

# Absence of Nucleoside Diphosphatase Activities in the Yeast Secretory Pathway Does Not Abolish Nucleotide Sugar-dependent Protein Glycosylation\*

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It is accepted that glycosyltransferase-generated nucleoside diphosphates are converted to monophosphates in the secretory pathway by nucleoside diphosphatases (NDPases) to provide substrates for antiport transport systems by which entrance of nucleotide sugars from the cytosol into the lumen is coupled to exit of nucleoside monophosphates. Working with *Saccharomyces cerevisiae* mutants affected in anterograde and/or retrograde endoplasmic reticulum (ER)-Golgi vesicular traffic and/or defective in one or both secretory pathway (Golgi) NDPases, we show that UDP-Glc: glycoprotein glucosyltransferase-mediated glucosylation is not dependent on the presence of NDPases or on ER-Golgi vesicular traffic and that GDP-Man-dependent *N*- and *O*-mannosylations are reduced but not abolished in the absence of NDPases in the secretory pathway. Further, the absence of the main Man-1-P transferase (a Golgi GMP-generating enzyme) does not modify the limited mannosylation observed in the absence of NDPases. Based on these results and on available additional information, we suggest that in the absence of NDPases, the already characterized nucleotide sugar transporters allow entrance of nucleotide sugars into the luminal compartments and that resulting nucleoside diphosphates exit to the cytosol by a still unknown mechanism. Further, an unexpected side result suggests that formation of Ser/Thr-Man<sub>2</sub> may occur in the ER and not exclusively in the Golgi.

Almost all nucleotide sugar-dependent glycosyltransferases in the secretory pathway generate nucleoside diphosphates (NDPs)<sup>2</sup> that are converted, via nucleoside diphosphatase (NDPase) activities, to nucleoside monophosphates (NMPs) to relieve inhibition of the transferring enzymes and to provide substrates for antiport transport systems by which entrance of nucleotide sugars from the cytosol into the lumen of the secretory pathway is coupled to exit of NMPs (1).

Several secretory pathway NDPases have been described already. There are two enzymes displaying such enzymatic activity in *Saccharomyces cerevisiae*, a GDPase/UDPase and an apyrase (denominated Gda1p and Ynd1p, respectively) (2, 3). Apyrases differ from proper

NDPases, since the former are able to degrade not only NDPs but also triphosphates. Both enzymatic activities localize to the Golgi, not a surprising finding, since no nucleotide sugar-dependent glycosyltransferases have been described in the *S. cerevisiae* endoplasmic reticulum (ER) so far. Irrespective of their cellular origin, all enzymes able to hydrolyze NDPs (NDPases proper and apyrases) share four highly similar sequences that are referred to as apyrase conserved regions. Analysis of the *S. cerevisiae* genome showed that Gda1p and Ynd1p were the only secretory pathway enzymes displaying those sequences (3). Three NDPases have been described so far in the mammalian cell secretory pathway, a Golgi apyrase and two ER GDPase/UDPases (soluble and membrane-bound) (4–7). The ER soluble enzyme is functional with Ca<sup>2+</sup>, Mg<sup>2+</sup>, or Mn<sup>2+</sup>, whereas the insoluble enzyme strictly requires the first cation for activity. It is worth mentioning that at least two nucleotide sugar-dependent glycosyltransferases have been described in the mammalian cell ER, the UDP-Glc:glycoprotein glucosyltransferase (GT) an enzyme involved in glycoprotein folding quality control that is also expressed in *Schizosaccharomyces pombe* and other fungi but not in *S. cerevisiae*, and the glucuronosyltransferase, that is expressed in the liver of higher eukaryotes (8–11).

The presence of NDPase and nucleotide sugar-dependent glycosyltransferase activities in the mammalian cell ER and Golgi compartments and the exclusive presence of the former activity in the *S. cerevisiae* Golgi (*i.e.* in the only secretory pathway compartment in which glycosyltransferases and NDPases were known to occur in this yeast) supported the notion that the presence of glycosyltransferase-generated NDPs in a subcellular compartment necessarily implied the presence of an enzyme able to hydrolyze them in the same compartment. However, we have recently reported that the yeast *S. pombe* only displayed, the same as *S. cerevisiae*, two NDPase activities in the secretory pathway (a GDPase/UDPase and an apyrase) and that both localized to the Golgi (12). This finding was rather surprising, since the fission yeast, contrary to what happens in *S. cerevisiae*, displays a robust GT activity in the ER that produces UDP (9, 12). It was suggested that perhaps ER-Golgi vesicular traffic could be involved in the conversion of UDP to UMP, thus providing antiporter metabolites for UDP-Glc entrance into the ER lumen.

To study the entrance mechanism of nucleotide sugars into the yeast secretory pathway in the absence of NDPases, we have now switched to *S. cerevisiae* cells, since in them, contrary to what happens in *S. pombe*, mutants lacking both NDPase activities are viable (3, 12), and moreover, several *S. cerevisiae* conditional mutants affected in ER-Golgi and Golgi-ER vesicular traffic are available. Synthesis and processing of *N*-glycans in wild type *S. cerevisiae* cells involves transfer of Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>2</sub> from a dolichol-P-P derivative to protein in the ER followed by deglycosylation and demannosylation to generate protein-linked Man<sub>3</sub>GlcNAc<sub>2</sub> (13). Further elongation of the glycan occurs in

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<sup>2</sup> The abbreviations used are: NDP, nucleoside diphosphate; DMJ, 1-deoxymannojirimycin; Endo H, endo- $\beta$ -N-acetylglucosaminidase H; ER, endoplasmic reticulum; GT, UDP-Glc:glycoprotein glucosyltransferase; KFN, kifunensin; NDPase, nucleoside diphosphatase; NMDNJ, *N*-methyldeoxymannojirimycin; NMP, nucleoside monophosphate.

TABLE ONE

## Strains used in this study

Strain	Genotype	Source
G2-12	MAT $\alpha$ <i>gda1::LEU2 ura3-52 lys2 ade2 his3 trp1 leu2</i>	C. Abeijón (29)
PRY225-3	MAT $\alpha$ <i>ynd1::kanMX4 alg5::HIS3 gls2::URA3 ura3-52 lys2 ade2 his3 trp1 leu2</i>	C. Abeijón (29)
RSY263	MAT $\alpha$ <i>sec12-4 ura3-52 his4</i>	R. Shekman (23)
XHW13	MAT $\alpha$ <i>mnn6-<math>\Delta</math>1::ADE2 leu2 ura3 trp1 his3 ade2 lys2</i>	Y. Jigami (27)
MLY101	MAT $\alpha$ , <i><math>\Delta</math>ufe1::TRP1 ura3-52 trp1 leu2 his3, containing pUT1 (CEN6, LEU2, ufe1-1)</i>	H. Pelham (24)
ASY-7B	MAT $\alpha$ <i>alg5::HIS3 sec12-4 ynd1::kanMX4 leu2 ura3-52 trp1</i>	This study
ASY2-14B	MAT $\alpha$ <i>alg5::HIS3 sec12-4 ynd1::kanMX4 gls2::URA3 leu2 lys2 ade2</i>	This study
AS-15C	MAT $\alpha$ <i>alg5::HIS3 sec12-4 lys2 leu2 ura3-52</i>	This study
ASG-19	MAT $\alpha$ <i>alg5::HIS3 sec12-4 gda1::LEU2 lys2 ura3-52</i>	This study
AG-17	MAT $\alpha$ <i>alg5::HIS3 gda1::LEU2 ura3-52 ade2 lys2 trp1</i>	This study
AY-21	MAT $\alpha$ <i>alg5::HIS3 ynd1::kanMX4 ura3-52 ade2 lys2 trp1</i>	This study
AYG-13	MAT $\alpha$ <i>alg5::HIS3 ynd1::kanMX4 gda1::LEU2 ura3-52 ade2 lys2 trp1</i>	This study
AU-36	MAT $\alpha$ <i>alg5::HIS3 <math>\Delta</math>ufe1::TRP1 ura3-52 containing pUT1 (CEN6, LEU2, ufe1-1)</i>	This study
AYGM-45	MAT $\alpha$ <i>alg5::HIS3 ynd1::kanMX4 gda1::LEU2 mnn6::ADE2 ura3-52 lys2 trp1</i>	This study
AYGS-1A	MAT <i>alg5::HIS3 ynd1::kanMX4 gda1::LEU2 ura3-52, sec12-4</i>	This study

the Golgi and involves the addition of Man from GDP-Man. O-Glycosylation is initiated in the ER upon transfer of Man residues from dolichol-P-Man (14). Further transfer of Man units from GDP-Man has been assumed to occur exclusively in the Golgi. An additional processing reaction, also occurring in the Golgi, is the transfer of Man-1-P from GDP-Man to both N- and O-glycans. This last reaction generates GMP as a by-product (15).

Results obtained show that entrance of UDP-Glc and GDP-Man into the yeast secretory pathway, probably mediated by the already characterized nucleotide sugar transporters, is not abolished in the absence of ER-Golgi vesicular traffic or of NDPases in the secretory pathway, although a lower extent of GDP-Man-dependent protein N- and O-mannosylation was detected under the last condition. This implies that there should be a transport mechanism allowing exit of NDPs from the secretory pathway.

## EXPERIMENTAL PROCEDURES

**Materials**—[<sup>14</sup>C]Glc (301 Ci/mol) was from PerkinElmer Life Sciences. NDPs, protease inhibitors, supplements for culture media, lysing enzyme, dithiothreitol, jack bean  $\alpha$ -mannosidase, and endo- $\beta$ -N-acetylglucosaminidase H (Endo H) were from Sigma. N-Methyl-1-deoxynojirimycin (NMDNJ), 1-deoxymannojirimycin (DMJ), and kifunensin (KFN) were from Toronto Biochemicals. Enzymes used for DNA procedures were from New England Biolabs. Zymolyase 100T was from Seikagaku Kogyo Co. Yeast culture media were from Difco, and amino acids and supplements for culture media were from Sigma.

**Strains and Media**—The *Escherichia coli* strains used were DH5 $\alpha$  or JA226. Bacteria were grown in LB medium (0.5% NaCl, 1% Tryptone, 0.5% yeast extract), with 100  $\mu$ g/ml ampicillin if necessary. *S. cerevisiae* cells were grown in YPDA medium (1% yeast extract, 2% bacto-peptone, 2% Glc, 20 mg/liter adenine) or SD medium (0.67% yeast nitrogen base without amino acids, 2% Glc) containing required supplements (adenine, uracil, tryptophan, and histidine at 20 mg/liter; leucine and lysine at 30 mg/liter). Solid media were made with 2% agar. *S. cerevisiae* mutants G2-12 and PRY225-3, RSY263, XHW13, and MLY101 were kindly provided by C. Abeijón (Boston University), R. Shekman (University of California, Berkeley), Y. Jigami (National Institute of Bioscience and Human Technology, Ibaraki, Japan), and H. Pelham (Medical Research Council, Cambridge, UK), respectively. Yeast strains used in this study are summarized in TABLE ONE.

**Strains Constructed via Tetrad Dissection of Diploids**—ASY-7B, ASY2-14B and AS-15C were obtained by mating PRY225-3 with RSY263 in YPDA. Diploid cells were selected in SD solid medium at 37 °C. Stationary phase cultures were washed twice with 0.3% potassium acetate, and sporulation was induced for 3 days in a 1:1000 dilution of the washed cultures in 0.3% potassium acetate. Asci were digested in water with 1 mg/ml Zymolyase 100T for 5 min at room temperature. The dissection was performed in YPDA with a manual micromanipulator (Singer Instruments Co). Spores were germinated at 24 °C and replica-plated to different media to be analyzed for auxotrophic markers, Geneticin resistance, and temperature sensitivity at 37 °C. Ura<sup>-</sup> mutants were chosen in all cases, since they could be transformed with p416 (URA3)-*gpt1*<sup>+</sup>. ASG-19 was obtained by mating AS-15C with G2-12. Diploids were selected in SD+Ade+Lys+Ura+Trp at 37 °C, and spores were obtained as described above. AG-17, AYG-13, and AY-21 were obtained from the tetrad dissection of the sporulated diploids obtained from mating G2-12 with PRY225-3 that were selected in SD+Ade+Lys+Ura+Trp at 28 °C. Thirteen colonies had a rough phenotype that could be easily distinguished from wild type or single mutant strains. AYGS-1A was obtained from the tetrad dissection of sporulated diploids resulting from the cross between ASY2-14B and ASG-19 that were selected in SD+Lys at 24 °C. Rough colonies were checked after dissection by replica-plating them to the appropriate auxotrophic marker media.

**Strains Constructed via Random Spore Selection of Sporulated Diploids**—To obtain AU-36, MLY101 was crossed with PRY225-3. Diploid cells were selected in SD+Ade+Lys and sporulated as described above. Sporulated cells were washed twice with water, resuspended in 5 ml of water, and incubated with agitation overnight at 28 °C with 0.6 mg/ml Zymolyase 100T in the presence of 5 mM  $\beta$ -mercaptoethanol. 5 ml of 1.5% Nonidet P-40 was added, and the suspension was incubated on ice for 20 min, vigorously shaken, and harvested. The spore suspension was resuspended in 400  $\mu$ l of 0.75% Nonidet P-40, transferred to Eppendorff tubes, and vigorously shaken during 3 min with 300 mg of acid-washed glass beads. The spore suspension was removed, and beads were washed with water. The resulting spores were plated in SD+Ade+Lys+Ura medium and incubated at 24 °C. Colonies were tested for ploidy by mating them with mating type tester strains. AYGM-45 was obtained by random spore selection from the cross

between AYG-13 and XHW13. The Mnn6p<sup>-</sup> phenotype was confirmed with the Alcian Blue test.

To confirm the presence of plasmid pUT1 (*ufe1-1*) in mutant strains, DNA was extracted, and *E. coli* strain JA226 was transformed. A plasmidic DNA extraction of ampicillin-resistant colonies was sequenced with primers T3 and T7.

**Expression of *S. pombe* GT in *S. cerevisiae* Mutant Strains**—Ura<sup>-</sup> mutants were chosen in all cases, since they could be transformed with the low copy number p416(URA3)-*gpt1*<sup>+</sup> shuttle vector, which was obtained as described before (16). Electrocompetent yeast cells were prepared from cells harvested from exponential phase cultures grown in YDA. Cells were then washed twice with 1 volume of cold water and twice with 1 volume of 10% glycerol. Cells were resuspended in 10% glycerol at a density of  $5 \times 10^9$  cells/ml, and 50  $\mu$ l was electroporated at 2.5 kV, 25 microfarads, and 200 ohms (Bio-Rad Gene Pulser II) with 400 ng of the GT-containing plasmid. Transformants were selected in appropriate SD selective medium lacking uracil at 28 °C or at 24 °C for *ufe1-1* and *sec12-4* thermosensitive strains. A DNA preparation of Ura prototrophs was tested for the presence of *S. pombe gpt1*<sup>+</sup> gene by PCR with primers GPD Promoter (Vector): 5'-CTTCTGCTCTCTGAT-TTG-3' and 1ASH2 (GT antisense) 5'-TATATCTAAGACCTTCGA-3').

***N*-Glycan Labeling**—For assessing Golgi *N*-glycan elongation, cells in the exponential growth phase were harvested, extensively washed with 1% yeast nitrogen base, resuspended in the same medium, and incubated (0.3 g in 1 ml) for 30 min at 28 °C (except where otherwise indicated) in the presence of 5 mM [<sup>14</sup>C]Glc (30.1 Ci/mol). After chasing cells with 50 mM Glc (final concentration), cells were incubated for 30 min more. Conditional mutant cells were incubated at 37 °C (restrictive temperature). *Sec12* cells were preincubated at that temperature for 30 min, after which they were incubated with 5 mM [<sup>14</sup>C]Glc (30.1 Ci/mol) for 30 min followed by a 30-min chase with 50 mM Glc. Cells bearing the *ufe1* mutation were first incubated for 30 min at 37 °C and then for 60 min at the same temperature in the presence of 5 mM unlabeled Glc and finally with 5 mM [<sup>14</sup>C]Glc (30.1 Ci/mol) for 60 min followed by a 60-min chase with 50 mM Glc. Where indicated, DMJ plus KFN (2.5 mM final concentration each) were added at the beginning of preincubations. For assessing ER, *N*-glucosylation incubations lasted for 30 min and were not chased. Where indicated, 2.5 mM NMDNJ plus 5 mM dithiothreitol were added to incubations, the first reagent 30 min and the second one 5 min before the label. For further details on the labeling procedure and preparation of whole cell Endo H-sensitive *N*-glycans, see Ref. 9.

***O*-Glycan Labeling**—Cells, previously extensively washed with 1% yeast nitrogen base, were resuspended in the same medium (0.16 g in 1 ml) and incubated for 60 min at 28 °C (except where otherwise indicated) in the presence of 5 mM [<sup>14</sup>C]Glc (30.1 Ci/mol), chased with 50 mM Glc (final concentration), and incubated for 60 min more. Conditional mutant cells were incubated at 37 °C (restrictive temperature). *Sec12* cells were preincubated at that temperature for 30 min and incubated with the label and chased as above. Cells bearing the *ufe1* mutation were first incubated for 30 min at 37 °C and then for 60 min at the same temperature in the presence of 5 mM unlabeled Glc and finally with the label for 60 min followed by a 60-min incubation with 50 mM Glc. Cells were then processed as above for obtaining *N*-glycans, but the denatured cell pellets obtained after organic solvent-water treatments were incubated with 0.4 ml of 0.1 N NaOH overnight at room temperature. Solutions were neutralized with 0.1 N HCl, and 0.3 ml of water was then added. The supernatants obtained upon the addition of 2 ml of methanol were dried, resuspended in water, and desalted with a mixed bead (H<sup>+</sup>/acetate) resin.

**Methods**—Paper chromatographies were performed on Whatman 1 papers with solvents A (1-propanol/nitromethane/water (5:2:4)), B (1-butanol/pyridine/water (10:3:3)), and C (1-butanol/pyridine/water (4:3:4)). *N*-Glycans were analyzed also by partition chromatography using a Glyco-Pak N column (Waters); <sup>14</sup>C-labeled glycans were dissolved in 200  $\mu$ l of CH<sub>3</sub>CN:H<sub>2</sub>O (75:25, v/v) and applied to the column equilibrated with CH<sub>3</sub>CN, 1 mM NaH<sub>2</sub>PO<sub>4</sub> in H<sub>2</sub>O (80:20, v/v). A linear gradient over 280 min was employed starting from the above mentioned initial condition and ending at CH<sub>3</sub>CN, 1 mM NaH<sub>2</sub>PO<sub>4</sub> in H<sub>2</sub>O (45:55, v/v) at a flow rate of 0.8 ml/min. Fractions of 1 ml were collected and dried in a SpeedVac. Radioactivity was quantified after solubilizing the eluted material in 200  $\mu$ l of water. The sizes of the main peaks in HPLC runs were ascertained by rerunning them on paper chromatography with appropriate standards at both sides of the samples. Strong acid hydrolysis was performed in 1 N HCl for 4 h at 100 °C. Acetolysis and jack bean  $\alpha$ -mannosidase treatments were performed as described before (17).

**Enzymatic Assays**—*S. cerevisiae* microsomes were prepared as described previously (9). NDPase activities were essentially assayed as described with slight modifications (18). Briefly, between 10 and 25  $\mu$ g of membrane proteins were incubated in a total volume of 100  $\mu$ l in 0.2 M imidazol buffer pH 7.2, 0.1% digitonin, 10 mM CaCl<sub>2</sub>, and a 2 mM concentration of the corresponding NDP and incubated for 5–10 min at 30 °C. Reactions were stopped upon the addition of 100  $\mu$ l of 10% SDS and 100  $\mu$ l of water. Liberated phosphate was assayed as described (19), employing 15-min incubations at 45 °C. Phosphates present either in microsomes and reagents or liberated during incubations at 45 °C were estimated for each tube with blanks in which the SDS was added before the membrane fractions. Glucosidase II and GDP-Man- and  $\alpha$ -methylmannoside-dependent activities were assayed as described before (12, 20). For the assay of the former enzyme either the labeled or unlabeled substrates indicated in Ref. 12 were employed. Fractionation of *S. cerevisiae* microsomal membranes by sucrose gradient centrifugations was performed as previously described (12).

## RESULTS

**Hydrolysis of NDPs by Mutant Cell Microsomes**—*Alg5* mutant cells were used throughout all experiments, since one of the purposes of the present work was to probe *in vivo* UDP-Glc entrance into the ER by assaying GT-mediated glycoprotein glucosylation. *Alg5* cells are defective in the dolichol-P-Glc synthetase and thence transfer to proteins Man<sub>9</sub>GlcNAc<sub>2</sub> instead of the complete glycan (21). In these mutants, therefore, monoglucosylated glycans can be formed only by GT-mediated activity. *Alg5*, *alg5 gda1*, *alg5 ynd1*, and *alg5 gda1 ynd1* mutant cells were obtained as described above. As already described for *gda1 ynd1* mutants, *alg5 gda1 ynd1* cells were severely impaired in the germination of spores but much less affected in vegetative growth (3). The doubling times of *alg5*, *alg5 gda1*, *alg5 ynd1*, and *alg5 gda1 ynd1* cells in YPD + Ade medium at 28 °C were 113, 116, 113, and 345 min, respectively. *Alg5 gda1 ynd1* cells looked under the light microscope as shown for *gda1 ynd1* mutants in Fig. 4 of Ref. 3. Further, *alg5 gda1 ynd1* cells were able to mate (see "Experimental Procedures"). UDPase and GDPase activities were assayed in microsomes derived from *alg5*, *alg5 gda1*, *alg5 ynd1*, and *alg5 gda1 ynd1* mutant cells. As mentioned above, the *GDA1* and *YND1* genes code for the GDPase/UDPase and apyrase activities, respectively. *Alg5 gda1 ynd1* mutant cells were totally devoid of UDPase and GDPase activities in the microsomal membranes (Fig. 1A). This result agrees with the analysis of *S. cerevisiae* genome that detected only two proteins of possible secretory pathway location (Gda1p and Ynd1p) bearing the so-called apyrase conserved regions (3). There are two

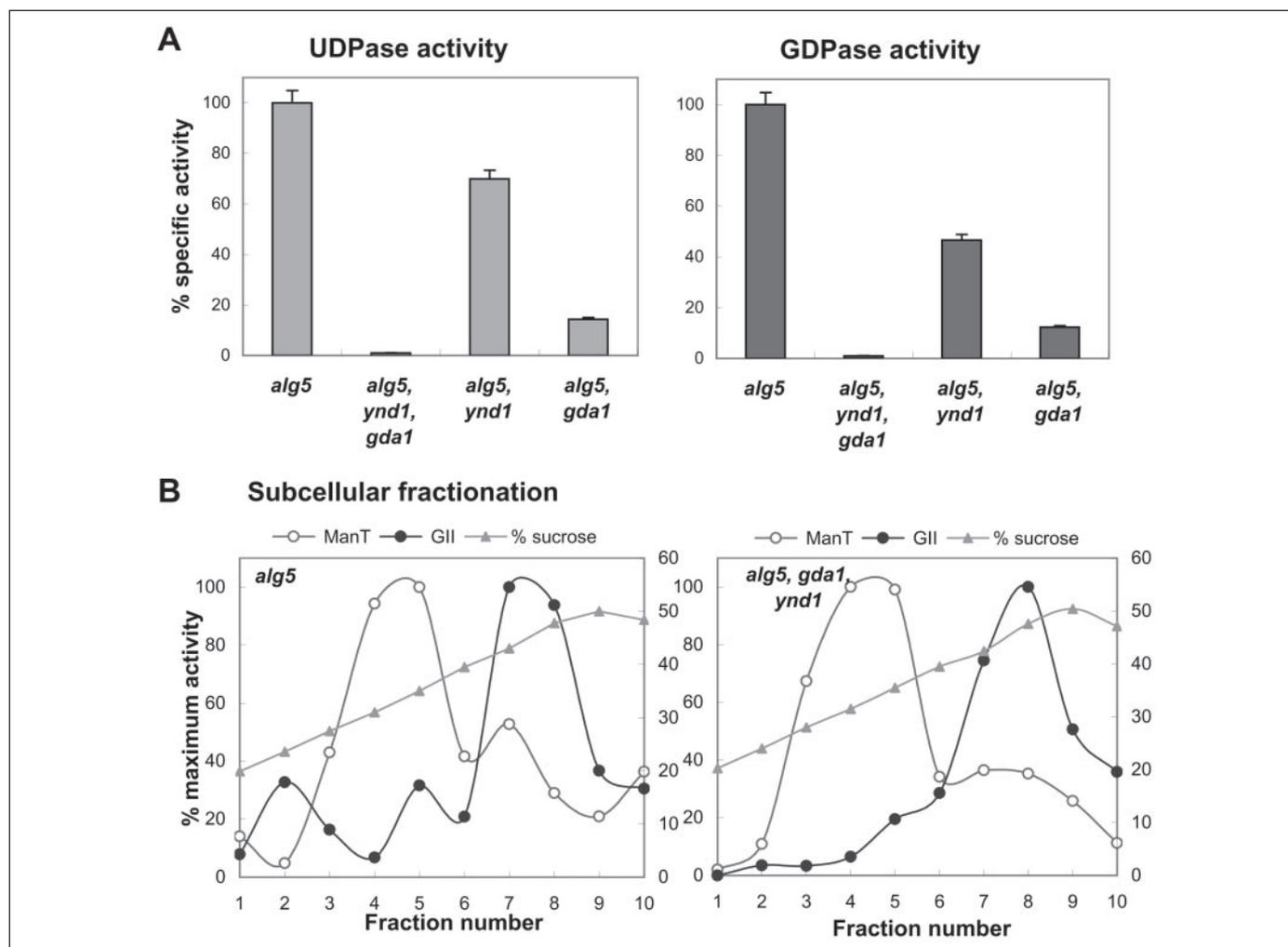
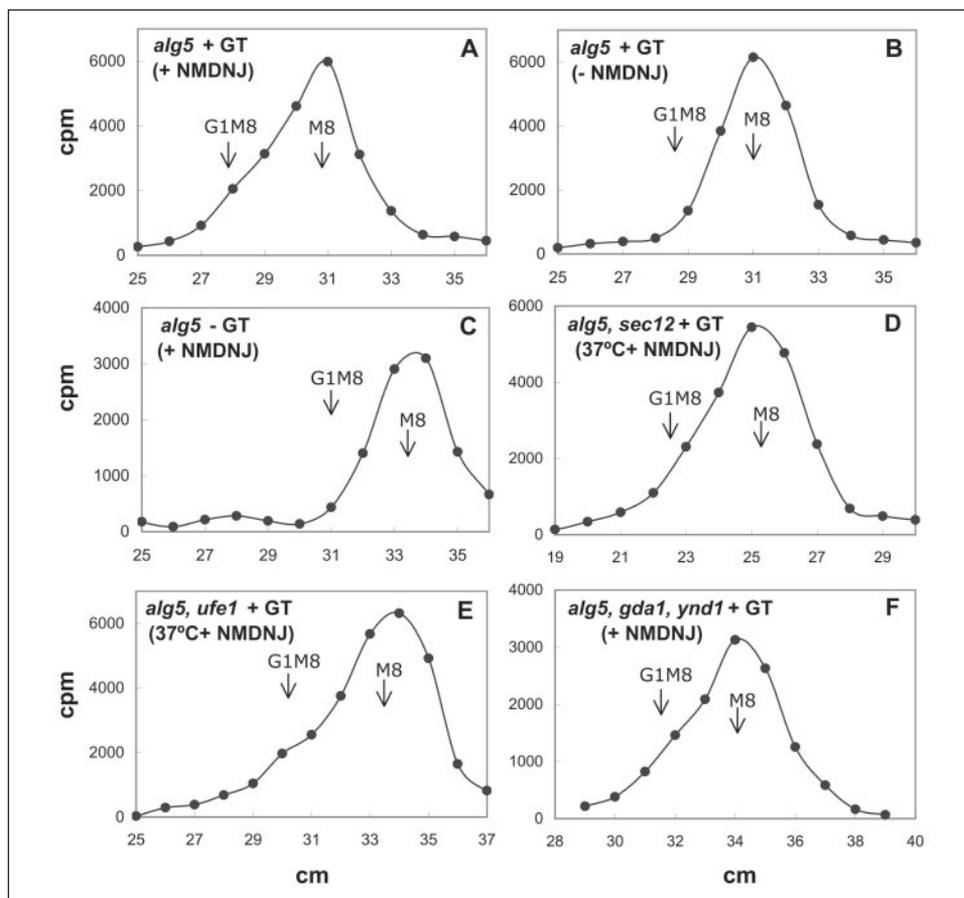


FIGURE 1. NDPases in wild type and mutant cell microsomes. Microsomes from indicated *alg5*, *alg5 gda1*, *alg5 ynd1*, and *alg5 gda1 ynd1* mutant cells were assayed for UDPase and GDPase activities (A). Microsomes derived from *alg5* and *alg5 gda1 ynd1* cells were submitted to sucrose gradient centrifugations and glucosidase II (full circles) and GDP-Man-dependent mannosyltransferase (empty circles) activities assayed (B). For further details, see "Experimental Procedures."

pieces of evidence indicating that *alg5 gda1 ynd1* mutants still conserved distinct ER and Golgi compartments: (a) similar separations between glucosidase II (an ER marker) and a GDP-Man dependent mannosyltransferase (a Golgi marker) were observed on submitting *alg5*- and *alg5 gda1 ynd1*-derived microsomes to sucrose gradient centrifugations (Fig. 1B), and (b) as will be described below (Fig. 4F), the presence of both *gda1* and *ynd1* mutations did not affect the restriction to the ER-Golgi anterograde vesicular traffic imposed at 37 °C by the *sec12* mutation.

*Expression of S. pombe GT in S. cerevisiae ER*—*S. cerevisiae alg5* mutant cells transfected with a low copy number expression vector coding for *S. pombe* GT were incubated for 30 min with 5 mM [<sup>14</sup>C]Glc in the presence of 2.5 mM NMDNJ, a glucosidase II inhibitor and 5 mM dithiothreitol. This reagent effectively prevents proper folding, and thus ER exit, of glycoproteins by interfering with disulfide bond formation (22). Secretion of disulfide-free proteins is not affected. The pattern of *N*-glycans obtained after submitting whole cell proteins to a drastic proteolytic degradation followed by Endo H treatment of resulting glycopeptides showed that the main peak thus liberated migrated as a Man<sub>8</sub>GlcNAc standard, but also material in the position expected for Glc<sub>1</sub>Man<sub>8</sub>GlcNAc was detected (Fig. 2A). Strong acid hydrolysis of the material shown in Fig. 2A followed by paper chromatography revealed the presence of labeled Glc and Man units (Fig. 3A). No material migrat-

ing as Glc<sub>1</sub>Man<sub>8</sub>GlcNAc and no labeled Glc residues appeared either when NMDNJ was omitted from the incubation or when the expression vector did not encode GT (Figs. 2, B and C, and 3, B and C). Patterns similar to those shown in Figs. 2A and 3A were obtained with *alg5 sec12* as well as with *alg5 ufe1* mutant cells at the restrictive temperature (Figs. 2, D and E, and 3, D and E). At this temperature, the *sec12* mutation totally impairs the initial stages in the formation of COPII-coated vesicles, responsible for ER to Golgi vesicular traffic (23). On the other hand, at the restrictive temperature, the *ufe1* mutation directly impairs Golgi to ER retrograde vesicular traffic and (indirectly and upon prolonged incubations, such as those employed in the present work) ER to Golgi vesicular traffic as well, presumably due to the impediment of recycling membrane proteins involved in anterograde transport (24). Cells bearing the *ufe1* mutation were first incubated for 30 min at 37 °C and then for 60 min at the same temperature in the presence of 5 mM unlabeled Glc and finally for 30 min with 5 mM labeled Glc also at the restrictive temperature. This procedure was followed to first block the retrograde vesicular traffic and then the anterograde one and to deplete the ER of any protein or NMPs that might had been transported from the Golgi by vesicular traffic (results shown further in Fig. 6 confirmed that both anterograde and retrograde vesicular traffic were completely blocked under the experimental conditions employed). Results shown in Figs. 2 and 3 indicate that *S. pombe* GT had been functionally expressed in *S.*



**FIGURE 2. Expression of *S. pombe* GT in *S. cerevisiae* ER.** A, N-glycans synthesized by *S. cerevisiae* *alg5* mutant cells transformed with a GT-encoding expression vector in the presence of NMDNJ; B, the same as A but without the addition of NMDNJ; C, the same as A but with a sham vector; D, the same as A but with *alg5 sec12* cells at 37 °C, or *alg5 ufe1* cells at 37 °C (E) or *alg5 gda1 ynd1* cells at 28 °C (F) were employed. Samples were run on paper chromatography with solvent A. Standards were as follows: M8, Man<sub>8</sub>GlcNAc; G1M8, Glc<sub>1</sub>Man<sub>8</sub>GlcNAc. For further details, see "Experimental Procedures."

*S. cerevisiae* ER, since detection of labeled Glc residues was dependent on an expression vector encoding the enzyme and upon inhibition of glucosidase II, a permanent ER-resident enzyme. Further indication that GT was functionally expressed in the ER was provided by two additional facts: (a) *S. pombe* GT, the same as GTs from other sources, is known to exclusively glucosylate folding intermediates and irreparably misfolded glycoproteins (9) (*i.e.* species that occur only in the ER), and (b) glucosylated glycoproteins were detected under conditions that totally precluded ER to Golgi vesicular traffic. Further, results shown indicate that entrance of UDP-Glc into the ER lumen was not dependent on the occurrence of ER-Golgi anterograde or retrograde vesicular traffic or of NDPases in the ER. Any of the NDPase molecules that might have been present in the ER as a consequence of ER-Golgi cycling would have been expected to be transported to the vacuole in the *ufe1* mutant at the restrictive temperature, as has been described for several Golgi proteins (25). It is worth noticing that the above mentioned conclusions were obtained with mutants still expressing both Golgi NDPases.

**Formation of Glc<sub>1</sub>Man<sub>8</sub>GlcNAc<sub>2</sub> in Other Mutant Cells**—To further confirm that entrance of UDP-Glc into the ER lumen did not require the presence of NDPases in the ER lumen, we expressed *S. pombe* GT in additional mutant cells lacking either one or both NDPases. Results indicative of Glc<sub>1</sub>Man<sub>8</sub>GlcNAc<sub>2</sub> formation similar to those shown in Fig. 2 (A, D, and E) and Fig. 3 (A, D, and E) were obtained under the following experimental conditions: (a) *alg5 gda1*, (b) *alg5 gda1 sec12* at the restrictive temperature, (c) *alg5 ynd1*, (d) *alg5 ynd1 sec12* at the restrictive temperature, and (e) *alg5 gda1 ynd1* at 28 °C (Figs. 2F and 3F). Results obtained confirmed, therefore, that the entrance of UDP-Glc into the ER lumen does not require the occurrence of NDPases in the secretory pathway. It is worth mentioning that the Glc/Man label ratio

in *alg5 gda1 ynd1* mutants (0.134; Fig. 3F) was similar to that detected in *alg5* cells (0.124; Fig. 3A), thus indicating that the absence of NDPases in the secretory pathway did not impair GT-mediated glycoprotein glucosylation.

**N-Mannosylation in Mutants Defective in NDPases**—The following experiments were performed with *alg5* mutants to maintain the same genetic background as those described above. To study whether the absence of NDPases in the secretory pathway affected GDP-Man-dependent N-mannosylation, we incubated *alg5*, *alg5 gda1*, *alg5 ynd1*, and *alg5 gda1 ynd1* mutants with 5 mM labeled Glc for 30 min followed by a 30-min chase with 10-fold higher Glc concentration. The N-glycan patterns obtained are shown in Fig. 4, A–D. In this case, N-glycans were not separated by paper chromatography but by HPLC in a Glyco-Pak N column in order to get better resolution of compounds. As outlined above, N-glycans were obtained by submitting whole cell proteins to proteolytic degradation followed by Endo H treatment. To further confirm that compounds thus liberated were canonical *S. cerevisiae* high mannose-type glycans, they were treated with jack bean  $\alpha$ -mannosidase (an exoglycosidase). In all cases, the isolated compounds were degraded to substances migrating as Man and ManGlcNAc, as judged by paper chromatography in solvent B.

It may be observed that *alg5*, *alg5 gda1*, and *alg5 ynd1* cells produced almost identical N-glycan patterns. On the other hand, that yielded by the *alg5 gda1 ynd1* mutant covered a more restrictive range of glycan sizes, since, besides Man<sub>8</sub>GlcNAc and Man<sub>9</sub>GlcNAc, only glycans with 10–11 Man residues were synthesized (Fig. 4D). The addition of mannosidase inhibitors DMJ plus KFN to *alg5 gda1 ynd1* cells somewhat modified the pattern, since lower proportions of Man<sub>8</sub>GlcNAc and higher proportions of Man<sub>10</sub>GlcNAc were obtained (Fig. 4E). DMJ plus

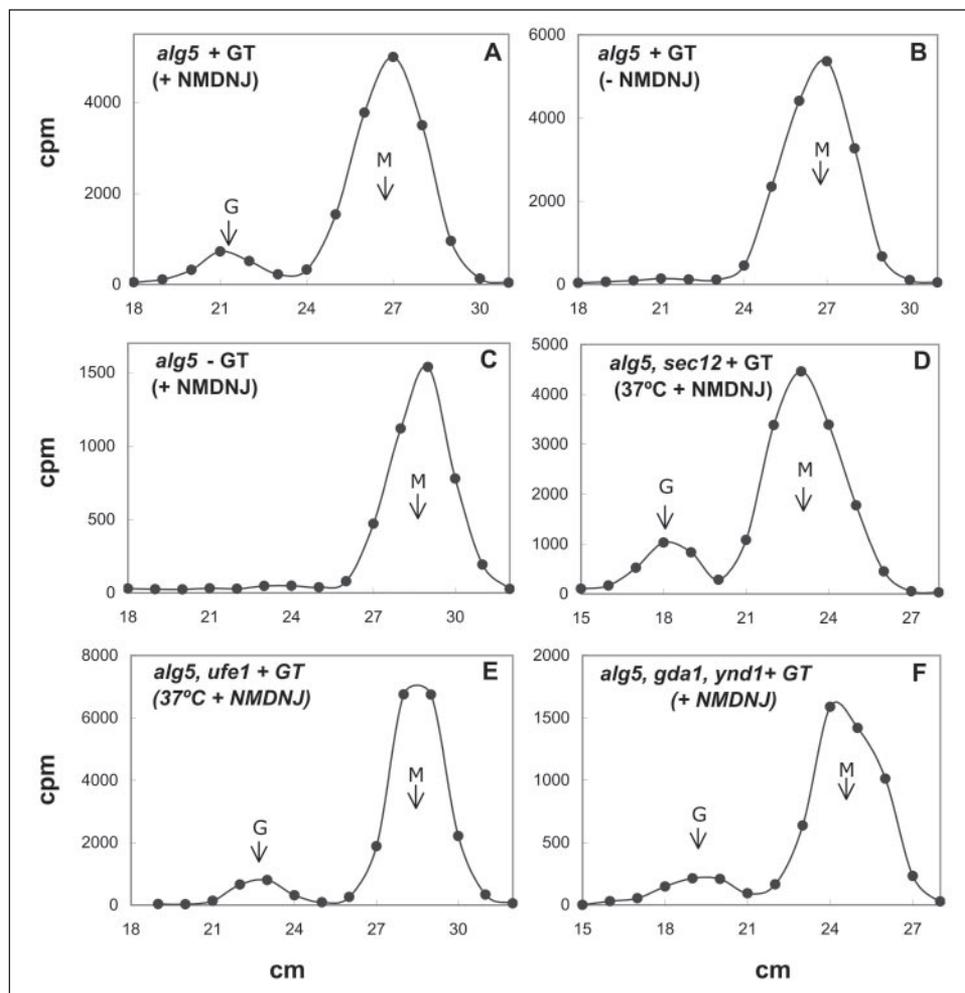


FIGURE 3. Monosaccharide composition of *N*-glycans. Materials shown in Fig. 2, A–F, were submitted to strong acid hydrolysis and run on paper chromatography with solvent B. The meaning of the lettering is the same as in Fig. 2. Standards were as follows: M, Man; G, Glc. For further details, see “Experimental Procedures.”

KFN was added to prevent as much as possible ER  $\alpha$ -mannosidase activity on *N*-glycans and thus to better detect GDP-Man-dependent glycan elongation. Labeling of *alg5 gda1 ynd1 sec12* mutant cells at the restrictive temperature (37 °C) in the presence of ER  $\alpha$ -mannosidase inhibitors yielded  $\text{Man}_9\text{GlcNAc}$  as the main *N*-glycan and minimal amounts of  $\text{Man}_{10}\text{GlcNAc}$  and  $\text{Man}_8\text{GlcNAc}$  (Fig. 4F). The low amount of the former compound (compare it with that shown in Fig. 4E) indicated that the absence of NDPases in the secretory pathway did not alter the restriction to the vesicular ER to Golgi traffic at 37 °C imposed by the *sec12* mutation. This is a further indication that *alg5 gda1 ynd1* mutant cells have distinct ER and Golgi compartments.

To further confirm that Man residues had been added to the glycan transferred from the lipid derivative to protein, we submitted the compound migrating as  $\text{Man}_9\text{GlcNAc}$  isolated from *alg5 gda1 ynd1* cells labeled in the presence or absence of mannosidase inhibitors to acetolysis followed by paper chromatography with solvent C. Acetolysis preferentially cleaves  $\alpha(1,6)$  bonds between Man residues. Since the  $\text{Man}_9\text{GlcNAc}$  glycan transferred from the dolichol-P-P derivative has two such bonds, it is expected to generate  $\text{Man}_2$  and  $\text{Man}_3$  upon acetolysis, in addition to  $\text{Man}_4\text{GlcNAc}$ . As mentioned above, the transferred glycan is demannosylated to  $\text{Man}_8\text{GlcNAc}$  by the ER  $\alpha$ -mannosidase. This last compound also has two  $\alpha(1,6)$  and yields  $\text{Man}_2$  and  $\text{Man}_4\text{GlcNAc}$  on acetolysis. According to Ref. 26, the first step in the elongation of  $\text{Man}_8\text{GlcNAc}$  is the addition of an  $\alpha(1,6)$ -linked Man unit to the  $\text{Man}_4\text{GlcNAc}$  arm of the glycan. Acetolysis of the  $\text{Man}_9\text{GlcNAc}$  isomer thus synthesized is expected then to generate Man,  $\text{Man}_2$ , and

$\text{Man}_4\text{GlcNAc}$  upon acetolysis (only one GlcNAc residue was indicated in the above structures, since acetolysis was performed on Endo H-released glycans; see Fig. 1 in Ref. 13 for a better understanding of the above explanation).  $\text{Man}_9\text{GlcNAc}$  obtained in the absence of DMJ plus KFN essentially yielded, in addition to  $\text{Man}_4\text{GlcNAc}$ ,  $\text{Man}_2$  and Man (Fig. 5A). This indicated, therefore, that in this case  $\text{Man}_9\text{GlcNAc}$  was the glycan formed upon a single Man addition to protein-linked  $\text{Man}_8\text{GlcNAc}_2$ . In the presence of the inhibitors, however, the main acetolysis products were, besides  $\text{Man}_4\text{GlcNAc}$ ,  $\text{Man}_2$  and  $\text{Man}_3$ , thus indicating that  $\text{Man}_9\text{GlcNAc}$  was the glycan transferred to protein from the dolichol-P-P derivative (Fig. 5B). Results shown in Figs. 4 and 5 indicate that about 2–3 Man residues had been transferred from GDP-Man to protein-linked  $\text{Man}_8\text{GlcNAc}_2$  in the absence of NDPases in the secretory pathway but that further glycan elongation absolutely required the presence of at least one of the enzymes displaying such activity.

**Subcellular Site of Initial *N*-Glycan Elongation**—To confirm that *N*-glycan elongation occurred in the Golgi, *alg5 sec12* cells were pulse-chased with labeled Glc at the restrictive temperature, in the absence or presence of DMJ plus KFN.  $\text{Man}_8\text{GlcNAc}$  was the main glycan formed under the former condition (Fig. 6A). Lower amounts of  $\text{Man}_9\text{GlcNAc}$  and  $\text{Man}_7\text{GlcNAc}$  also appeared. This last glycan reflected the long permanence of glycoproteins in the ER imposed by the *sec12* mutation at the restrictive temperature.  $\text{Man}_9\text{GlcNAc}$  and lower  $\text{Man}_8\text{GlcNAc}$  amounts were the glycans formed in the presence of the mannosidase

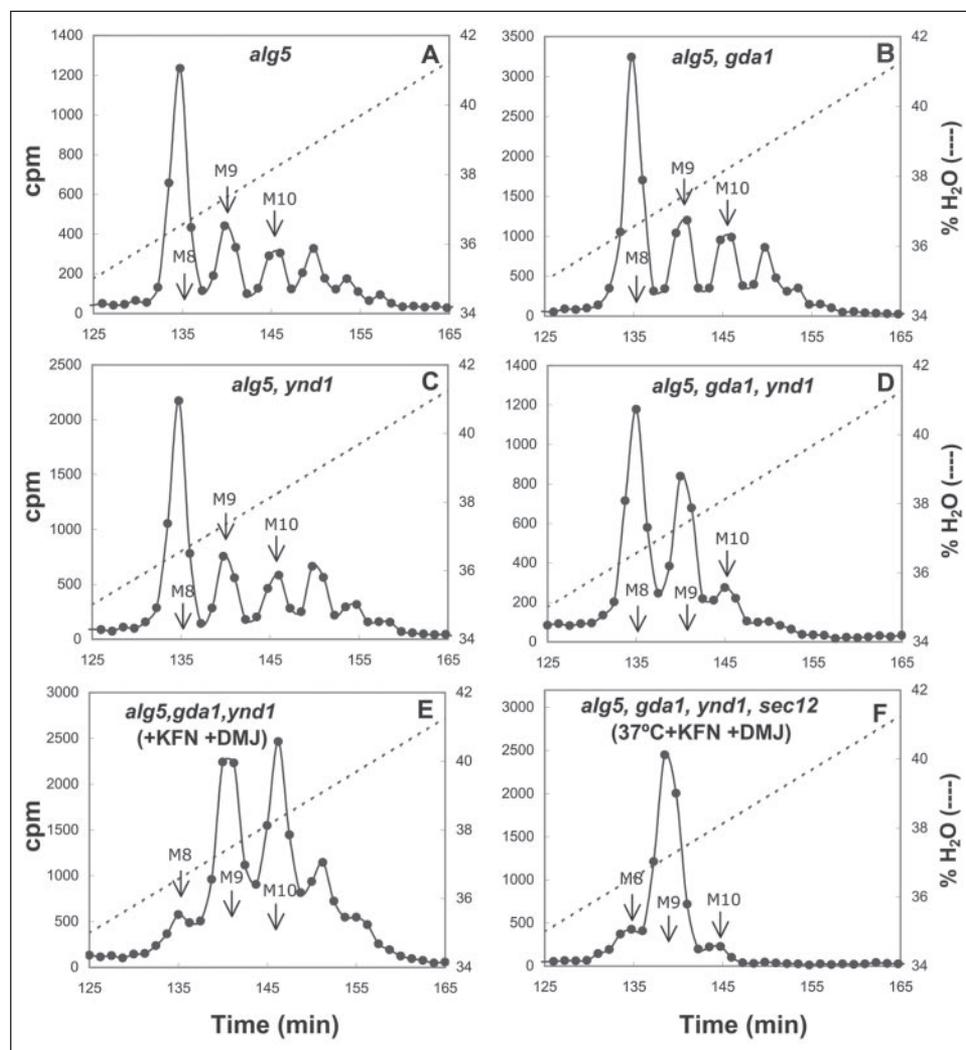


FIGURE 4. *N*-mannosylation in mutants defective in NDPases. Cells were pulse-chased with [ $^{14}$ C]Glc and Endo H-sensitive, whole cell *N*-glycans separated by HPLC. Cells used were as follows: *alg5* (A), *alg5 gda1* (B), *alg5 ynd1* (C), *alg5 gda1 ynd1* (D and E). In E, cells were incubated with DMJ plus KFN. In F, *alg5 gda1 ynd1 sec12* cells were preincubated for 30 min at 37 °C and further pulse-chased at the same temperature in the presence of DMJ plus KFN. Standards were as follows: M8, Man<sub>8</sub>GlcNAc; M9, Man<sub>9</sub>GlcNAc; M10, Man<sub>10</sub>GlcNAc. For further details, see "Experimental Procedures."

inhibitors (Fig. 6B). A pattern similar to that shown in Fig. 6B was obtained when *alg5 ufe1* cells were employed at the restrictive temperature in the presence of ER  $\alpha$ -mannosidase inhibitors (Fig. 6C). In this case, cells had been previously preincubated for 30 min at 37 °C and then for 60 min with 5 mM unlabeled Glc before adding the label, as described under "Experimental Procedures." Since no glycans larger than Man<sub>9</sub>GlcNAc were obtained in any case, results shown in Fig. 6 indicate that *sec12* and *ufe1* mutations effectively blocked anterograde and retrograde vesicular traffic, respectively, and that initial *N*-glycan elongation does not occur in the ER but in the Golgi. From results shown in Figs. 4–6, it may be concluded, therefore, that GDP-Man may enter into the lumen of the Golgi in the absence of NDPases in the secretory pathway.

*O*-Mannosylation in Mutants Defective in NDPases—To study how the absence of NDPases in the secretory pathway affected GDP-Man-dependent *O*-mannosylation, we incubated *alg5*, *alg5 gda1*, *alg5 ynd1*, and *alg5 gda1 ynd1* with 5 mM labeled Glc for 60 min followed by a similar time chase with a 10-fold higher concentration of the unlabeled monosaccharide yielded the *O*-linked glycan patterns shown in Fig. 7, A–D. It may be observed that the proportions of Man, Man<sub>2</sub>, Man<sub>3</sub>, Man<sub>4</sub> and even the shoulder in the position of Man<sub>5</sub> were very similar in glycans produced by the first three strains. On the other hand, *alg5 gda1 ynd1* cells mainly produced Man and a low proportion of Man<sub>2</sub>. As in the case of *N*-glycans, to ensure that only  $\alpha$ -linked mannose residues

had been incorporated into *O*-glycans, the latter were treated with jack bean  $\alpha$ -mannosidase and further run on paper chromatography with solvent B. In all cases, *O*-glycans were fully degraded to mannose. It may be concluded that cells lacking NDPases in the secretory pathway are able to transfer a single Man unit from GDP-Man to Ser/Thr-Man but cannot further elongate the disaccharide. Results obtained on studying nucleotide sugar-dependent *N*- and *O*-mannosylation were in complete agreement, since only mutant cells lacking both NDPases yielded significant differences in the glycan patterns.

*Subcellular Site of Initial O-Glycan Elongation*—*Alg5 sec12* and *alg5 ufe1* were pulse-chased with labeled Glc at the restrictive temperature. The patterns of *O*-linked glycans formed in both strains were very similar, since in both, Man and Man<sub>2</sub> and low amounts of Man<sub>3</sub> were formed, but the proportion of the disaccharide was higher in the *sec12* mutant (Fig. 8, A and B). The formation of Man<sub>2,3</sub> cannot be ascribed to leakiness of either *sec12* or *ufe1* mutants, since none of them showed detectable Golgi elongation of *N*-glycans, even in the presence of ER  $\alpha$ -mannosidase inhibitors (Fig. 6). Since many Golgi proteins continuously recycle between that organelle and the ER (see below), it may be speculated that one or both NDPases may have accumulated in the ER due to the impediment in anterograde vesicular traffic imposed by the *sec12* mutation at the restrictive temperature. To discard the possibility that a Golgi NDPase could have been responsible for the entrance of

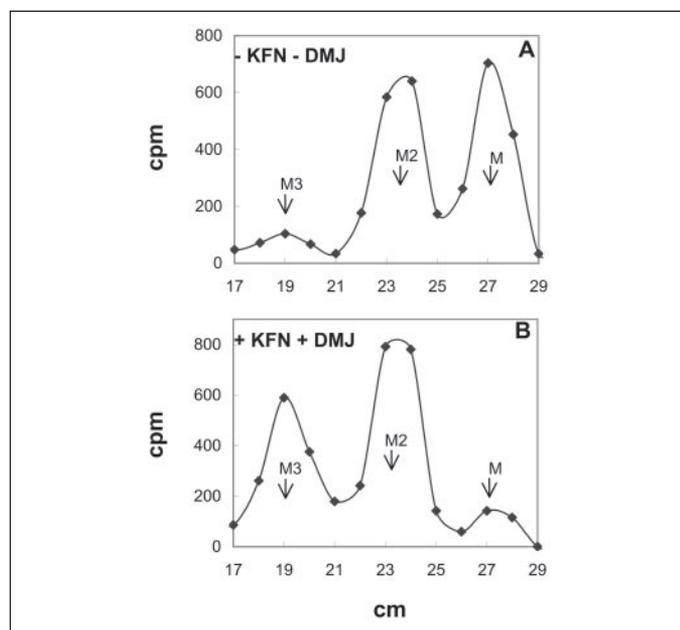


FIGURE 5. **N-glycan structures.**  $\text{Man}_6\text{GlcNAc}$  synthesized by *alg5 gda1 ynd1* cells in the absence (A) or in the presence (B) of DMJ plus KFN were submitted to acetolysis and run on paper chromatography with solvent C. Standards were as follows: M, Man; M2,  $\text{Man}_2$ ; M3,  $\text{Man}_3$ . In both samples, the peak corresponding to  $\text{Man}_4\text{GlcNAc}$  was omitted. For further details, see "Experimental Procedures."

GDP-Man into the ER lumen, *alg5 gda1 ynd1 sec12* cells were pulse-chased with labeled Glc at the restrictive temperature. As shown in Fig. 8C,  $\text{Man}_{2,3}$  were also formed under those conditions.

The most straightforward interpretation of the results shown in Fig. 8 is that nucleotide sugar-dependent O-glycan elongation may occur in the ER and that, therefore, GDP-Man may enter into the lumen of that organelle in the absence of NDPases in the secretory pathway.

It is worth noting that evidence indicating that GDP-Man-dependent N- and O-mannosylations start in the Golgi and the ER, respectively, was obtained with traffic mutants expressing both NDPases and in which the subcellular structure of the secretory pathway known for wild type cells is not expected to be affected.

**Transfer of Man-1-P Does Not Generate GMP Required for GDP-Man Luminal Entrance**—As mentioned above, an additional processing reaction occurring in the Golgi is the transfer of Man-1-P from GDP-Man to Man units in N- and O-glycans, thus generating GMP (15, 26). The Man-1-P transferases are probably distributed along all of the Golgi cisternae, since phosphate units are found linked to Man units both close to and distal from the Asn residue. Not all Man-1-P transferases have been identified yet, but Mnn6p has been found to account for over 80% of total cellular activity and to be able to phosphorylate  $\text{Man}_8\text{GlcNAc}_2$ , the glycan present in glycoproteins transported from the ER to the Golgi (15, 27). We have assayed the *alg5 gda1 ynd1 mnn6* mutant for N-glycan elongation in the presence of DMJ plus KFN and for O-glycan synthesis and found no difference with patterns obtained with *alg5 gda1 ynd1* cells under the same experimental conditions, thus suggesting that GMP generated by Man-1-P transfer is not responsible for GDP-Man luminal entrance and thus of mannosylation observed in *alg5 gda1 ynd1* mutant cells (Fig. 9, A and B). Further, formation of  $\text{Man}_2$  in the ER of *alg5 gda1 ynd1 sec12* mutant cells at 37 °C (Fig. 8C) cannot be explained by the entrance of GDP-Man into the ER lumen mediated by GMP generated by the Man-1-P transferase, since the enzyme(s) localizes to the Golgi, not to the ER.

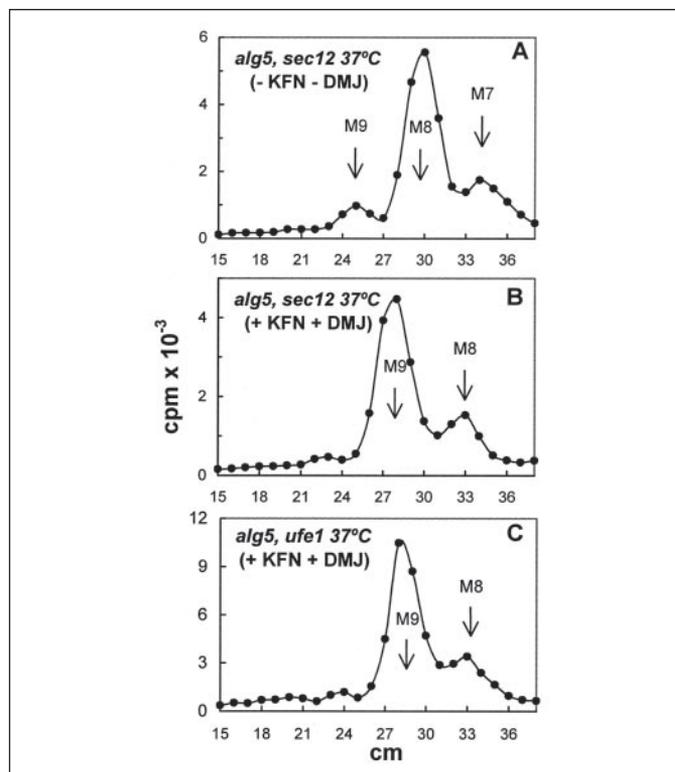


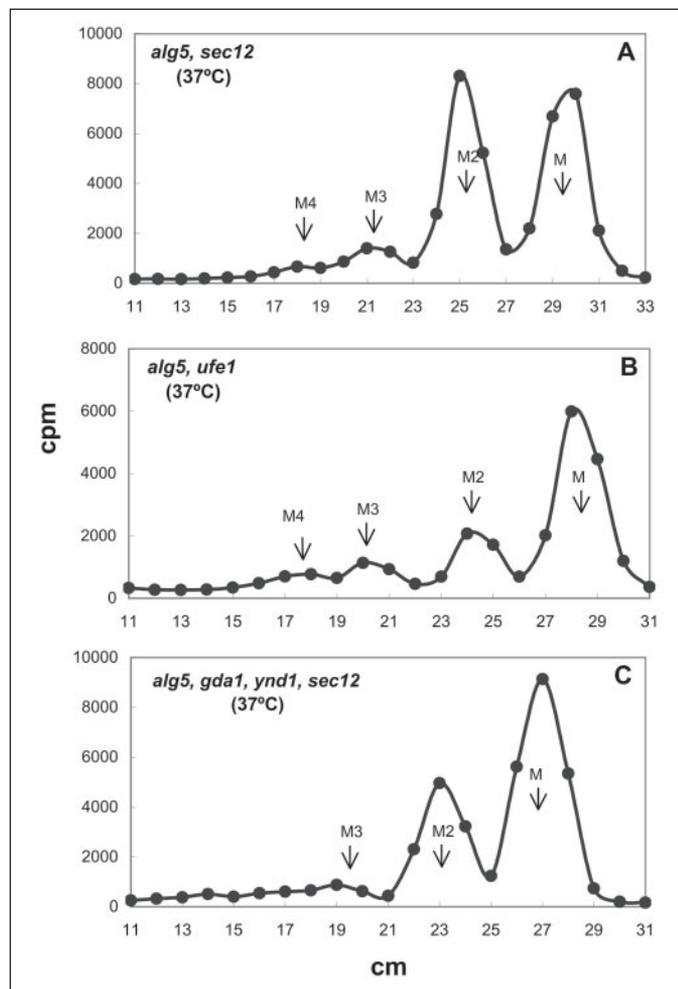
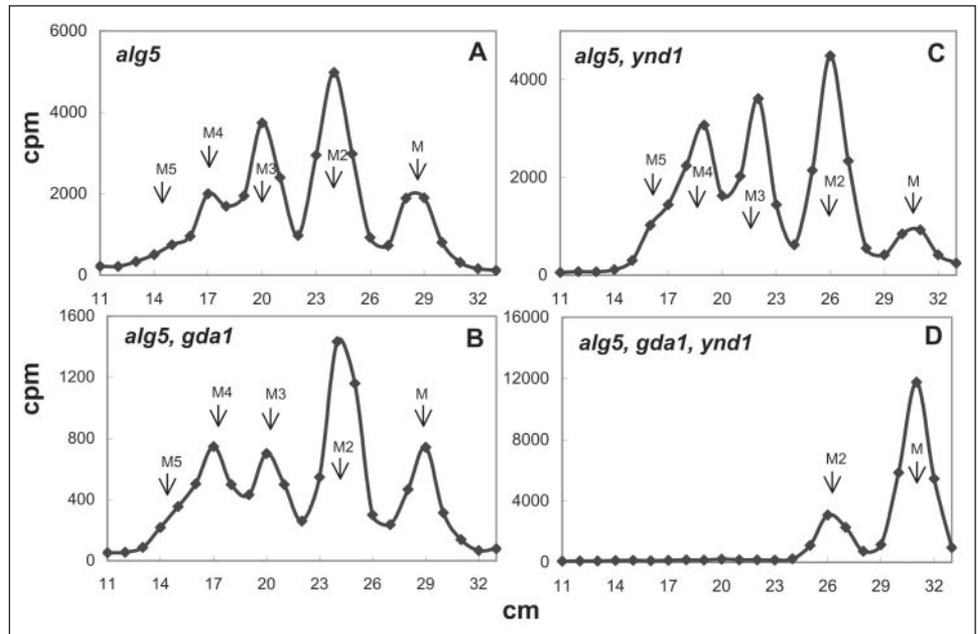
FIGURE 6. **Subcellular site of initial N-glycan elongation.** A, N-glycans formed by *alg5 sec12* cells pulse-chased with [ $^{14}\text{C}$ ]Glc at the restrictive temperature; B, the same as A but in the presence of 2.5 mM DMJ plus 2.5 mM KFN; C, the same as B, but *alg5 ufe1* cells were employed. N-Glycans were run on paper chromatography with solvent A. Standards were as follows: M7,  $\text{Man}_7\text{GlcNAc}$ ; M8,  $\text{Man}_8\text{GlcNAc}$ ; M9,  $\text{Man}_9\text{GlcNAc}$ . For further details, see "Experimental Procedures."

## DISCUSSION

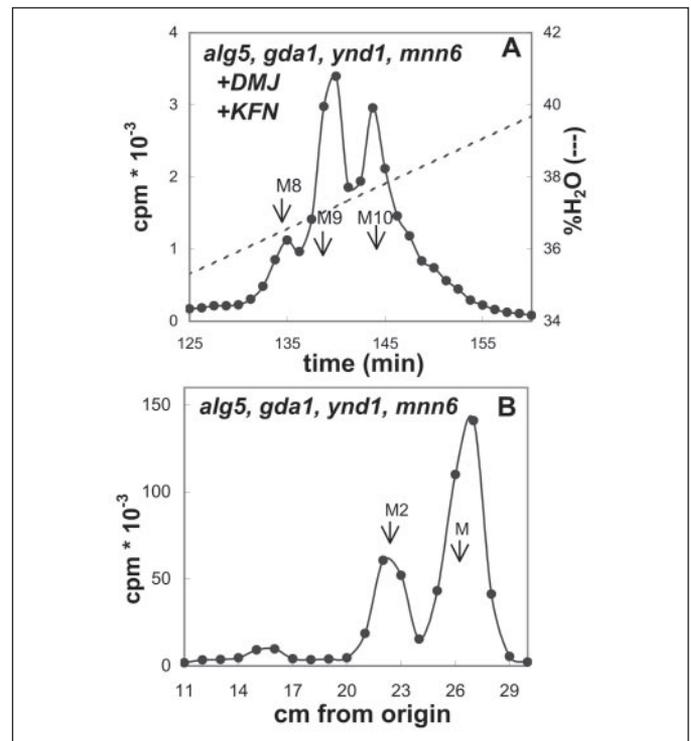
*S. pombe* GT was functionally expressed in *S. cerevisiae* ER as detection of protein-linked  $\text{Glc}_1\text{Man}_8\text{GlcNAc}_2$  in *alg5* mutants was dependent on transformation with a GT-encoding vector and on inhibition of the ER-resident enzyme that removes the GT-added residue (glucosidase II). Moreover, formation of the glucosylated glycan occurred in *alg5 sec12* and *alg5 ufe1* mutant cells at the restrictive temperature (*i.e.* under conditions that totally prevented anterograde and both anterograde and retrograde ER-Golgi vesicular traffic, respectively). This suggests that Golgi NDPases do not participate in the entrance of UDP-Glc into the secretory pathway lumen. Since formation of  $\text{Glc}_1\text{Man}_8\text{GlcNAc}_2$  was detected not only in *alg5 gda1* or *alg5 ynd1* mutants but also in *alg5 gda1 ynd1* mutants, it may be concluded that UDP-Glc may enter into the secretory pathway in the total absence of NDPase activities. Surprisingly, the same extent of glucosylation was detected in *alg5* and *alg5 gda1 ynd1* cells. The role of UDP-Glc in the *S. cerevisiae* ER lumen is unknown for the moment, but indirect evidence suggests that  $\beta(1,6)$ -glucan synthesis might be initiated at that subcellular location (28).

Results presented show that similar size patterns were obtained on GDP-Man-dependent N-glycan elongation in *alg5*, *alg5 gda1*, and *alg5 ynd1* cells. It has been reported that invertase, a glycoprotein only displaying N-glycans, isolated from *gda1* mutants showed an increased mobility on SDS-PAGE (29). On the other hand, glycosylation defects observed in carboxypeptidase Y synthesized also in *gda1* mutants were much more pronounced than those detected in invertase (29). It has been reported also that invertase isolated from *ynd1* mutant cells had an increased mobility on SDS-PAGE (3). Our results apparently contradict these reports, since *gda1* and *ynd1* cells synthesized patterns of N-glycans indistinguishable from that produced by wild type cells. The con-

**FIGURE 7. O-Mannosylation in mutants defective in NDPases.** Cells were pulse-chased with [ $^{14}$ C]Glc, and whole cell O-glycans were run on paper chromatography with solvent C. Cells used were *alg5* (A), *alg5 gda1* (B), *alg5 ynd1* (C), and *alg5 gda1 ynd1* (D). Standards were as follows: M, Man; M<sub>2</sub>, Man<sub>2</sub>; M<sub>3</sub>, Man<sub>3</sub>; M<sub>4</sub>, Man<sub>4</sub>; and M<sub>5</sub>, Man<sub>5</sub>. For further details, see "Experimental Procedures."



**FIGURE 8. Subcellular site of initial O-glycan elongation.** O-Glycans were synthesized at 37 °C by the following cells: *alg5 sec12* (A), *alg5 ufe1* (B), and *alg5 gda1 ynd1 sec12* (C). Glycans were run on paper chromatography with solvent C. Standards were as follows: M, Man; M<sub>2</sub>, Man<sub>2</sub>; M<sub>3</sub>, Man<sub>3</sub>; and M<sub>4</sub>, Man<sub>4</sub>. For further details, "Experimental Procedures."



**FIGURE 9. N- and O-glycan synthesis in the absence of a Man-1-P transferase.** N-Glycans synthesized by *alg5 gda1 ynd1 mnn6* mutant cells pulse-chased with [ $^{14}$ C]Glc in the presence of 2.5 mM DMJ plus KFN (A) and O-glycans synthesized by the same strain (B) were separated on HPLC or on paper chromatography with solvent C, respectively. Standards were as follows: M, Man; M<sub>2</sub>, Man<sub>2</sub>; M<sub>8</sub>, Man<sub>8</sub>GlcNAc; M<sub>9</sub>, Man<sub>9</sub>GlcNAc; M<sub>10</sub>, Man<sub>10</sub>GlcNAc. For further details, see "Experimental Procedures."

tradition is perhaps more apparent than real, since the mentioned reports address N-glycans of single glycoproteins, whereas ours addresses whole cell glycans (this issue will be further discussed below). An unexpected finding was that nucleotide sugar-dependent elongation of N-glycans occurred also in *alg5 gda1 ynd1* mutants, although to a limited extent when compared with cells displaying both or only one

## Nucleoside Diphosphatases and Glycosylation

NDPase activity. *N*-Glycan elongation did not take place in the ER but probably in the Golgi, since it was detected neither in *alg5 sec12* nor in *alg5 ufe1* mutant cells at the restrictive temperature. Results obtained with the former mutant were rather surprising, since it has been reported that two complexes containing mannosyltransferases and other proteins involved in *N*-glycan elongation that normally reside in the Golgi actually cycle between that compartment and the ER (25). Since the *sec12* mutation produces a total inhibition of anterograde vesicular traffic, incubation of cells at the restrictive temperature resulted in the ER accumulation of said proteins. However, it was observed that, whereas Mnn9p, a mannosyltransferase responsible for the addition of the second Man residue in the elongation process was among the cycling proteins, that responsible for the addition of the first Man unit (Och1p) was not present in that group (25). Absence of Och1p in the ER might be then responsible for our failure to observe *N*-glycan elongation in the ER of *sec12* mutants at the restrictive temperature. It is worth mentioning the cycling proteins mentioned above are diverted to the vacuole in *ufe1* mutants at the restrictive temperature (25). It is the capacity of cells to retrieve the proteins to the ER that maintains them in the Golgi, since this process prevents their migration to the vacuole. From results presented, it may be concluded that GDP-Man may enter into the Golgi in the absence of NDPases in the secretory pathway but that, in agreement with previous reports, a full elongation of *N*-glycans absolutely requires the presence of either the GDPase/UDPase or the apyrase (3, 29).

In agreement with results obtained upon studying *N*-glycan elongation in cells lacking only one NDPase, very similar *O*-linked glycan patterns were formed in *alg5*, *alg5 gda1*, and *alg5 ynd1* mutant cells as in the three strains Man, Man<sub>2</sub>, Man<sub>3</sub>, Man<sub>4</sub> and Man<sub>5</sub> were formed. On the contrary, *alg5 gda1 ynd1* mutant cells had a much more restricted size range, since only Man and Man<sub>2</sub> were synthesized in them. The fact that the full complement of *O*-linked glycans were synthesized in *alg5 gda1* cells is in contrast with reports informing that chitinase isolated from *gda1* mutant cells only displayed Ser/Thr-linked Man and Man<sub>2</sub> (3, 29).

A possible explanation for the apparent conflict observed when studying nucleotide sugar-dependent *N*- and *O*-mannosylations in whole cell and individual glycoproteins occurring in single NDPase null mutants may be that the individual glycoproteins studied in previous reports (chitinase, invertase, and carboxypeptidase Y) are relatively minor components of the glycoprotein pool, not representative of the bulk of whole cellular species. It has been reported that disruption of the *GDA1* gene reduced 5-fold the entrance rate of GDP-Man into Golgi-derived vesicles (30). Since individual glycoproteins may display different rates of transit through the Golgi cisternae, the presumably lower GDP-Man concentrations occurring in the Golgi lumen of *gda1* cells may affect them differently. In addition, glycan structural and biosynthetic studies revealed that not the same complement of mannosyltransferases is involved in the glycan elongation of all glycoproteins. Since different mannosyltransferases have distinct  $K_m$  values for GDP-Man, variations in GDP-Man concentrations are likely to affect individual glycoproteins differently. The differential extent of *N*-glycan modification of invertase and carboxypeptidase Y observed in *gda1* mutants reported in Ref. 29 supports the notion that the absence of one of both NDPases may differently affect glycan elongation in distinct glycoproteins. Another source of the discrepancy observed could be that *alg5* mutant cells were used in the present work. These mutants transfer (inefficiently) Man<sub>5</sub>GlcNAc<sub>2</sub> to proteins and thence produce underglycosylated *N*-glycoproteins. The lower amounts of glycans to be elongated may result in higher GDP-Man levels in the Golgi lumen when

compared with *ALG5* cells, which in turn may result in larger glycans synthesized.

Both *alg5 sec12* and *alg5 ufe1* mutant cells synthesized Man<sub>2</sub> at the restrictive temperature. This is consistent with previous results obtained with *sec18* mutant cells, which are affected in the fusion process of COPII-coated vesicles with the Golgi cisternae at the restrictive temperature (31). Nevertheless, the authors tentatively ascribed Man<sub>2</sub> formation to leakiness of the mutation and/or to mannosylation of glycoproteins that were already at the Golgi upon shifting up the temperature and that had remained at that subcellular location until the addition of the label. In the present report, we show that both explanations are highly unlikely, since neither the *alg5 sec12* nor the *alg5 ufe1* mutants were leaky (no Golgi *N*-glycan elongation was observed in them at 37 °C), and the *alg5 ufe1* mutant cells had been incubated first for 30 min at 37 °C and then for 60 min more at the same temperature with unlabeled Glc before label addition. Proteins in the Golgi are expected to have left the organelle after such a relatively long time period. Further, according to Refs. 23–25, labeling protocols employed ensured total hindering of anterograde transport in the case of both *alg5 sec12* and *alg5 ufe1* mutants and of retrograde transport in the case of the last one. Our results, obtained with both *alg5 sec12* and *alg5 ufe1* mutants, show that, contrary to what has been assumed, the addition of the second Man unit (and also a minor amount of the third one) may take place in the ER. Since formation of Man<sub>2</sub> also occurred in *alg5 gda1 ynd1 sec12* at the restrictive temperature, results presented indicate that GDP-Man may enter not only into the Golgi but also into the ER lumen in the absence of NDPases in the secretory pathway. It is worth mentioning that it has been recently reported that nucleotide sugar-dependent protein *O*-glycosylation also occurs in the mammalian cell ER (32). Further, our results show that GMP generated by Man-1-P transfer in the Golgi cannot account for the NMPs required for GDP-Man entrance into the ER or *cis*-Golgi lumen.

It is not necessary to postulate the occurrence of nucleotide sugar transporters using NDPs as antiporters to explain results presented, since studies performed with pure nucleotide sugar transporters, derived from species as distant in evolution as rat and *Leishmania*, inserted in proteoliposomes, showed that the transporters are able to sustain the entrance of nucleotide sugars into such vesicles in the absence of NDPases or of NMPs in their interior until inner and outer concentrations reach equilibrium (33, 34). The entrance rate was, however, about one-third of that attained when preloading proteoliposomes with the corresponding NMPs. Preloading vesicles with NDPs had no effect, thus indicating that NDPs are not antiporters for the already characterized transporters. Why are not NDPs the antiporters of nucleotide sugar luminal entrance? As it has been observed that nucleotide sugar concentrations may be 20–50-fold higher in the Golgi than in the cytosol, it may be concluded that additional NMPs over those produced by hydrolysis of the NDPs generated by the glycosyltransferases must enter directly from the cytosol into the lumen to allow nucleotide sugars reaching such high concentrations (35). Energy required for concentrating nucleotide sugars in the Golgi would be then provided by the hydrolysis of NDPs coming from the cytosol. It follows that the absence of NDPases in the secretory pathway would lead to luminal concentrations of nucleotide sugars similar to those present in the cytosol. On the other hand, the fact that nucleotide sugars may enter into the secretory pathway and glycosylate glycoproteins in the absence of NDPases implies that NDPs should be able to exit the luminal compartments. If NDPs entered into the luminal compartments by facilitated diffusion, then their luminal accumulation would lead to their exit through the same NDP transporter once their concentrations surpass those in the

cytosol. An alternative possibility could be the entrance of nucleoside triphosphates into the secretory pathway lumen followed by their successive conversion first to NDPs by nucleoside triphosphatases and/or apyrases and then to NMPs by the last enzymes and/or by proper NDPases. NMPs thus generated would also allow luminal nucleotide sugar concentration.

Similar cytosolic and luminal nucleotide sugar concentrations, as would be expected to occur in the absence of NDPases in the secretory pathway, are not necessarily an impediment for glycosyltransferase activities, since there are several such enzymes in the cytosol (glycogenin, glycogen synthase, and chitin synthases in *S. cerevisiae*; the first two and the UDP-GlcNAc:Ser/Thr-protein *N*-acetylglucosaminyltransferase in mammalian cells). Lower  $K_m$  values for nucleotide sugars in glycosyltransferases may obviate the need for luminal concentration of the donor substrates. For instance, the cytosolic concentrations of UDP-Gal and UDP-GalNAc have been reported to be 60 and 180  $\mu\text{M}$ , respectively, which is within the same range of the  $K_m$  values of Golgi glycosyltransferases for nucleotide sugars, thence the need for concentrating them in the Golgi lumen (36). On the other hand, a value of 170  $\mu\text{M}$  has been reported for cytosolic UDP-Glc concentration (36). This value is about 10-fold higher than that reported for the ER GT  $K_m$  for the same compound (18  $\mu\text{M}$ ) (37). The sharp difference between the  $K_m$  value and the cytosolic UDP-Glc concentration obviates, therefore, the need for concentrating this last nucleotide sugar in the ER and thence of the occurrence of NDPases in that subcellular location. This interpretation agrees with the reduced GDP-Man-dependent *N*- and *O*-mannosylations (Golgi or Golgi-like reactions) observed in *alg5 gda1 ynd1* mutant cells and the similar GT-dependent glucosylation (an ER reaction) detected in that mutant and *alg5* cells. Although the observed absence of NDPases in the *S. pombe* ER (12) fits into this explanation, a resulting open question is then why there are two NDPases in the mammalian cell ER (6, 7).

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## REFERENCES

- Hirschberg, C. B., Robbins, P. W., and Abeijón, C. (1998) *Annu. Rev. Biochem.* **67**, 49–69
- Yanagisawa, K., Resnick, D., Abeijón, C., Robbins, P. W., and Hirschberg, C. B. (1990)

- J. Biol. Chem.* **265**, 19351–19355
- Gao, X.-D., Kaigorodov, V., and Jigami, Y. (1999) *J. Biol. Chem.* **274**, 21450–21456
- Zhong, X., and Guidotti, G. (1999) *J. Biol. Chem.* **274**, 32704–32711
- Wang, T.-F., and Guidotti, G. (1998) *J. Biol. Chem.* **273**, 11392–11399
- Trombetta, E. S., and Helenius, A. (1999) *EMBO J.* **18**, 3282–3292
- Failor, B. U., Braun, N., and Zimmermann, H. (2002) *J. Biol. Chem.* **277**, 36978–36986
- Parodi, A. J. (2000) *Annu. Rev. Biochem.* **69**, 69–93
- Fernandez, F. S., Trombetta, S. E., Hellman, U., and Parodi, A. J. (1994) *J. Biol. Chem.* **269**, 30701–30706
- Jakob, C. A., Burda, P., te Heesen, S., Aebi, M., and Roth, J. (1998) *Glycobiology*. **8**, 155–164
- Bossuyt, X., and Blanckaert, N. (1997) *Biochem. J.* **323**, 645–648
- D' Alessio, C., Trombetta, E. S., and Parodi, A. J. (2003) *J. Biol. Chem.* **278**, 22379–22387
- Dean, N. (1999) *Biochim. Biophys. Acta* **1426**, 309–322
- Strahl-Bolsinger, S., Gentzsch, M., and Tanner, W. (1999) *Biochim. Biophys. Acta* **1426**, 297–307
- Jigami, Y., and Odani, T. (1999) *Biochim. Biophys. Acta* **1426**, 335–345
- Castro, O. A., Chen, L. Y., Parodi, A. J., and Abeijón, C. (1999) *Mol. Biol. Cell* **10**, 1019–1030
- Engel, J. C., and Parodi, A. J. (1985) *J. Biol. Chem.* **260**, 10105–10110
- Abeijón, C., Orlean, P., Robbins, P. W., and Hirschberg, C. B. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 6935–6939
- Ames, B. (1966) *Methods Enzymol.* **8**, 115–117
- Chappell, T. G., Nasser Hajibagheri, M. A., Ayscough, K., Pierce, M., and Warren, G. (1994) *Mol. Biol. Cell* **5**, 519–528
- Burda, P., and Aebi, M. (1999) *Biochim. Biophys. Acta* **1426**, 239–257
- Simons, J. F., Ferro-Novick, S., Rose, M. D., and Helenius, A. (1995) *J. Cell Biol.* **130**, 41–49
- Kaiser, C. A., and Schekman, R. (1990) *Cell* **61**, 723–733
- Lewis, M. J., and Pelham, H. R. B. (1996) *Cell* **85**, 205–215
- Todorow, Z., Spang, A., Carmack, E., Yates, J., and Schekman, R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 13643–13648
- Hernandez, L. M., Ballou, L., Alvarado, E., Gillette-Castro, B. L., Burlingame, A. L., and Ballou, C. E. (1989) *J. Biol. Chem.* **264**, 11849–11856
- Wang, X.-H., Nakayama, K., Shimma, Y., Tanaka, A., and Jigami, Y. (1997) *J. Biol. Chem.* **272**, 18117–18124
- Shahinian, S., Dijkgraaf, G. J. P., Sdicu, A.-M., Thomas, D. Y., Jakob, C. A., Aebi, M., and Bussey, H. (1998) *Genetics* **149**, 843–856
- Abeijón, C., Yanagisawa, K., Mandon, E. C., Hausler, A., Moreman, K., Hirschberg, C. B., and Robbins, P. W. (1993) *J. Cell Biol.* **122**, 307–323274
- Berninsone, P., Miret, J. J., and Hirschberg, C. B. (1994) *J. Biol. Chem.* **269**, 207–211
- Haselbeck, A., and Tanner, W. (1983) *FEBS Lett.* **158**, 335–338
- Luo, Y., and Haltiwanger, R. S. (2005) *J. Biol. Chem.* **280**, 11289–11294
- Puglielli, L., and Hirschberg, C. B. (1999) *J. Biol. Chem.* **274**, 35596–35600
- Segawa, H., Soares, R. P., Kawakita, M., Beverley, S. M., and Turco, S. J. (2005) *J. Biol. Chem.* **280**, 2028–2035
- Waldman, B. C., and Rudnick, G. (1990) *Biochemistry* **29**, 44–52
- Chiaromonte, M., Koviach, J. L., Moore, C., Iye, V. V., Wagner, C. R., Halcomb, R. L., Miller, W., Melancon, P., and Kuchita, R. D. (2001) *Biochemistry* **40**, 14260–14267
- Trombetta, E. S. (1992) *Identification, Partial Characterization and Purification of Rat Liver UDP-Glc: Glycoprotein Glucosyltransferase. A New Glycoprotein Processing Reaction*. Ph.D. thesis, University of Buenos Aires