Scheme IC).

In Vivo Glucosylation—NMR-derived structures of secreted invertase glycans in Δalg9 yeast indicate that ~7% of Hexα1,6GlcNAc2 isomers retained residue G1 (Scheme II). To ascertain whether the persistence of G1 is a product of nominal glucosylation and processing, Δalg9 cells were pulse-chase labeled with [2-3H]Man in the absence (Fig. 8, A–C) or presence (Fig. 8, D–F) of CST, and the labeled glycans were released from glycoprotein pellets by endo H and analyzed as described under “Experimental Procedures.” At 0 min of chase, greater than 95% of endo H released glycans migrated as Glc3Man6GlcNAc (Fig. 8A, peak centered at 31 min) and ~5% migrated as Man6GlcNAc (Fig. 8A, peak centered at 8 min) in the absence of CST. The glycans migrated as the expected sizes, Hex11 and Hex9, respectively, on Bio-Gel P-4 (data not shown). The Glc3Man6GlcNAc oligosaccharide lost the equivalent of two hexose units upon digestion with jack bean α-mannosidase as revealed by Bio-Gel P-4 chromatography (data not shown), all data being consistent with its assigned identity. In the presence of CST, 0 min of chase revealed that virtually all detectable glycans released from protein by endo H were Glc3Man6GlcNAc (Fig. 8D, peak centered at 31 min). The lack of Man6GlcNAc in the presence CST and its trace amount in the absence of CST clearly demonstrate that Glc3Man6GlcNAc is the main if not the only glycan transferred to protein in the Δalg9 background.

At 1 min of chase in the absence of CST smaller glycans form,
while in the presence of CST only a trace amount of these smaller forms are detectable. From these results it is evident that the smaller glycan species (Fig. 8B, peak centered at 20 min and shoulder at 16.5 min) are largely generated by glucosidases I and II in the absence of CST but cannot be formed in the presence of the glucosidase inhibitor. In the presence of CST at 1 min of chase larger glycans are formed (Fig. 8E, peak centered at 43 min), which is expected as the GlcMnGlcNAc present on accessible regions of the peptide backbone would be expected to be elongated by Golgi mannosyltransferases.

At 10 min of chase nearly all of the [2-3H]Man-labeled GlcMnGlcNAc (Fig. 8C, peak centered at 30 min) has been processed to ManGlcNAc (Fig. 8C, peak centered at 8 min) in the absence of CST, while in the presence of CST the majority of endo H-released oligosaccharides remain GlcMnGlcNAc (Fig. 8F, peak centered at 30 min). This result clearly demonstrates that GlcMnGlcNAc is transferred to protein and largely processed to ManGlcNAc in the Δalg9 background.

DISCUSSION

The structure of the alg9 core oligosaccharide and its Golgi-modified elongation products on secreted invertase have been defined through methylation analysis, HPAEC, MALDI-TOF MS, endo H sensitivity, pulse-chase-[2-3H]Man labeling, and 

The Δalg9 core ManGlcNAc2 N-glycan transferred to protein in vitro, via its dolichol-linked intermediate, and that present on secreted invertase, are one and the same structure. This is the first endo H-sensitive oligosaccharide formed during OSL synthesis and is the Alg3p-directed product (Scheme IC). Examining the synthesis and processing of Δalg9 N-linked glycans reveals an important role for the Alg3p phosphorodolichol pathway step in downstream processing events leading to mannan on secreted S. cerevisiae invertase.

Previous studies have shown that a significant proportion of the glycan chains on alg3 sec18 invertase retained one or more Glc residues transferred from OSL during glycosylation in the ER (14, 15, 25). Furthermore, the level of OSL glucosylation in the alg3 background was very low, with only ~7% of the chains transferred to protein containing the normal glucotriose unit. Thus, the ManGlcNAc2-PP-Dol translocated into the yeast ER in alg3 is both poorly glucosylated, and the proportion that is glucosylated becomes a poor substrate for glucosidases I and II once transferred to protein. Although little residual Glc survived on Δalg9 invertase glycans, initially making it difficult to assess the potential level of glucosylation and subsequent processing, [2-3H]Man pulse-chase labeling demonstrated convincingly that the Δalg9 fully glucosylates the truncated ManGlcNAc2-PP-Dol, and that GlcMnGlcNAc is the major glycan transferred to protein from OSL in this mutant (Fig. 8). Furthermore, the complete absence of NMR signals for the terminal α-1,2-linked Glc residue, G3, or the penultimate α-1,3 Glc residue, G2, and the only small residual level of G1 (Table I) on the invertase oligosaccharides imply that both glucosidases I and II have nearly wild-type activity on the glucosidase-like alg9 oligosaccharides. It is worth noting that wild-type N-glycans retain a similar amount of G1 as seen here in the Δalg9 strain (34). Thus, an important conclusion of the current work is that the middle arm α-1,3-linked Man specified by the ALG3 locus provides structural information that potentiates the Alg3p, Alg5p, and Alg10p ER glucosyltransferases and Gls1p and Gls2p trimming glycosidases.

The NMR data show the complete absence of the upper arm Manα1,2Manα1,6-residues, indicating that, without the addition of the α-1,2-linked Man 10 to residue 7 by Alg9p (Scheme IA), no further Man additions can occur on the OSL precursor by downstream Man-P-Dol dependent mannosyltransferases. The reason such strict dependence on the Alg9p step evolved is not known with certainty, but may relate to the order of subsequent processing reactions. It is known that this disaccharide, consisting of the upper arm Manα1,2Manα1,6, –residues 9 and 6, respectively, is required for efficient ER α-1,2-mannosidase (Mns1p) trimming of the middle arm α-1,2-Man (38), and removal of this residue in conjunction with glucose trimming appears to be an integral part of protein quality control editing in the S. cerevisiae ER (39).

Mannan outer chain synthesis begins with the addition of α-1,6-linked Man 12 to the lower arm core α-1,3-linked Man 5 (Scheme ID) (7, 10) catalyzed by Och1p in the cis-Golg1, whose in vitro substrate specificity has been defined (36). Pyridylmethylated ManGlcNAc2-PA derived from the ald3och1mnna1 triple mutant (Scheme IB), formed only 9% of the Och1p product compared with Manα9GlcNAc2-PA as substrate. Another glycan, a ManGlcNAc2-PA, differing from the core alg9 structure (Scheme IC) by the presence of the upper arm α-1,6-linked Man (residue 6, see Scheme IA), converted 60% of input to product compared with the wild-type Manα9GlcNAc2-PA substrate in this study. Although the Δalg9 core Manα9GlcNAc2 was not tested as a substrate in this study (36), it appears from the current work that the addition of residue 7 does help potentiate the activity of Och1p. When comparing the alg3 and Δalg9 glycans that have one Golgi-type hexose addition (HexαGlcNAc2 for alg3; HexαGlcNAc2 for Δalg9), only 3% of the former’s pool isomers have the Och1p addition (15), while 22% of the latter’s have this addition (Scheme II). This clearly implies that in vivo the addition of Man residue 7 to the alg3 core Manα9GlcNAc2 (Scheme IC) significantly increases the proportion of glycans that are substrates for Och1p activity compared with those without this residue.

A novel series of core-filled structures was assigned containing terminal Manα1,3Manα1,3-disaccharides (see Scheme II). These structures have been identified previously on S. cerevisiae wild-type Manα1,GlcNAc (16) and to a greater extent on larger alg3 core glycans (15). Currently, little is known regarding the enzyme activity catalyzing this addition. Studies on the mnn1 mutant noted the absence of terminal Manα1,3Manα1,3-disaccharide on O-linked glycans (40), suggesting that at least the penultimate α-1,3-linked Man is added by the MNN1-encoded mannosyltransferase (40, 41). Three genes in the protein sequence data base, yil014w, ygl257c, and ynr059w, closely related to MNN1, have been implicated in the terminal α-1,3-Man addition to O-glycans. Furthermore, a new family of enzymes that add α-1,3-Man to α-1,3-Man termini in O-linked glycans has been described recently in S. cerevisiae (42). It is possible that one of these genes, yil014, ygl257, and/or ynr059w, may be responsible for decorating existing N-linked α-1,3-Man caps with a 3-O-substituting Man under certain metabolic conditions or in isolated genetic backgrounds.

The interrelationship of the alg9 structures deduced in this study is summarized in Scheme II. Kinetically, those glycans that are good substrates for mannan elongation will be depleted preferentially from the isomer pools. Outer chain elongation to mannan begins with the Och1p addition of α-1,6-linked Man 12 to the core α-1,3-linked Man residue 5 (Scheme ID). The major difference between wild-type core-filled mannan and that of alg9 is the accumulation of isomer 7b (Scheme II), which escapes the Och1p α-1,6-Man addition (residue 12), although to a much lesser extent than described earlier for alg3 glycans (15, 25). While the ALG3-directed Manα1,3-addition enhances to nearly wild-type levels OSL glucosylation and

2 GenBank™ accession numbers are: NC_001141 (yil014w), NC_001139 (ygl257c), NC_001146 (ynr059w).
subsequent removal of those added Glc residues from glycoprotein, the Och1p activity clearly did not reach wild-type levels on the basis of residual isomers 6a, 7b, and 8d (Scheme II). This suggests that completing the upper arm Manα1,2Man1,6-branched (residues 9 and 6; Scheme IA) provides additional structural information recognized by the Och1p enzyme. An important next step in understanding the role of a given mannose addition on the subsequent OSL processing steps will be to analyze glycan structures in alg mutants that accumulate OSL intermediates further on the phosphorodolichol pathway. To this end, we have begun a structural analysis of the alg12 mutant (39), which accumulates the alg9 product.

While yeast are primitive eukaryotes, the conserved aspects of their N-glycosylation pathways and homology of many biosynthetic components with those of higher eukaryotes has yielded important understanding concerning errors in metabolism leading to OSL truncation in humans. This has proven to be the case in several forms of carbohydrate-deficient glycoprotein syndrome. Indeed, a lesion in the human homolog of the ALG6 defines a new form of carbohydrate-deficient glycoprotein syndrome type I (43–45). It is likely that, while diagnosis of CDGS may fall into a limited range of recognizable phenotypes, the underlying genomic lesions may be numerous, and defects in many of the ALG genes are clear candidates to be causative agents in this emerging disease.

REFERENCES
1. Burda, P., and Aebi, M. (1998) Glycobiology 8, 455–462
2. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
3. Snider, M. D., and Robbins, P. W. (1981) Methods Cell Biol. 23, 89–100
4. Roth, J. (1987) Biochim. Biophys. Acta 906, 405–436
5. Trimble, R. B., Byrd, J. C., and Maley, F. (1982) J. Biol. Chem. 255, 11892–11895
6. Byrd, J. C., Tarentino, A. L., Maley, F., Atkinson, P. H., and Trimble, R. B. (1982) J. Biol. Chem. 257, 14657–14666
7. Herscovics, A., and Orlean, P. (1993) FASEB J. 7, 540–550
8. Hufnaker, T. C., and Robbins, P. W. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 7466–7470
9. Tanner, W., and Lehle, L. (1995) in Glycoproteins (Montreuil, J., Vliegenthart, J. F. G., and Schachter, H., eds) pp. 475–509, Elsevier, New York
10. Trimble, R. B., and Verostek, M. F. (1993) Trends Glycosci. Glycotechnol. 1, 1–30
11. Reiss, G., te Heesen, S., Zimmerman, J., Robbins, P. W., and Aebi, M. (1996) Glycobiology 6, 493–498
12. Reiss, G., te Heesen, S., Gilmore, R., Zafferey, R., and Aebi, M. (1997) EMBO J. 16, 1164–1172
13. Jakob, C. A., Burda, P., te Heesen, S., Aebi, M., and Roth, J. (1998) Glycobiology 8, 155–164
14. Verostek, M. F., Atkinson, P. A., and Trimble, R. B. (1991) J. Biol. Chem. 266, 5547–5551
15. Verostek, M. F., Atkinson, P. A., and Trimble, R. B. (1993) J. Biol. Chem. 268, 12104–12115
16. Trimble, R. B., and Atkinson, P. H. (1986) J. Biol. Chem. 261, 9815–24
17. Ito, H., Fukuta, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
18. Trimble, R. J., Robbins, P. W., Belfort, M., Ziegler, F., Maley, F., and Trimble, R. B. (1985) J. Biol. Chem. 260, 5683–5690
19. Plummer, T. H., Jr., and Tarentino, A. L. (1991) Glycobiology 1, 257–283
20. Waechter, C. J., Lucas, J. J., and Lennarz, W. J. (1973) J. Biol. Chem. 248, 7570–7579
21. Bearden, J. C., Jr. (1978) Bioclin. Biophys. Acta 533, 523–29
22. Jensen, J. W., and Schutzbach, J. S. (1982) J. Biol. Chem. 257, 9025–9029
23. Tarentino, A. L., Trimble, R. B., and Maley F. (1979) Methods Enzymol. 50, 574–580
24. Tarentino, A. L., and Plummer, T. H., Jr. (1975) Methods Enzymol. 138, 770–778
25. Verostek, M. F., Atkinson, P. H., and Trimble, R. B. (1993) J. Biol. Chem. 268, 12095–12103
26. Chu, F. K., Trimble, R. B., and Maley, F. (1978) J. Biol. Chem. 253, 8691–8693
27. Ciucanu, F., and Kerek, F. (1984) Carbohydr. Res. 131, 209–217
28. States, D. J., Haberkorn, R. A., and Ruban, D. J. (1982) J. Magn. Reson. 48, 296–292
29. Stanley, P., and Atkinson, P. H. (1986) J. Biol. Chem. 261, 11374–11381
30. Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., and Wuthrich, K. (1983) Bioclin. Biophys. Res. Commun. 117, 479–485
31. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Anal. Chem. 28, 350–356
32. Aebi, M., Gassenhuber, J., Horst, D., and te Heesen, S. (1996) Glycobiology 6, 439–444
33. Ballou, C. E. (1990) Methods Enzymol. 185, 440–470
34. Trimble, R. B., and Atkinson, P. H. (1992) Glycobiology 2, 57–75
35. Tsai, P. K., Ballou, L., Esmon, B., Schekman, R., and Ballou, C. E. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6340–6343
36. Nakayama, K., Nakaniishi-Shindo, Y., Tanaka, A., Haga-Toda, Y., and Jigami, Y. (1997) FEBS Lett. 412, 547–550
37. Trimble, R. B., Atkinson, P. H., Tschopp, J. F., Townsend, R. R., and Maley, F. (1991) J. Biol. Chem. 266, 22807–22817
38. Ziegler, F. D., and Trimble, R. B. (1991) Glycobiology 1, 605–614
39. Jakob, C. A., Burda, P., Roth, J., and Aebi, M. (1998) J. Cell Biol. 142, 1223–1233
40. Jars, M. U., Osborn, S., Forstrom, J., and MacKay, V. L. (1995) J. Biol. Chem. 270, 24810–24817
41. Graham, T. R., Verostek, M. F., MacKay, V., Trimble, R. B., and Emr, S. D. (1992) Yeast 8, (suppl.), 458
42. Romero, P. A., Lussier, M., Veronoune, S., Sidia, A. M., Herscovics, A., and Bussey, H. (1997) Glycobiology 7, 1045
43. Burda, P., Borsig, L., Derijkvanandel, J., Wevers, R., Jaeken, J., Carchon, H., Berger, E. G., and Aebi, M. (1998) J. Clin. Invest. 102, 647–652
44. Corner, C., Knauer, R., Holzbach, U., Hanefeld, F., Lehle, L., and Venfigura, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13200–13205
45. Imbach, T., Burda, P., Kuhnert, P., Wevers, R. A., Aebi, M., and Hennet, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6982–69875

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