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Guanosine Diphosphatase Is Required for Protein and Sphingolipid Glycosylation in the Golgi Lumen of *Saccharomyces cerevisiae*

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Abstract. Current models for nucleotide sugar use in the Golgi apparatus predict a critical role for the lumenal nucleoside diphosphatase. After transfer of sugars to endogenous macromolecular acceptors, the enzyme converts nucleoside diphosphates to nucleoside monophosphates which in turn exit the Golgi lumen in a coupled antiporter reaction, allowing entry of additional nucleotide sugar from the cytosol. To test this model, we cloned the gene for the *S. cerevisiae* guanosine diphosphatase and constructed a null mutation. This mutation should reduce the concentrations of GDP-mannose and GMP and increase the concentration of GDP in the Golgi lumen. The alterations should in turn decrease mannosylation of proteins and lipids in this compartment. In fact, we found a partial block in *O*- and *N*-glycosylation of proteins such as chitinase and carboxypeptidase Y and underglycosylation of invertase. In addition, mannosylinositolphosphorylceramide levels were drastically reduced.

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THE lumen of the Golgi apparatus is the subcellular organelle where proteins and lipids become terminally glycosylated. In mammalian cells, nucleotide sugars are transported from the cytosol, their site of synthesis, into the lumen of the Golgi apparatus, via specific carrier proteins. After transport into the lumen and transfer of the sugars to the appropriate macromolecular acceptors, nucleoside diphosphates, the other reaction products are presumably substrates for a nucleoside diphosphatase, which converts them to nucleoside monophosphates. These exit the lumen of the Golgi in a coupled, equimolar, exchange with additional, cytosolic, nucleotide sugar (Hirschberg and Snider, 1987). Nucleoside diphosphates are inhibitors of glycosyltransferases while the monophosphates are not (Khatra et al., 1974; Kuhn and White, 1977; Brandan and Fleischer, 1982). The Golgi membrane transporters for nucleotide sugars appear to be of physiological significance: mutant CHO and MDCK cells have been described in which a specific defect in nucleotide sugar transport leads to the corresponding defect in glycosylation of proteins and lipids in vivo (Hirschberg and Snider, 1987).

S. cerevisiae contains heavily mannosylated proteins, and a highly specific guanosine diphosphatase (GDPase)[1] has been described and purified to homogeneity (Yanagisawa et al., 1990). This enzyme appears to be localized in the lumen of Golgi vesicles (Abeijon et al., 1989). Addition of mannose to outer chains of N- and O-linked oligosaccharides in glycoproteins as well as mannosylation of inositolphosphorylceramides (IPC) occurs in this compartment (Kukuruzinska et al., 1987; Puoti et al., 1991). In all these reactions, GDP-mannose, the mannosyl donor, is synthesized in the cytosol and translocated into the Golgi lumen via a specific membrane carrier (Abeijon et al., 1989). After GDP-mannose entry into the lumen and mannosylation of endogenous protein and lipid acceptors, GDP, is converted, presumably by the above GDPase, to GMP which, by analogy to the mammalian system, is the nucleotide which exits the Golgi lumen and allows entry of additional cytosolic GDP-mannose. In the absence of GDPase, a decrease of GMP, the putative antiporter, should lead to a decrease of GDP-mannose in the Golgi lumen and therefore to substantially reduced Golgi mannosylation.

To determine the in vivo role for the above described *S. cerevisiae* Golgi GDPase, its gene (*GDA1*) was cloned and a null mutation was constructed. The recovered strain was viable and showed no in vitro membrane-bound GDPase ac-

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; GDPase, guanosine diphosphatase; IPC, inositolphosphorylceramides; MIPC, mannosylinositolphosphorylceramides; $M(IP)_2C$, mannosyldiphosphorylinositolceramide; ORF, open reading frame; PI, phosphatidylinositol.

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tivity. As predicted, the null strain showed a block of O- and N-mannosylation of proteins such as chitinase and carboxypeptidase Y and underglycosylation of external invertase. In addition, a significant impairment of mannosylation of IPC was also found.

Materials and Methods

Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA) or GIBCO BRL (Gaithersburg, MD). pUC13, SmaI cut, and phosphatase treated was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Radiochemicals: [2-^{3}H]Mannose, 14 Ci/mmol (American Radiolabeled Chemicals, Inc., St. Louis, MO); Tran^{35}S-labeling mixture (ICN Biomedicals, Inc., Costa Mesa, CA). [^{3}H]myoinositol and [α-^{32}P]dCTP (DuPont, New England Nuclear Boston, MA).

Strains and Growth Conditions

S. cerevisiae strains were grown in YEPD or SD medium supplemented with the required amino acids (Sherman et al., 1986). Cells were grown at 30°C. Solid media was made by adding 2% agar to the liquid stock. Sporulation and tetrad dissection were carried out according to published procedures (Sherman et al., 1986). *E. coli* DH5α and DH5α FIQ (GIBCO BRL) were used in the cloning experiments. The *E. coli* strains were grown in LB medium (Maniatis et al., 1982). Ampicillin [50 μg/ml] was added when needed. All nucleic acid manipulations and bacterial transformations were carried out according to Maniatis et al. (1982). Yeast transformations were carried out by the lithium acetate method of Ito et al. (1983).

Peptide Sequence Analysis

Approximately 80 μg of highly purified GDPase obtained after the second Mono Q column (Yanagisawa et al., 1990; see Fig. 5 *a*, lane *5*) was subjected to SDS-PAGE, electroblotted to nitrocellulose, and stained with Ponceau S in 1% acetic for 1 min; the 48-kD band was then cut, immediately destained with 0.1% acetic acid, washed with water, and frozen as described in detail by Aebersold et al. (1987).

Digestion of the 48-kD GDPase with trypsin was performed in situ according to Aebersold et al. (1987), omitting the NaOH wash, and subjected to HPLC fractionation. Peptides were separated by a narrow-bore reverse phase HPLC (Hewlett-Packard 1090 HPLC equipped with a 1040 iodide assay detector, using a Vydac 2.1 × 150 mm C_{18} column as described by Camirand et al., 1991). While monitoring absorbance at 210 nm, fractions were collected manually by peak and stored at −20°C. Several peptides were then selected for sequencing by automated Edman degradation on a model 470 sequenator (Appl. Biosystems Inc., Foster City, CA). Tryptic digests, HPLC, and peptide sequencing were done by William S. Lane (Harvard Microchemistry Facility, Harvard University, Cambridge, MA).

Synthetic Oligonucleotide Design and PCR

Regions of least degeneracy were selected in two of the internal peptides of the GDPase for the design of oligonucleotides (See Table I). Sense and antisense 26-mers were designed for each peptide according to Moremen (1989). All oligonucleotides were designed with mixed bases (64–128-fold mixtures overall for oligonucleotides ls/la and 2s/2a, respectively), and contained deoxyinosine residues (3 and 4 residues in oligonucleotides ls/la and 2s/2a, respectively) at positions of degeneracy. Oligonucleotides were synthesized on an Applied Biosystems model 380 B DNA synthesizer.

For the PCR experiments, every sense-antisense primer combination was tested [3 μM each] using genomic DNA of *S. cerevisiae* PRY123 as template (0.1–0.2 μg) in 25 μl reactions. 35 cycles were conducted as follows: 1 min at 92°C, 1 min at 50°C and 3 min at 72°C, and extensions of 5 min at 72°C were carried out after the last cycle. Primers ls and 2a (Table I) gave a 140-bp amplification product. This product was phosphorylated with T4 polynucleotide kinase, blunted with T4 polymerase, and cloned into the SmaI site of pUC13. The insert was then cloned into M13 and sequenced. The authenticity of the product was confirmed by the presence of coding sequence for amino acids E and S (Table I, peptide 2) left out of the primer design for confirmation purposes. The PCR amplification product was labeled by extension of the ls and 2a primers with [α-^{32}P]dCTP (New En-

S. Cerevisiae Strains Used

Strain	Genotype	Source
PRY 123	*MATa, gal2*	P. Robbins
PRY 223	*MATa, ura3-52, lys 2-801am, ade2-101oc, trpl-1, his3-Δ200, leu2-Δ1*	P. Robbins
PRY 304	*MATα, mnn1, mnn10, Trp 1*	P. Robbins
CGY 2332	*MATα, ade2, his3, leu2, ura3*	G. Fink
CGY 2333	*MATα, ade2, his3, leu2, ura3, pmr 1-1::LEU2*	G. Fink
YPH 274	*MAT a/α, ura3-52/ura3-52, Lys 2-801 am/lys 2-801 am, ade2-101 oc/ade 2-101 oc, trpl-1Δ1/trp- 1-1Δ1 his3-Δ200/his3-Δ200, leu2-Δ1/leu2-Δ1*	P. Robbins
G2	*MATa/α, ura3-52/ura3-52, lys 2-801 am/lys 2-801 am, ade2-101 oc/ade 2-101 oc, trpl-1Δ1/trp 1-1Δ1, his3-Δ200/his3-Δ200, leu2-Δ1/leu2-Δ1, GDA1/gda1::LEU2*	This study
PRY 238	*MAT α/a, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112, lys2-801/+,+/his4-619*	P. Robbins
G11	*MAT α/a, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112 lys2-801/+, +/his4-619, GDA1/gda1::LEU2*	This study
G2-9	*MAT a, ura3-52, lys2-801 am, ade2-101 oc, trp1-Δ1, his3-Δ200,leu2-Δ1*	This study
G2-10	*MAT α, ura3-52, lys2-801 am, ade2-101 oc, trp1-1Δ, his3-Δ200, leu2-Δ1*	This study
G2-11	*MAT α, ura3-52, lys2-801 am, ade2-101 oc, trp1-Δ1, his3-Δ200, leu2-Δ1, gda1::LEU2*	This study
G2-12	*MAT a ura3-52, lys2-801 am, ade2-101 oc, trp1-Δ1, his3-Δ200,leu2-Δ1, gda1::LEU2*	This study
G11-5	*MAT a, ura3-52, leu2-3, 112, lys2-801, gda1::LEU2*	This study
G11-6	*MAT α, ura3-52, leu2-3, 112, lys 2-801*	This study
G11-7	*MAT α, ura3-52, leu2-3, 112, his4-619, gda1::LEU2*	This study
G11-8	*MAT a, ura3-52, leu2-3, 112, his4-619*	This study

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gland Nuclear) and Klenow fragment of DNA polymerase I. The labeled product was used as a probe for screening a *S. cerevisiae* genomic library in YEp13.

Screening of S. cerevisiae Genomic Library and Overexpression of the GDA 1 Gene

The *S. cerevisiae* genomic library in YEp13 was plated on nitrocellulose filters on LB-ampicillin plates (~20,000 clones were screened). Two sets of replicas were prepared on nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH). Both sets were prehybridized and hybridized according to Maniatis et al. (1982). The labeled PCR product was used as a probe. Positive clones were picked, purified, and plasmids isolated and subjected to restriction analysis. Southern hybridizations confirmed the presence of PCR product sequences in the isolated plasmids.

Five independent clones were isolated and used to transform *S. cerevisiae* PRY223 strain. Cells containing YEp13 vector without insert were used as controls. Independent transformants were grown to early logarithmic phase and a crude membrane extract (Orlean et al., 1988) was tested for overexpression of GDPase activity.

Subcloning, Plasmid Construction, and DNA Sequence Analysis

Restriction analysis of the five independent YEp13 clones showed that four had a 3.7-kb XhoI fragment in common, which hybridized with the PCR amplification product. This 3.7-kb XhoI fragment was subcloned in the SalI site of the vector YEp352 (Hill et al., 1986) to generate p13X (see Fig. 1).

The other YEp13 clone had a 3.5-kb HindIII fragment hybridizing with the PCR probe; it was cloned into the multiple cloning site of the vector YEp352 to generate p13H (see Fig. 1). This 3.5 HindIII fragment was also cloned into the vector pBluescript to generate p13HB, needed in the construction of the plasmid used in the gene disruption experiments (see below). A 3-kb PstI–XhoI genomic fragment was cloned into the PstI–XbaI sites at the multiple cloning site of the vector YEp352 to generate p13 PX (see Fig. 1).

Plasmids p13X, p13H, p13PX, and YEp352 without insert were used to transform *S. cerevisiae* PRY223 and overexpression of GDPase activity was tested as described in the previous section. Based on these results, a 2.2-kb, HindIII–NheI genomic fragment (see Fig. 1) was selected for sequencing and cloned in both orientations into M13 mp19. Sequential overlapping deletions were introduced on the M13 clones with T4 polymerase using the Cyclone biosystem kit (International Biotechnology, Inc., New Haven, CT). Sequence was obtained by the "dideoxy" chain termination method (Sanger et al., 1977), using deoxyinosine triphosphate in place of dGTP and Sequenase (Un. States Biochem. Corp., Cleveland, OH) as described by the manufacturer. DNA sequence data were assembled into a contiguous sequence data base with the SeqMan, DNAstar program. Each strand was sequenced at least twice, and gaps were filled by sequencing from synthetic oligonucleotide primers (see Fig. 2).

Sequence comparisons against the GenBank or GenPept sequence data bases were performed using the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) programs. The hydrophobicity plot was generated by the method of Kyte and Doolittle (1982) with a window of 20 amino acids.

Disruption of the GDA1 Gene

Construction of pGDΔ1. The pGDΔ1 plasmid contains the *GDA1* gene, with 128 codons in the center of the GDAp coding region were deleted and replaced by the *LEU2* gene (see Fig. 1). It was constructed in three steps for technical reasons. (Step 1) The 1,550-bp PstI–NheI fragment of p13HB (containing part of the GDA 1-ORF) was cloned into the PstI and XbaI sites of pUC18 to give plasmid A. (Step 2) The 650-bp HindIII–PstI fragment of p13HB (containing part of the GDA1-ORF) was cloned into the multiple cloning site of pJH-L1 to obtain plasmid B. pJH-L1 has a 3-kb, BglII *S. cerevisiae* genomic fragment containing the *LEU 2* gene cloned into the BamH1 site of pUC18.

(Step 3) A 3.7-kb Hind II–SmaI segment of plasmid B (containing the first 172 codons of the GDA1-ORF and the entire *LEU2* gene) was cloned into plasmid A cut with HindIII and HpaI to produce pGDΔ1 (Fig. 1).

Construction of G2 and G11 Strains. The heterozygous *gda1::LEU2/GDA1* strains G2 and G11 were made via one-step gene replacement (Rothstein, 1983) by transforming the parent strains PRY225 and PRY238, respectively, with a 4.8-kb HindIII–BamHI fragment from pGDΔ1 containing the *LEU2* marked gda1 deletion. Leu^+ transformants were selected and allele replacement was confirmed by Southern analysis (Maniatis, 1982). Northern blot analyses showed a single 2-kb message in wild-type strains; this band was absent in RNA from disrupted strains. Five- to sevenfold overexpression of the same 2-kb message was detected in RNA derived from a strain which overexpressed the GDPase activity to a similar extent. These studies were done by applying similar amounts of total RNA to every lane (based on ethidium bromide staining and hybridization with a probe for the actin message).

Eight independent diploid transformants were sporulated from each genetic background, and 12 asci from four of them were dissected. Tetrad analysis showed 2:2 segregation of the *LEU2* marker on every case. Transformants from strain PRY 225 are called G1–G8, and transformants from strain PRY238 are called G9 through G16.

Strains G2-9, G2-10, G2-11, and G2-12 represent one tetrad from the G2 diploid and strains G11-5, G11-6, G11-7, and G11-8 represent one tetrad from the G11 diploid. These strains were used throughout this study.

Guanosine Diphosphatase Assay

GDPase activity was assayed essentially as described previously (Yanagisawa et al., 1990). Briefly, incubation mixtures contained in a final volume of 0.1 ml, enzyme (10–50 μg of total membrane extract, Orlean et al., 1988), $CaCl_2$ (1 μmol), Triton X-100 (100 μg), GDP (0.2 μmol), and imidazole-HCl buffer pH 7.6, (20 μmol). For UDPase and ADPase assays, GDP was replaced by UDP or ADP at the same concentration. Incubations were done for 5 min at 30°C.

Protein concentration was determined using the BCA protein assay reagent as described by the manufacturer (Pierce, Rockford, IL).

Radiolabeling of S. cerevisiae Cells for the Analysis of O-linked Carbohydrates

Exponentially growing cultures (YEP 2% Sucrose) with an initial OD_{600} = 1, were used. 30 min before labeling, 2 OD_{600} of cells were harvested and suspended in 1 ml of YEP 0.5% sucrose. Labeling was done with [2-^{3}H]mannose (specific activity 14 Ci/mmol); 25 μCi/ml were used when total membrane fraction was isolated and 1 mCi/ml when labeled chitinase was isolated. Cells were labeled during 60 min.

O-linked Carbohydrate Analyses

The method of Haselbeck and Tanner (1983) was followed for the isolation of total O-linked carbohydrates from radiolabeled cells. The β-elimination was achieved in 0.1 M NaOH for 24 h at room temperature after which the reaction was stopped by addition of HCl to a final concentration of 0.15 M and the protein was removed by centrifugation. Radiolabeled species in the supernatant were subjected to paper chromatography (Whatman 1 paper) in ethyl acetate/butanol/acetic acid/water (3:4:2.5:4, by vol) during 24 h, or thin layer chromatography on silica gel 60 plates with two ascents in butanol-ethanol-water (5:3:2, by vol). The standard sugar mixture contained mannose, maltose, and raffinose which was detected by silver staining. To determine the distribution of radioactivity, paper chromatograms were cut into 1-cm strips, placed in a vial with 1 ml of H_2O to elute the radioactivity. Then 9 ml of liquid scintillation cocktail were added and the radioactivity measured in a liquid scintillation counter. The thin layer chromatograms were treated with EN^{3}HANCE reagent (New England Nuclear) for fluorography, and then exposed to Kodak X-OMAT X-ray film at −70°C. When quantitation was required, the area corresponding to the bands was scraped (based on the autoradiogram) and the radioactivity determined as described for the paper strips.

Isolation of Chitinase from Culture Media by Chitin Binding

Native chitinase was isolated from saturated *S. cerevisiae* cultures grown in YEPD basically as described by Kuranda and Robbins (1991). Chitinase from 10 ml of media from a saturated culture was allowed to bind ~30 mg of purified chitin (Sigma Chem. Co., St Louis, MO) for 4 h at 4°C. Chitin was then pelleted by centrifugation and washed three times with PBS. The washed pellet was suspended on 100 μl of SDS sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol) heated to 100°C for 10 min, and analyzed by SDS-PAGE on 6% resolving gels with the discontinuous buffer system described by Laemmli (1970). The gel was then stained with Coomassie brilliant blue.

Radiolabeled chitinase was isolated from 1 ml of [2-^{3}H]mannose la-

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beled cells as described by Orlean (1990). The material was then subjected to β-elimination and paper chromatography as described in the O-linked carbohydrate analysis section.

Immunoprecipitation of Carboxypeptidase Y

The method described by Orlean (1990) was followed. Briefly, logarithmically growing cultures (OD 600 ~1) in methionine-free minimal medium and 2% glucose were used as starting material. Between 2 and 4, OD_{600} of cells were harvested and suspended in 1 ml of the same medium. Labeling was done for 30 min with 100 mCi/ml Trans ^{35}S-labeling mix. After labeling, the cells were washed and suspended on 200 μl PBS with 1% SDS, and broken with glass beads. Triton X-100 was added to give a final concentration of 0.4%. Samples were precleared with preimmune serum and protein A-Sepharose (2 h at 4°C), and then immunoprecipitated with 5 μl of anti-CPY serum (a gift from R. Scheckman, University of California, Berkeley, CA) overnight at 4°C. Protein A conjugates were washed and boiled for 10 min in SDS-sample buffer, and run on SDS-PAGE. Acrylamide concentration was 10% on the resolving gel; the discontinuous system described by Laemmli (1970) was used. The gel was then treated with EN^{3}HANCE (New England Nuclear) and exposed to Kodak X-OMAT x-ray film at −80°C.

Analysis of External Invertase

Preparation of the invertase extracts, native PAGE of the external invertase, and activity staining were done as described by Ballou (1990) with minor modifications. Five OD_{600} of cells growing logarithmically on YEPD (2% glucose) were harvested and suspended in 3 ml of YEP containing 0.05% glucose to induce invertase during 4 h at 30°C. Further treatment was as described (Ballou, 1990) and 10 μl of total lysate was loaded on the gel. The crude extracts were heated to 50°C for 30 min before loading onto the native gel in order to convert the invertase to a single oligomeric form (Esmon et al., 1987). The concentration of acrylamide was raised to 8% and 30-cm long slab gels were run for 10 h at 10 mA constant current. These changes were introduced in order to better resolve the highly glycosylated forms of invertase.

Partial Purification of Invertase

To increase the production of invertase, wild-type (G2-9) and gda 1 null cells G2-11 were transformed with the plasmid pRD58 containing the *SUC2* gene (Carlson and Botstein, 1982) After transformation, the strains produced 4–5 times more invertase than the parents. Invertase was induced by growing cells for 3 h in YPD medium containing 0.1% glucose. Cells were harvested (80 g) and broken with a Bead Beater (Bio Spec Products, Inc., Bartlesville, OK) using 0.5-mm, acid washed, glass beads. Invertase purification was done as described previously by Verostek et al. (1991) up to the DE 52 cellulose column step. Invertase activity was measured as previously described by Goldstein and Lampen (1975).

SDS-PAGE and Western Blotting of Purified Invertase

Active fractions (0.5 U) of enzyme from wild-type and gda 1 null strains from the DE 52 cellulose column were combined into three pools and subjected to SDS-PAGE on 11% gels with the discontinuous buffer system described previously by Laemlli (1970). After electrophoresis, samples were blotted to PVDF membranes with CAPS buffer, pH 11. Equivalent membranes were probed with affinity-purified rabbit IgG antibodies against invertase core protein and against α1,6 and α1,3 mannose linkages (antibodies were a generous gift of Dr. Randy Schekman, University of California, Berkeley). Final visualization was obtained with anti-rabbit IgG conjugated with alkaline phosphatase and development with the stabilized substrate for alkaline phosphatase Western Blue™ from Promega.

Radiolabeling of S. cerevisiae Cells for the Analysis of Mannosylinositol Phosphosphigolipids

Exponentially growing cultures (OD_{600}=1) in Wickerham's minimal medium (Wickerham, 1946) were used with 2% glucose as carbon source but omitting myoinositol. Cells (OD_{600}=1) were labeled with 5 μCi/ml of [^{3}H]myoinositol (20 Ci/mmol; New England Nuclear) or with 1 mCi/ml of [2-^{3}H] mannose (14 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) for 10 min. Routinely, 3 OD_{600} of cells per time point were used. Cultures were then diluted with 4 vol of minimal medium containing 40 μg/ml of cold myoinositol and incubated further for the periods indicated. At the end of the incubations, NaN_3 (3 mM) was added, cells were washed four times, and the pellet was resuspended in 100 μl of 10 mM NaN_3. Cells were broken by vortexing with glass beads (three times for 1 min each). 10 μl were used to determine protein concentration and total incorporation of substrate. Chloroform/methanol (1:1) was added to the remaining suspension to achieve a final concentration of chloroform/methanol/aqueous cell suspension of 10:10:3 (vol/vol/vol). After centrifugation, the pellet was extracted two more times with chloroform/methanol/water, 10:10:3 (vol/vol/vol). The pooled lipid extracts were dried under N_2 and desalted by butanol extraction (Krakow et al., 1986).

Mannosylinositol Phosphosphingolipid Analysis

Lipid extracts were analyzed by ascending TLC on 0.2-mm thick silica gel 60 plates (Merck Sharpe & Dohme/Isotopes, St. Louis, MO) using $CHCl_3/CH_3OH$/0.22% KCl in water (55:45:10) as solvent system. Approximately 200,000 cpm per lane were applied. The developed TLC plates were sprayed with EN^{3}HANCE (New England Nuclear) and fluorograms were obtained after exposure of Kodak X-OMAT film for 3 d at −80°C. When quantitation was required, it was done as described for the O-linked carbohydrate analyses. Identification of the lipids was based on (*a*) reported Rf; (*b*) comigration with authentic standards (PI and lyso PI); (*c*) sensitivity to mild alkaline hydrolysis; and (*d*) analysis of head groups (Puoti et al., 1991).

Results

Cloning of the Luminal Golgi Guanosine Diphosphatase GDA1 Gene

In *S. cerevisiae*, GDP-mannose is the sugar nucleotide used for all known Golgi glycosylation reactions (Kukuruzinska et al., 1987; Puoti et al., 1991). A luminal, highly specific GDPase was previously found associated with this subcellular compartment (Abeijon et al., 1989). The Golgi GDPase was purified to homogeneity (Yanagisawa et al., 1990) and tryptic peptides derived from the deglycosylated 48-kD enzyme were used to obtain amino acid sequence (Table I). Degenerate synthetic oligonucleotides corresponding to peptides 1 and 2 were designed in sense and antisense orientations because the relative positions of the peptides in the GDPase

Table I. Amino Acid Sequence of Tryptic Peptides from GDAp and PCR Primer Design

Peptide	Primer	aa Position in the protein
1 s	5′ TT^T_C T^T_CTI ACI GA^T_C GA^G_A ATI T^T_CTI AA^T_C AA 3′	
1	F L T D E I L N K	366–374
1 a	3′ AA^A_G A^A_GAI TGI CT^A_G CT^C_T TAI A^A_GAI TT^A_G TT 5′	
2 s	5′ GA^T_C ATI TA^T_C ATI TT^T_C T$^G_{AC}$I TA^T_C TT^T_C TA 3′	
2	E S N D I Y I F S Y F Y D R	399–412
2 a	3′ TT^A_G CT^A_G TAI AT^A_G TAI AA^A_G A$^C_{TG}$I AT^A_G AA 5′	
3 i	L [P or D] T [L or M] A [V or E] F [D or P] L G G G [T or S] T Q I V F E P	238–257
3 ii	F [D or L] M [A or S] E [P or S] G [L or S] S S F D [S or T] D (S) V (G) A (H) N	125–144
4	F G D E N Y	276–282

Amino acid sequence of tryptic peptides from purified GDAp was obtained as described in Materials and Methods. Peptides 3i and 3ii were obtained from the sequencing of a complex peak; boxes represent positions where primary/secondary identifications were not possible; ( ) indicate tentative identification of amino acids. *I*, inosine; *s*, sense; *a*, antisense.

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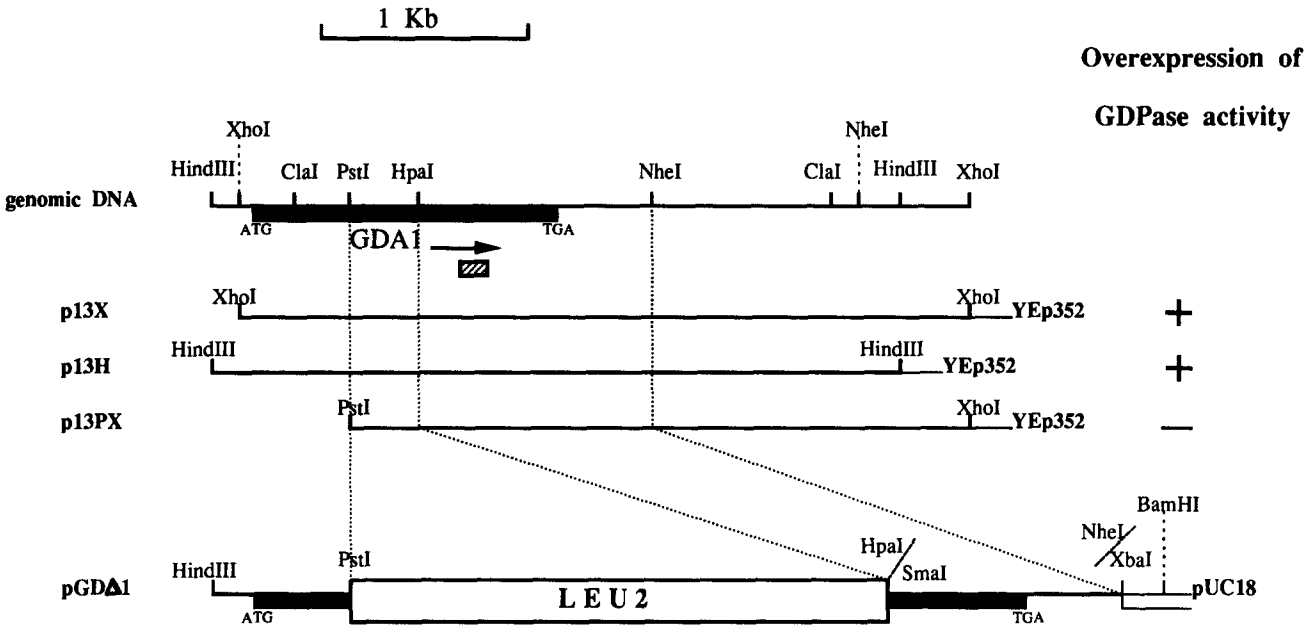


Figure 1. Restriction endonuclease cleavage pattern or *S. cerevisiae* genomic DNA isolated from the YEp13 genomic library. The 140-bp amplification product of the PCR reaction is indicated with a *hatched bar.* Restriction fragments were subcloned into YEp352 to generate p13X, p13H, and p13PH. Their ability to overexpress GDPase activity is shown. The 2.2-kb HindIII-NheI fragment was sequenced, and the GDA1-ORF is denoted as a *black box* flanked by ATG and TGA-codons. Disruption of GDA1-ORF was performed using the HindIII-BamHI fragment of pGDΔ1, which contains a *LEU2* insertion and deletion of the PstI-HpaI (381 bp) fragment of the GDA1-ORF. The HpaI/SmaI ligation and the NheI/XbaI ligation in pGDΔ1 did not regenerate either of the restriction sites.

protein were not known (Table I). The oligonucleotides were then used in a PCR amplification with genomic DNA of *S. cerevisiae* PRY123 as template. Only when oligonucleotides 1s and 2a (Table I) were combined in the reaction, was a product obtained (~140 bp). This product was cloned into an M13 vector and its DNA sequence determined. Coding for the additional amino acid sequence of peptide 2, not used in primer design, was found within the PCR product, as expected. Both peptides, 1 and 2, were found in the same open reading frame (ORF) in the PCR product and there was a lysine residue upstream of peptide 2, consistent with the tryptic digestion used to generate the peptides. These results strongly suggested that a specific DNA fragment of the GDPase gene (*GDA1*) had been amplified.

The 140-bp PCR product was labeled by primer extension and used to screen a *S. cerevisiae* genomic library in YEp13. A total of 20,000 clones were screened from which five positive colonies were isolated. These clones contained 7–9-kb inserts. Restriction endonuclease cleavage showed that a 3.7-kb XhoI fragment and a 3.5-kb HindIII fragment hybridized with the PCR probe. Both of these fragments and a smaller PstI–XhoI piece (also hybridizing with the PCR probe) were subcloned into YEp352 giving plasmids p13X, p13H, and p13PX (Fig. 1).

Since YEp352 is a 2-μm type vector which replicates with high copy number in *S. cerevisiae*, "overexpression" of GDPase was measured upon transformation of strain PRY223 with p13X, p13H, and p13PX. Both p13X and p13H gave 6–8-fold overexpression of GDPase activity. This result showed that the 0.6-kb Hind III–PstI fragment missing in p13PX (Fig. 1) was required for activity. Based on this information, a 2.2-kb HindIII–NheI fragment, which hybridized with the PCR probe, was selected for sequencing.

Sequence of the GDA1 Gene

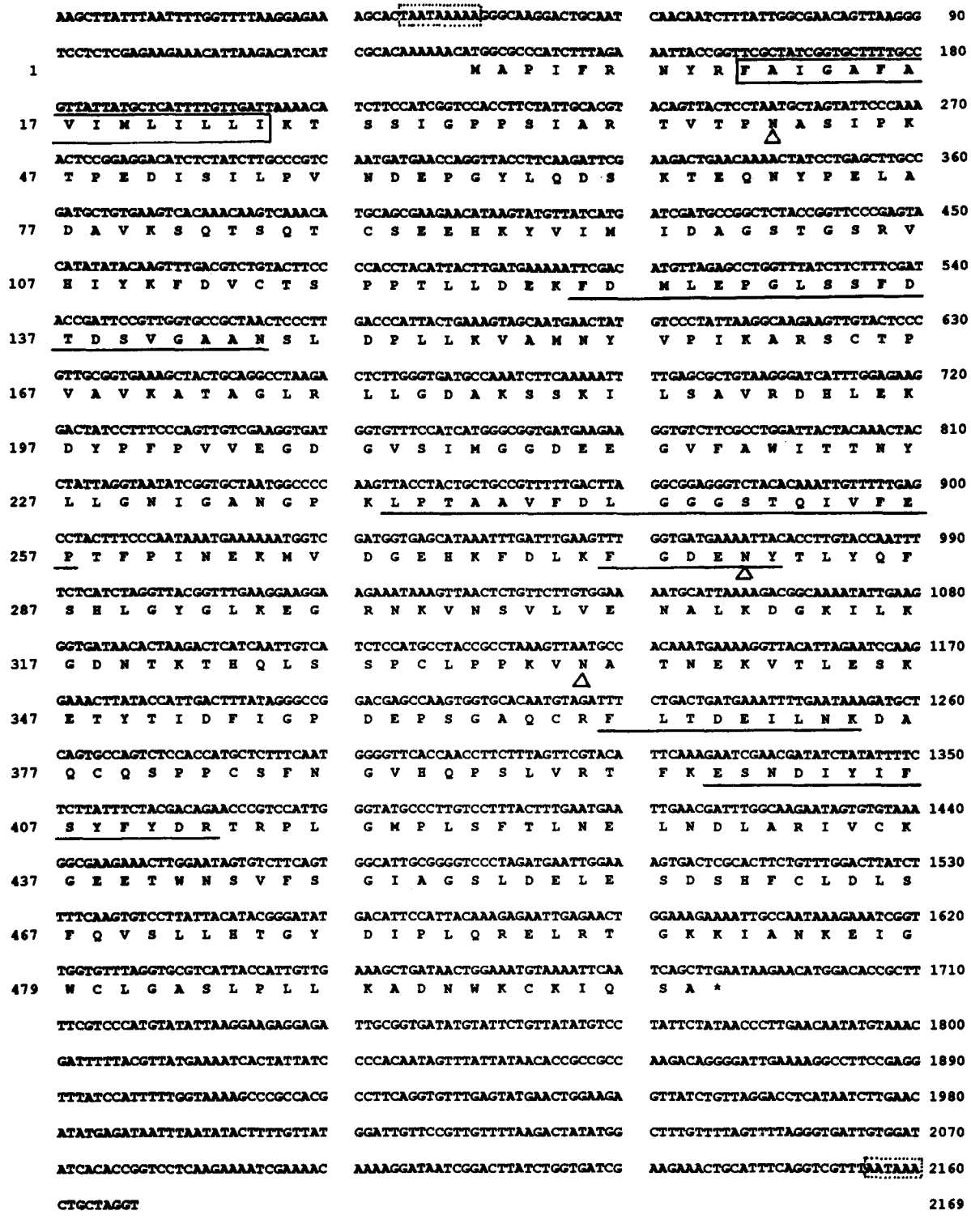
Sequence information was obtained from both DNA strands for the complete 2.2-kb HindIII–NheI fragment. A 1,557 bp ORF was found starting at a methionine residue 133 bp downstream of the HindIII cloning site (Fig. 2 *A*). The predicted protein contains the sequence corresponding to all the peptides obtained from the GDPase protein (Fig. 2 *A*). No NH_2-terminal protein sequence was obtained. The hydrophobicity plot of the predicted protein shows a single putative membrane-spanning region of 15 amino acids, starting 9 amino acids after the first methionine (Fig. 2 *B*). Although this putative transmembrane domain is short, Adams and Rose (1985) demonstrated that mutants of vesicular stomatitis virus glycoprotein G containing 14 or more amino acids of the original 20-amino acid transmembrane domain assumed a transmembrane orientation and were correctly targeted. The deduced protein sequence of the *GDA1* gene encodes a 519-amino acid polypeptide with a calculated molecular weight of 56,817 D. Three potential N-glycosylation sites are present within the coding region (Fig. 2 *A*).

These results are in agreement with the observation that GDPase was purified as a luminal Golgi glycoprotein. The hydrophobic plot suggests a typical type II membrane protein with a single hydrophobic stretch acting as an uncleaved signal sequence and anchor to the membrane (Parks and Lamb, 1991) preceded by a short hydrophilic, cytosolic tail (Fig. 2 *B*). Most mammalian (Paulson and Colley, 1989; Moremen

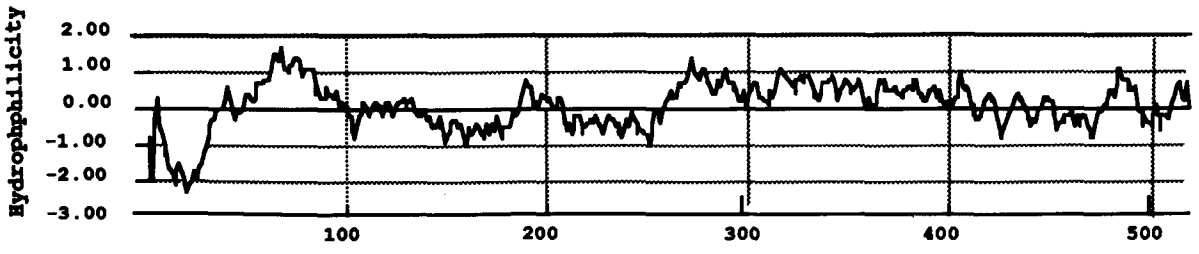
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B



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Table II. Guanosine Diphosphatase Activity after Disruption of GDA1 ORF

Strain	Relevant genotype	GDPase	UDPase
		(*U*/mg protein*)	
G2-9	*GDA1*	1.68	0.12
G2-10	*GDA1*	1.46	0.12
G2-11	gda1::*LEU2*	0	0
G2-12	gda1::*LEU2*	0	0

Total membrane fractions were assayed for their ability to hydrolize GDP, ADP, and UDP. GDPase and UDPase activity are defined as GDPase or UDPase minus ADPase. ADPase activity was 0.22 U/mg protein for every fraction. Results are the average of three independent determinations.
* One unit is the amount of enzyme that releases 1 μmol of inorganic phosphate per min under the standard assay conditions described in Materials and Methods.

and Robbins, 1991) and some yeast (Carmirand et al., 1991) glycosyltransferases and glycosidases sequenced to date share this characteristic; the bulk of the globular carboxy-terminal domain, with the potential N-glycosylation sites and catalytic activity, face the lumen of the organelle. The GDPase is known to be a "high mannose" glycoprotein since it binds to Con A and is deglycosylated by endo H (Yanagisawa et al., 1990). The discrepancy in the apparent MW of the purified, deglycosylated GDPase (48 kD on SDS-PAGE) and that predicted based from the ORF (57 kD) is probably the result of proteolytic cleavage of the enzyme during purification.

GDA1 Is Not Essential for Cell Viability and Growth

The GDPase gene (*GDA1*) was disrupted by replacing the 381 bp Pst–HpaI fragment in the center of the coding region with a fragment encoding *LEU2* (Fig. 1). A linear 4.8-kb HindIII–BamHI fragment containing the gda1::*LEU2* allele was used for transformation of PRY225 and PRY238. A schematic representation of the disrupted allele is shown in Fig. 1. Eight independent diploid transformants from each background were sporulated and 12 asci each from 4 of them were dissected. Viable colonies could be generated from each of the tetrad spores, indicating that the *GDA1* gene is not essential for viability. Tetrad analysis showed that Leu$^+$:Leu$^-$ segregated 2:2 in all cases. One tetrad from each genetic background was analyzed in more detail. Southern analyses confirmed the correct replacement of the *GDA1* gene by the gda1::*LEU2* allele (data not shown).

Assays of GDPase confirmed the loss of this activity in the Leu$^+$ strains. Table II shows the result of a typical experiment. GDPase activity is not detected in membranes derived from the disrupted strains. All values are corrected for production of inorganic phosphate from ADP, which is not a substrate for the enzyme (Yanagisawa et al., 1990). ADPase values were constant in the four samples from each tetrad (see legend of Table II).

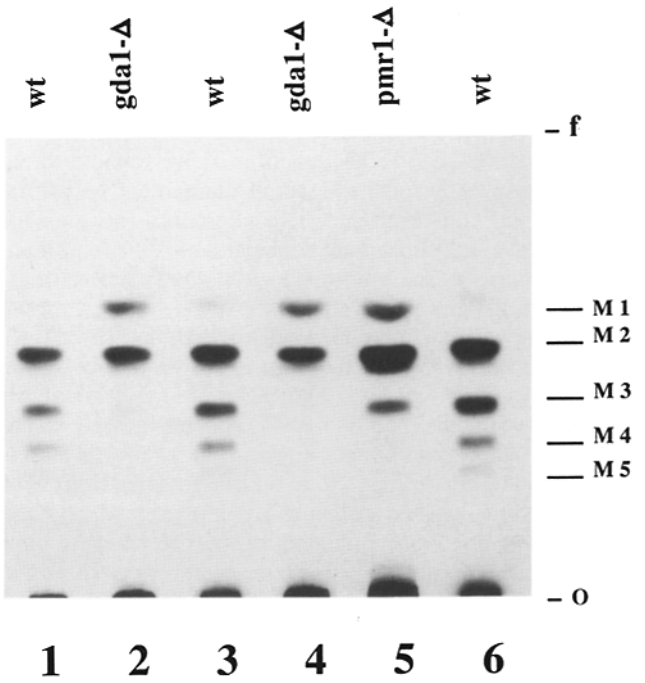


Figure 3. O-glycosylation: total alkali releasable saccharides. Total base sensitive oligosaccharides from various strains radiolabeled with [2-^{3}H]mannose were separated on thin layer chromatography (butanol, ethanol, water-5:3:2) and subjected to autoradiography. Lanes *1* and *3* are strains G11-6 and G11-8, both wild type for *GDA1*; lanes *2* and *4* are strains G11-5 and G11-7 in which the GDA1-ORF is disrupted. Lane *5* is strain CGY2333 in which the PMR1-ORF is disrupted; lane *6* is strain CGY2332, the corresponding wild type. Approximately 100,000 cpm β-eliminated from 15 to 30 μg of protein were loaded on lanes *1–4*, and *6*. On lane *5*, 160,000 cpm β-eliminated from 90 μg of protein were loaded. M_1, mannose; M_2, mannobiose; M_3, mannotriose; M_4, mannotetraose; and M_5, mannopentose.

GDA1 Is Required to Complete the O-Glycosylation of Proteins

Analysis of the O-linked carbohydrate chains was performed by labeling cells in vivo with [2-^{3}H]mannose for 60 min. Total O-linked carbohydrates were then released by β-elimination and radiolabeled carbohydrate chains were resolved by either paper or thin layer chromatography. The oligosaccharide pattern from the wild-type strains agreed with those previously published (Häusler et al., 1992; Haselbeck and Tanner, 1983) and showed five species (Man_1–Man_5) (Fig. 3, lanes *1*, *3*, and *6*). The gda1::*LEU2* strains showed accumulation of M_1 and almost normal amounts of M_2. There

Figure 2. (*A*) Nucleotide sequence of the HindIII–Nhe I fragment (Fig. 1) containing the *GDA1* gene and (*B*) hydrophobicity plot of the GDA1 ORF Genebank accession number L19560. The aa sequence of the GDA1p is shown below the nucleotide sequence. The putative membrane-spanning domain is boxed and the experimentally determined peptide sequences are underlined. The *open triangles* indicate the asparagine residues of the potential N-glycosylation sites. *Dotted boxes* indicate probably regulatory sequences for transcription initiation and termination. The hydrophobicity profile of the GDA1 ORF was generated using the algorithm of Kyte and Doolittle (1982) with a window of 20 amino acids.

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was an almost complete absence of chains with 3, 4, and 5 mannose residues (Fig. 3, lanes *2* and *4*). To extend these findings, four tetrads from each genetic background were subjected to the same labeling protocol, total O-linked sugar chains were released, resolved by paper chromatography, and labeled M_1–M_5 species quantitated. We found a three-fold increase of M_1 and a 1.2-fold increase of M_2 in the gda1Δ strains from both genetic backgrounds (not shown). The decrease in O-linked sugar chains with 3, 4, in 5 mannose residues was more pronounced in gda1Δ strains originated from the G2 diploid that in those originated from the G11 diploid strain. In the G2 derived disrupted strains, we detected a decrease of 4–5-fold in M_3, 10-fold in M_4, and 6–10-fold in the M_5 species. The decline was 2–3-fold in M_3 species, 4–5 in M_4, and 3–5 in M_5 species in the G11 derived disrupted strains (not shown). The wild-type strains corresponding to tetrads from both genetic backgrounds were indistinguishable (not shown).

Mutations in PMR1, a member of the Ca^{++} ATPase family, have been reported to perturb the secretory pathway and Golgi N-glycosylation in yeast (Rudolph et al., 1989). The O-glycosylation phenotype of this mutant has not been reported. As with the gda1Δ deletion, we found that the pmr1::*LEU2* mutants cannot complete the O-linked sugar chains; species with 4 and 5 mannoses were not found (Fig. 3, lane *5*). We measured a 2.4-fold increase in the amount of M_1, a 1.3-fold increase in M_2, and 20% decrease of M_3 in the pmr1Δ. The almost normal amounts of M_3 in pmr1Δ are a significant difference from gda1Δ, where almost none was found.

Secreted, O-Mannosylated Chitinase Is Underglycosylated in gda1 Mutants

S. cerevisiae chitinase is secreted into the growth medium and is extensively and exclusively O-mannosylated (Orlean et al., 1986; Kuranda and Robbins, 1991). The enzyme can be purified from culture media by chitin binding and its mobility on SDS-PAGE depends on the amount and length of the O-linked attached sugar chains (Kuranda and Robbins, 1991). As seen in Fig. 4, lanes *1* and *2* vs lanes *3* and *4*, similar amounts of chitinase were isolated by chitin binding from the medium of saturated cultures of wild-type and gda1Δ strains indicating that mutant cells do not have a defect in

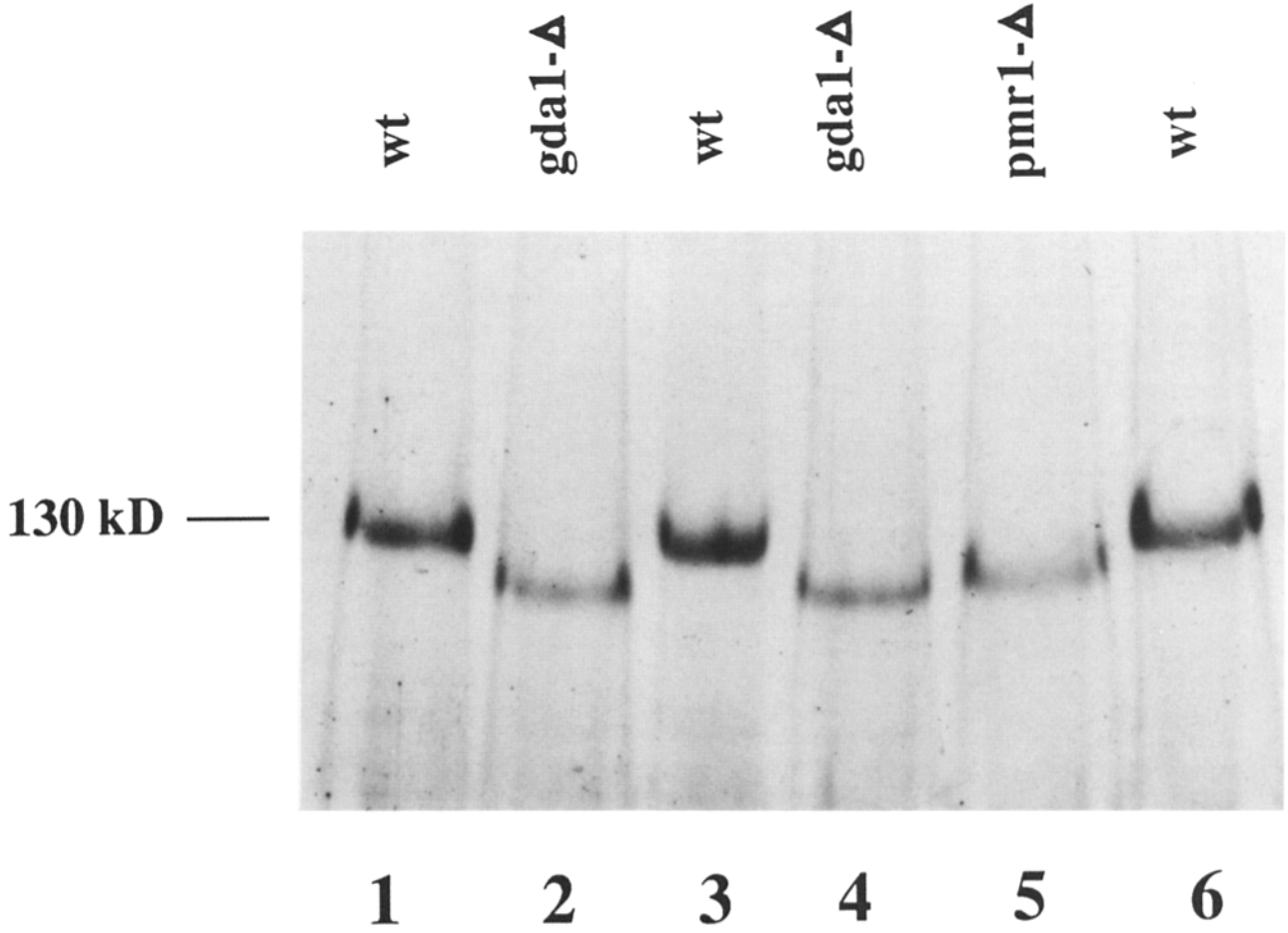


Figure 4. Effect of GDA1 disruption on O-glycosylated secreted chitinase. Coomassie blue stained SDS-PAGE of secreted chitinase from various strains of *S. cerevisiae* is shown. Secreted chitinase was isolated from 15 ml of medium of saturated cultures by chitin binding. Bound protein was eluted by boiling in sample buffer. Samples were then analyzed on 6% SDS polyacrylamide gels. Lanes *1* and *3* are strains G2-9 and G2-10, wild type for *GDA1*; lanes *3* and *4* are strains G2-11 and G2-12, both having the GDA1-ORF disrupted; and lanes *5* and *6* correspond to strains CGY2332 and CGY2333, respectively, described in the legend to Fig. 3.

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secretion. The mobility on SDS-PAGE was greater for the chitinase produced by the gda1Δ strains (Fig. 4, lanes *1* and *3* vs lanes *2* and *4*). A smaller difference was observed when comparing chitinase produced by the pmr1Δ strain to the wild-type enzyme (Fig. 4, lane *6* vs lane *6*).

To further substantiate the assumption that differences in apparent mobility of chitinase produced by wild-type and mutant strains were the result of differences in glycosylation, cells were labeled in vivo with [2-^{3}H]mannose, chitinase was isolated by chitin binding, sugar chains were isolated by β-elimination and separated by paper chromatography (Orlean et al., 1991). The pattern seen in the O-linked chains released from wild-type chitinase was similar to that previously published (Häusler et al., 1992; Kuranda and Robbins, 1991; Orlean et al., 1991) (Fig. 5, *A* and *B*). Chitinase produced by the gda1Δ strains contained 35% of the radiolabel in the M_1 species (relative to total M species); M_1 was almost completely absent in chitinase from the wild-type. M_2 had the remaining 65% of the radiolabel released from gda1Δ chitinase while M_3, M_4, and M_5 were absent (Fig. 5 *A*). Chitinase produced by the pmr1Δ strain also showed a significant accumulation of the M_1 species; M_2 and M_3 were also present, but in this case only M_4 and M_5 were absent (Fig. 5 *B*).

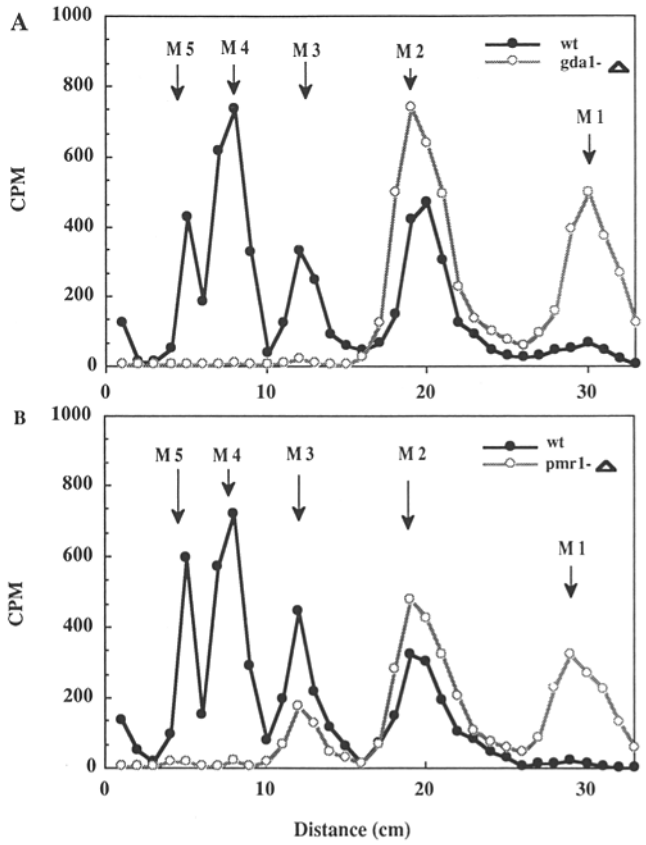


Figure 5. O-linked sugar chains released from secreted chitinase. Paper chromatography of mannoligosaccharides β-eliminated from secreted chitinase isolated by chitin binding from the medium of the experiment shown in Fig. 3. The solvent system was ethyl acetate/butanol/acetic acid/water (3:4:2.5:4). 1-cm strips were cut and subjected to liquid scintillation counting. (*A*) (*closed circles*) wild type, values represent the average from strains G11-6 and G11-8; (*open circles*) gda1Δ, represents the average from strains G11-5 and G11-7. (*B*) (*closed triangles*) wild type, represents strain GCY-2332, and (*open triangle*) pmr1Δ represents strain GCY2333. Strains are described in the legend of Fig. 3.

gda1 Mutants Cannot Synthesize the Golgi p2 Form of Carboxypeptidase Y

The vacuolar enzyme Carboxypeptidase Y (CPY) has been extensively studied; it is a glycoprotein with four N-linked carbohydrate chains (Stevens et al., 1982). Three forms of the enzyme have been identified: the mature (proteolytically processed) vacuolar form, the core glycosylated ER form of the proenzyme (p1), and the more extensively glycosylated p2 form which is found in the Golgi (Stevens et al., 1982). The three forms were immunoprecipitated from wild-type extracts (Fig. 6, lanes *3* and *5*); the *GDA1* disrupted strain, however, showed only the ER precursor p1, and a smaller than wild-type mature form, while the Golgi modified p2 was not detected (Fig. 6, lanes *2* and *4*). It had previously been shown that the conversion of p1 to p2 is solely the result of further mannosylation of the N-linked core in the Golgi, and is not required for accurate proteolytic processing and targeting (Stevens et al., 1982). Thus, the mature form of CPY made by the gda1Δ strain lacks the additional carbohydrates normally added in the Golgi. The core protein of gda1::*LEU2* was not significantly altered because immunoprecipitation of CPY from wild-type and gda1Δ strains followed by treatment with endo H and electrophoresis showed that proteins from both strains migrated equally (not shown). To further support this finding we immunoprecipitated CPY

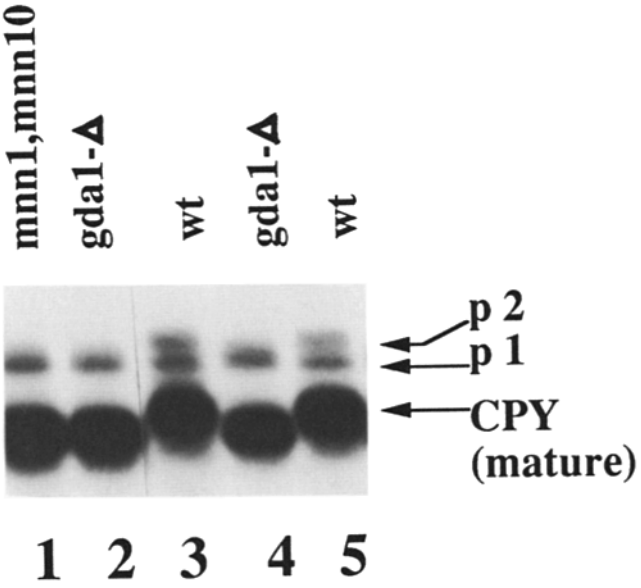


Figure 6. N-glycosylation of CPY: effect of GDA1 disruption. Cells were labeled with $^{35}SO_4$ for 60 min followed by immunoprecipitation, SDS-PAGE, and radioautography. (Lane *1*) represents strain PRY 304; (lane *2*) strain G11-5; (lane *3*) strain G11-6; (lane *4*) strain G11-7; and (lane *5*) strain G11-8. Strains are described on the legend to Fig. 3. p1 (precursor 1) and p2 (precursor 2) refer to the ER and Golgi forms of CPY, respectively. Mature CPY originates by proteolytic cleavage of p2.

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from mnn1, mnn10. The structure of the oligosaccharides of CPY from this strain has been reported (Ballou et al., 1990) and lacks outer chain α1-3 linked mannoses which are added by the mannosyltransferase encoded by the MNN1 gene (Nakajima and Ballou, 1975). The enzyme has been localized to an intermediate Golgi compartment (Graham and Emr, 1991). The electrophoretic pattern obtained from this strain, with an apparently normal p1 form, a mature CPY smaller than wild type, and no observable p2 form, appears identical to that obtained for the gda1Δ strain (Fig. 6, lane *1* vs lanes *2* and *4*).

Invertase Is Underglycosylated in gda1 Mutants

Invertase is a highly glycosylated protein whose rate of migration on a native gel depends on the number and size of the N-linked oligosaccharide chains (Ballou, 1990). Moreover, the crude extracts were heated to 50°C for 30 min before loading on to the native gels in order to convert the invertase to a single oligomeric form (Esmon et al., 1987). Fully glycosylated external invertase from wild-type strains migrated as a diffuse band in the upper third of the gel (Fig. 7, lanes *2*, *3*, and *7*) as reported by Ballou (1990). Invertase produced by pmr1Δ strains has been shown to migrate faster, at a rate similar to invertase from strains carrying the mnn9 mutation (Rudolph et al., 1989) consistent with the absence of outer mannan chains. This behavior was confirmed by the present study (Fig. 7, lanes *1* and *6*). Invertase produced by the gda1Δ strains showed an intermediate phenotype (Fig. 7, lanes *4* and *5*, vs lanes *1* and *6* and lanes *2*, *3*, and *7*); it clearly is underglycosylated, but not completely devoid of outer mannan chains. This result was independent of the genetic background in which the null mutations were made (not shown).

To further determine the degree of underglycosylation of invertase, wild-type and gda 1 null cells were transformed with a multicopy plasmid carrying the external invertase gene (*SUC 2*). The enzyme was partially purified from these overproducing strains. Invertase from the wild-type strain eluted from the DEAE cellulose column as a sharp peak with maximum activity at 90–100 mM NaCl (Fig. 8 *A*). Invertase from the gda 1 null mutant was more heterogeneous eluting from the DEAE column as a broad peak with maximum activity between 95 and 130 mM NaCl (Fig. 8 *A*). This behavior is consistent with either underglycosylation, hyperphosphorylation, or both. Internal nonglycosylated invertase binds very tightly to DEAE, requiring 2 M NaCl for elution (Fig. 8 *A*). Fractions across the activity peak of each strain were divided into three pools (P_1, P_2, and P_3 for wild-type enzyme and P_4, P_5, and P_6 for gda 1 null mutant enzyme; Fig. 8 *A*) and subjected to SDS-PAGE followed by blotting to PVDF membranes as described in Materials and Methods. One membrane was stained with antiinvertase antibody (Fig. 8 *B*). Invertase from the wild-type strain migrated as a broad band with an apparent mobility of 100–150 kD (Fig. 8, lanes *1*, *2*, and *3*); this is similar to the previously described behavior of invertase by Trimble et al. (1991). The mobility of the invertase from the gda 1 mutant strain was clearly more heterogenous, appearing as a diffused ladder of species migrating between 80 and 130 kD (Fig. 8 *B*, lanes *4*, *5*, and *6*). Moreover, invertase which elutes later from the DEAE column (lane *6*) was of the size

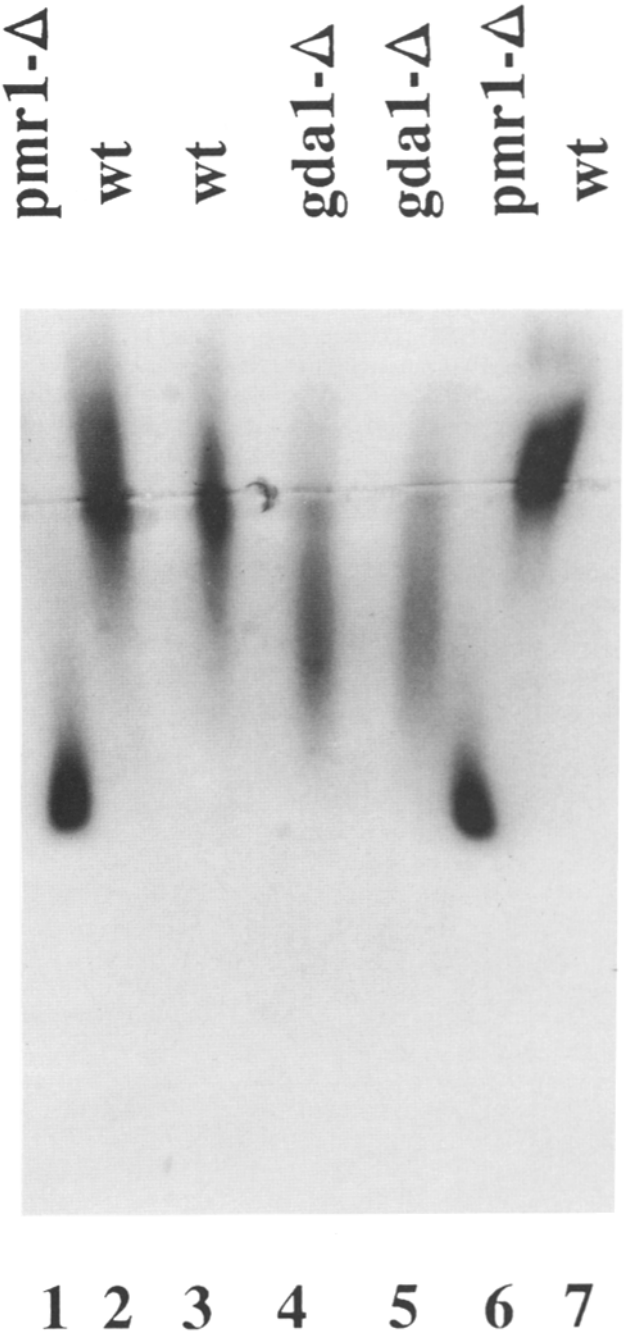


Figure 7. N-glycosylation of invertase I: effect of GDA1 and PMR1 deletion on native invertase glycosylation. Cultures were derepressed for production of external invertase, samples were subjected to native electrophoresis and stained for invertase activity. (Lanes *2*, *3*, and *6*) wild type, fully glycosylated secreted invertase from strains G2-9, G2-10, and CGY2332; (lanes *1* and *6*) pmr1Δ, underglycosylated secreted invertase from strain CGY2333; (lanes *4* and *5*) gda1Δ, underglycosylated invertase produced by strains G2-11 and G2-12, respectively. Strains are described in the legend of Fig. 4.

range of invertase from *Pichia pastoris* and *S. cerevisiae* pmr 1 mutant strains (Fig. 8, lanes *7* and *8*; *B*). Preparations of invertase from *Pichia pastoris* have been shown on SDS-PAGE as a ladder of species of the same size (85–95 kD) as the ER form of invertase from *S. cerevisiae* Sec 18 mutants

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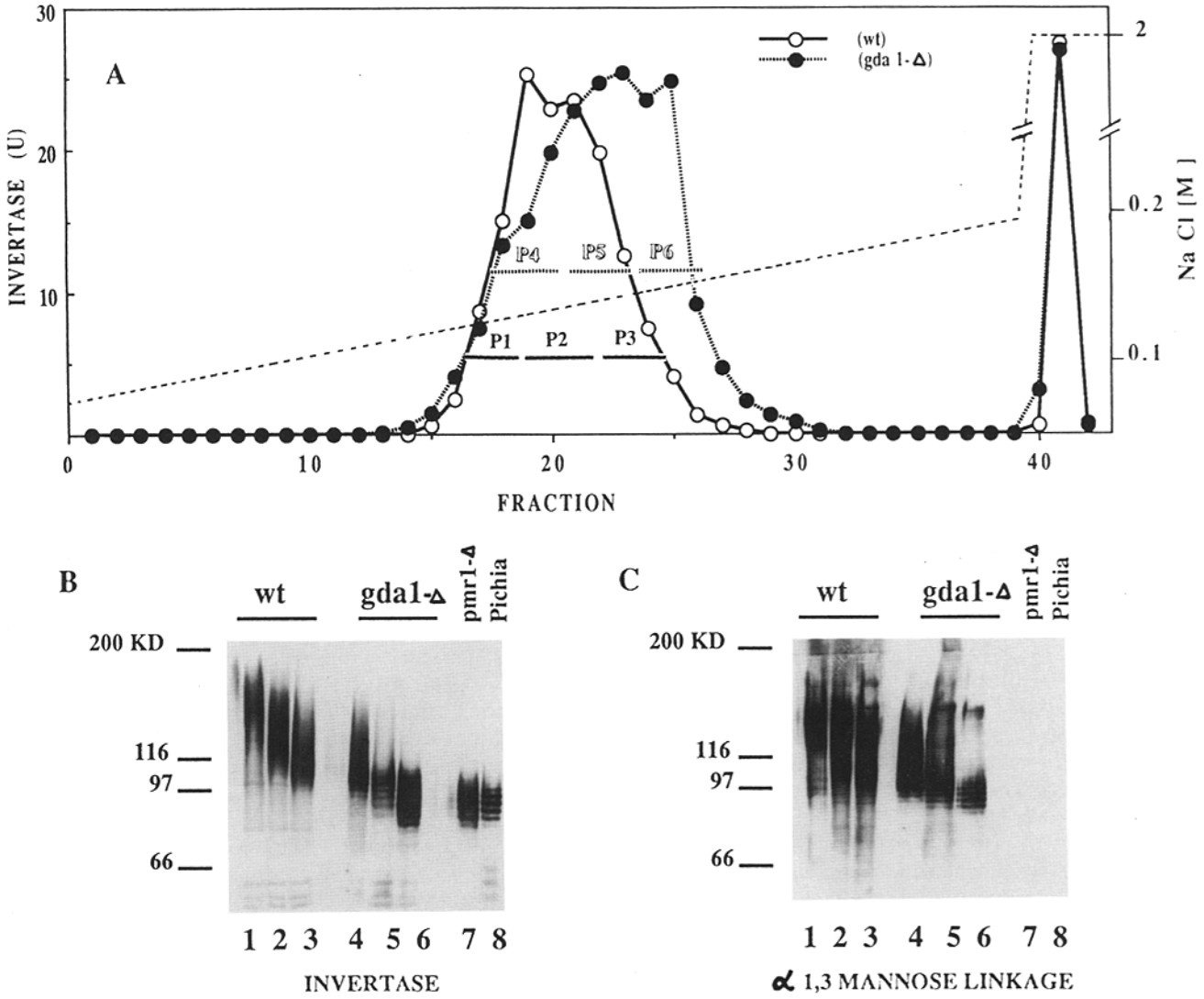


Figure 8. N-glycosylation of invertase II: chromatographic behavior and analyses of mannose linkages. The wild-type stain (G2-9) and gda 1 null strain (G2-11) were transformed with pRD 58 containing the *SUC2* gene; cultures were derepressed for invertase production and the enzyme was purified as described in Materials and Methods. (*A*) Profile from the DEAE-cellulose column. P_1, pooled fractions 16–18; P_2, pooled fractions 19–21; P_3, pooled fraction 22–24, all from wild-type invertase; P_4, pooled fraction 18–20; P_5, pooled fractions 21–23; and P_6, pooled fractions 24–26 all from gda 1 null mutants. Pooled fractions (0.5 U of activity) were resolved on 11% SDS-PAGE, Western blotted with rabbit anti-invertase antibodies (*B*) or rabbit anti-α1,3 linked mannose antibodies (*C*), and visualized with alkaline phosphase conjugated to anti-rabbit IgG. In *B* and *C*, lanes *1*, *2*, and *3* are P_1, P_2, and P_3 from wild-type invertase; lanes *4*, *5*, and *6* are P_4, P_5, and P_6 from gda 1 null invertase; lane *7* is invertase from pmr 1 null strain (CGY 2333); lane *8* is invertase from *Pichia pastoris* strain GS115.

at 37°C (Trimble et al., 1991). Chromatography of *P. pastoris* oligosaccharides released by endo H showed that over 90% of the species were Man_{8-11} GlcNac (Trimble et al., 1991). Invertase produced by pmr 1 mutants have also been shown to be devoid of outer mannan chains.

All the invertase fractions from the wild-type and gda 1 null mutant strains showed reactivity with antibodies against the α1, 6-mannose linkage (data not shown). Staining for the presence of terminal α1, 3 linked mannose showed that this linkage occurred, as expected, in the wild-type invertase (Fig. 8 *C*, lanes *1*, *2*, and *3*). Invertase secreted by the gda 1 null mutants showed a wide range of reactivity varying in amounts similar from wild-type (Fig. 8 *C*, lane *4*) to very reduced levels (lane *6*), further supporting the heterogeneity of this enzyme's mannan chains. Invertase from *P. pastoris* (lane *8*) does not contain such linkage in agreement with previous biochemical studies (Trimble et al., 1991).

The Cell Wall Is Altered in gda1 Mutants

The *MNT1* gene (Häusler et al., 1992) is identical to *KRE2* (Hill et al., 1992), a gene isolated based on resistance of *S. cerevisiae* to killer toxin K_1. Wild-type *S. cerevisiae* strains are sensitive to K_1 while gda1 mutants were partially resistant (Bussey, H., personal communication) indicating a structural alteration of the cell wall. Although β-1,6 glucans are components of the K_1 killer toxin receptor on the cell

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wall (Boone et al., 1990), other components must play a role since the structure and amounts of β-1,6 glucan is normal in mnt 1/kre2 null mutants (Hill et al., 1992). β-glucans are known to be crosslinked with mannoproteins in the *S. cerevisiae* cell wall. Perhaps the basis for toxin resistance is a reduced number of attachments between these two types of chains.

Biosynthesis of Mannosylinositolphosphorylceramides Is Severely Impaired in gdal Mutants

About one third of *S. cerevisiae* lipids contain inositol; of these, 60% is phosphatidylinositol (PI) which is essential for cell viability and growth (Culbertson and Henry, 1975; Henry, 1982; Nikawa et al., 1982). The remaining 40% consists of three classes of related inositolphosphate-containing sphingolipids, they are: (*a*) IPC containing a single inositolphosphate; (*b*) mannosylinositolphosphorylceramides (MIPC), containing a single inositolphosphate with a mannose attached; and (*c*) the major sphingolipid, mannosyldiphosphorylinositolceramide (M[IP]$_2$C) which contains a second inositolphosphate attached to the mannose of MIPC (Steiner et al., 1969; Smith and Lester, 1974). Diversity in the types of sphingoid bases and in the degree of hydroxylation and chain lengths of the fatty acids gives rise to many molecular species of these three lipid classes (Smith and Lester, 1974).

Yeast membranes contain activities which transfer (*a*) inositolphosphate from PI onto endogenous ceramides yielding IPC, and (*b*) mannose, from GDP-mannose, onto IPC to yield MIPC (Becker and Lester, 1980). It is also known that the biosynthesis of mannosylinositolsphinogoids is dependent on genes controlling the flow of secretory vesicles from the ER to the Golgi apparatus (Puoti et al., 1991). Previous results suggest that IPC is the critical substrate transported between ER and Golgi vesicles, because ER blocked mutants like sec17 and sec18 accumulate IPC and cannot synthesize MIPC at the restricted temperature (Puoti et al., 1991).

From knowledge that MIPC is likely to be made in the Golgi, that GDP-mannose is the mannosyl donor, and that MIPC and M(IP)$_2$C are normally found in the plasma membrane facing the periplasmic space, we speculated that mannosylation of IPC probably occurs in the Golgi lumen and would be affected by mutations in the *GDA1* gene. To test this hypothesis, we determined the kinetics of [^{3}H] myo-inositol incorporation into lipids of wild-type and *GDA1* disrupted strains of *S. cerevisiae.* After a 10-min labeling and 10-min chase, the major labeled species in the wild type was PI (Fig. 9, lane *1*) in agreement with previous in vivo and in vitro results (Angus and Lester, 1972; Becker and Lester, 1980). After a 90-min chase (Fig. 9, lane *3*), the label in PI decreased while that of IPCs, MIPC, and M(IP)$_2$C increased, also in agreement with previous observations (Angus and Lester, 1972; Becker and Lester, 1980; Pouti et al., 1991). In the gdal Δ strains, the kinetics of [^{3}H] myo-inositol incorporation into PI was not affected and formation of IPC was normal or slightly increased (Fig. 9, lanes *2* and *4* vs lanes *1* and *3*), as expected for processes occurring in the ER. However, significant differences were seen with MIPC and M(IP)$_2$C; a dramatic decrease in the amount of these mannosylated ceramides in the mutants was observed after a 90-min chase (Fig. 9, lane *4* vs lane *3*). Quantitation showed a 60% decrease in the amount of mannosylinositol-sphingoids in the gdal Δ strain (Table III) compared to wild type; moreover, the ratio of MIPC/IPC changed drastically, from 1.06 in the wild type to 0.28 in the gdal Δ strain. The only step affected is the addition of mannose from GDP-mannose to IPC to yield MIPC. The subsequent reaction, addition of a second inositol phosphate to MIPC, to yield M(IP)$_2$C occurs at a slightly higher rate in the gdal Δ strain compared to wild type as shown by the ratio 0.42 vs 0.32 (Table III). Also, the ratio of IPC/PI is elevated in gdal Δ cells from 0.23 to 0.32 (Table III). This accumulation of precursor indicates independently that the addition of mannose is the limiting step. Results very similar to the ones shown on Fig. 9 and Table III were obtained when the experiment was repeated with tetrads derived from both genetic backgrounds G2 and G11 (not shown).

Discussion

We have shown in the gdal null mutant that, (*a*) elongation of O-linked carbohydrate chains is blocked at the mannobiose step in bulk cell mannoproteins and in secreted chitinase; (*b*) N-linked carbohydrates of CPY are not elongated beyond the pl (ER) stage; (*c*) invertase is underglycosylated; and (*d*) biosynthesis of MIPC is severely impaired. All these processes have in common a requirement for GDP-mannose in the Golgi lumen for completion of glycosylation. *GDA1* is the structural gene for a GDPase, previously localized by subcellular fractionation, to a Golgi-like compartment (Abeijon et al., 1989). *GDA1* deletion was not expected to be lethal because mutants which can only complete glycosylation in the ER (like mnn9) are viable (Ballou, 1990).

We shall briefly review the current status of *S. cerevisiae* Golgi subcompartmentation before interpreting the phenotype of gdal strains in relation to this Golgi structure. The complexity of the *S. cerevisiae* Golgi is just unravelling; few genes have been cloned whose products are specific markers of this compartment. Among these are integral membrane proteins responsible for Golgi functions, such as (*a*) mntlp, an α1,2 mannosyltransferase involved in O-glycosylation (Häusler and Robbins, 1992; Häusler et al., 1992); (*b*) kex2p, an endoproteinase that processes α-factor in a late Golgi compartment (Redding et al., 1991); and (*c*) pmrlp, a P-type ATPase, homologous to mammalian Ca^{++} pumps (Rudolph et al., 1989; Antebi, 1991). A null mutation in PMR1 exhibits pleiotropic Golgi dysfunctions (Antebi, 1991).

Other specific Golgi markers are peripheral membrane proteins such as sec7p involved in vesicle-mediated protein sorting and traffic through the Golgi segment of the secretory pathway. Anti sec7p IgG blocks ER to Golgi transport in vitro (Franzusoff et al., 1992) and conditional sec7 mutants accumulate Golgi-like structures (Novick et al., 1981). Other peripheral Golgi membrane proteins include small (20–25 kD) ras-like GTP-binding proteins such as arf1p and yptlp. These are localized on the cytosolic surface of the Golgi complex and function in protein transport to and/or within the organelle (Stearns et al., 1990; Segev et al., 1988). Sec14p is a peripheral membrane and cytosolic protein which cofractionates with the putative late Golgi marker kex2p and has PI/PC transfer (exchange) activity; condi-

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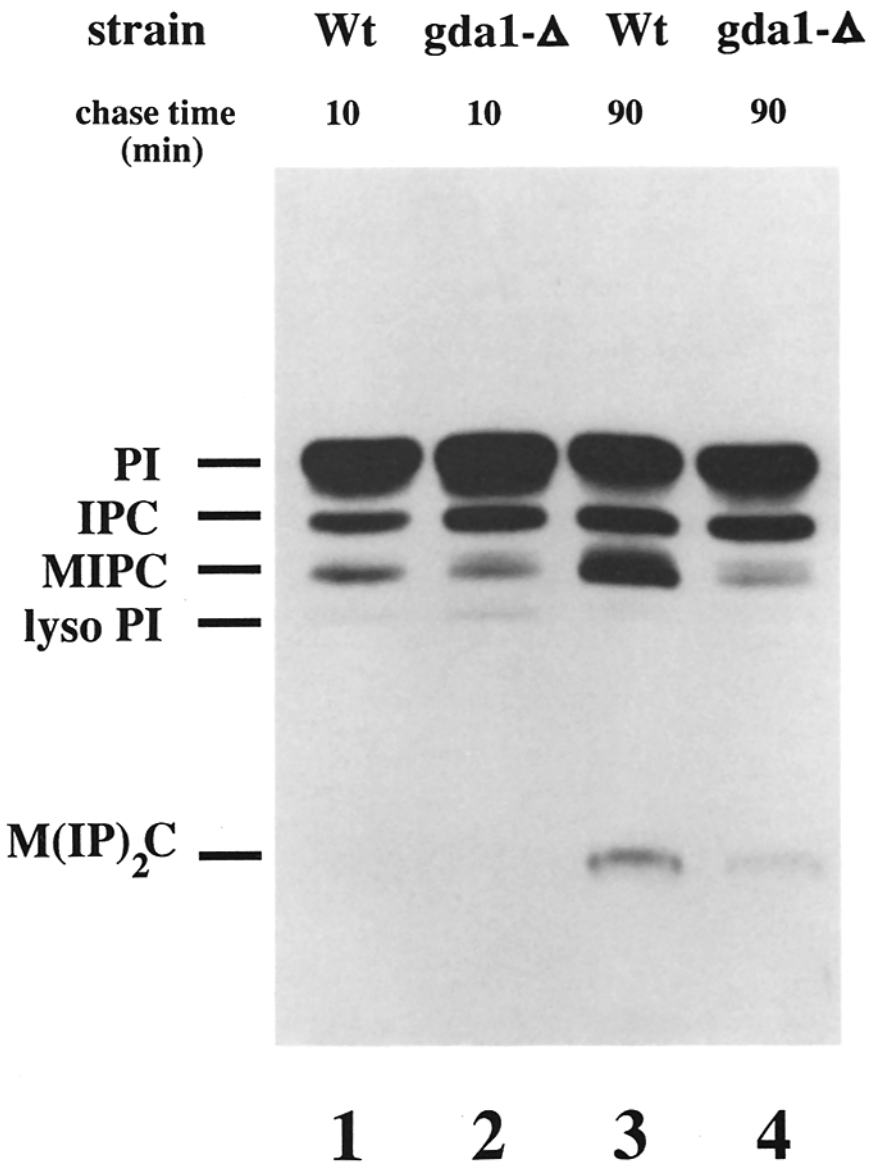


Figure 9. Effect of *GDA1* disruption on the biosynthesis of MIPC. Wild-type (G2-10 strain, lanes *1* and *3*) and gda1Δ (G2-12 strain, lanes *2* and *4*) cells were incubated with [$_3$H]myoinositol for 10 min, followed by addition of high concentration of unlabeled myoinositol and further incubation for 10 min (lanes *1* and *2*) or 90 min (lanes *3* and *4*). Labeled lipids, PI, IPC, MIPC, $M(IP)_2C$ were extracted and analyzed by thin layer chromatography ($CHCl_3/CH_3OH/0.22\%$ aqueous KCl, 55:45:10) and fluorography. Quantitation is presented in Table III.

Table III. Effect of gda1Δ on the Biosynthesis of Inositol-containing Phospholipids

	Incorporation of [^{3}H]inositol (*cpm/mg protein/90 min*)		
Inositol phospholipid	Wild-type	gda1Δ	gda1Δ/wt
PI	42,700	47,700	1.12
IPC	10,000	15,500	1.55
MIPC	10,600	4,300	0.40
$M(IP)_2C$	3,400	1,800	0.53

	Ratios of radioactivity of inositol-containing phospholipids	
	Wild-type	gda1Δ
IPC/PI	0.23	0.32
MIPC/IPC	1.06	0.28
$M(IP)_2CC/IPC$	0.34	0.12
$M(IP)_2C/MIPC$	0.32	0.42

Results were taken from the 90-min time point of the experiment described in Fig. 8.

tional sec14 mutants accumulate Golgi structures and 80–100 nm vesicles (Cleves et al., 1991).

Biochemical, functional, and morphological results support Golgi subcompartmentalization in *S. cerevisiae*. Golgi markers have been separated from those of other organelles and from each other. Subcellular fractionation can clearly dissociate kex2 endoprotease from GDPase-containing compartments (Bowser and Novick, 1991); kex2p and α1,3 mannosyltransferase can be partially separated (Cunningham and Wickner, 1989) while GDPase and the α1,2 mannosyltransferase, responsible for the addition of the third O-linked mannose (mntlp, Häusler et al., 1992) cosediment (Abeijon et al., 1989). Furthermore, the bulk of pmrlp comigrates with kex2p and GDPase, clearly separating from markers of the ER, plasma membrane, vacuole and ERD2, and the yeast HDEL receptor (Antebi, 1991). However, peak and shoulder fractions of pmrlp, GDPase, and kex2p do not overlap completely (Antebi, 1991). At least three functionally distinct Golgi subcompartments have been postulated in *S. cerevisiae* containing from *cis* (early) to *trans* (late) (*a*)

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α1,6-mannosyltransferase, (*b*) α1,3-mannosyltransferase, and (*c*) kex2 endopeptidase (Graham and Emr, 1991). *S. cerevisiae* strains which lack clathrin heavy chains mislocalized Golgi proteases involved in α factor maturation like kex2p and dipeptidylaminopeptidase A to the cell surface, while other Golgi integral membrane proteins such as GDPase were not affected (Seeger and Payne, 1992).

Recently, improved ultrastructural EM analyses with permanganate staining and serial sectioning, showed that wild-type *S. cerevisiae* cells contain ~30 Golgi-like structures (Preuss et al., 1992) slightly concave disks, typically observed as single isolated cisternae (60% of cases). Occasionally two (30% of cases) or up to three (10% of cases) of these structures are in parallel stacks, often associated with nearby 50-nm vesicles. Golgi structures are always discrete, not contiguous with ER or other organelles. The yeast Golgi compartments are equidistant from the nucleus and the cell wall (Preuss et al., 1992), and different from the mammalian Golgi complexes which are near the nucleus as a consequence of their association with the microtubule organizing center (Kreis, 1990). Permanganate staining in *S. cerevisiae* revealed morphological features and structures similar to the cisternae and vesicles labeled with antibodies against yptlp, sec7p, and α1,6-mannose residues (Preuss et al., 1992) the latter recognizing proteins which have gained access to the Golgi (Ballou, 1982). Unfortunately, no colocalization of these antigens was done in this EM study.

The above ultrastructural studies agree with previous immunofluorescent experiments which showed yeast Golgi associated antigens dispersed in many locations within the cell, but not overlapping with other organelles. By immunofluorescence sec7p colocalizes with kex2p in most instances (60–80%) (Franzusoff et al., 1991). Kex2p is thought to occupy a late Golgi subcompartment whereas sec7 appears to be associated with several Golgi subcompartments (Franzusoff et al., 1991; Franzusoff and Schekman, 1989). PMR may be in another subcompartment because indirect immunofluorescence showed a punctate pattern resembling Golgi staining, but double-labeling experiments in wild-type cells (Antebi, 1991) revealed only a small degree of congruence (although in close juxtaposition) between pmrlp and sec7p (17%) or pmrlp and kex2p (26%); pmrlp accumulated in aberrant Golgi-like structures that occur in sec7 mutants.

How does knowledge of the above Golgi subcompartments relate to the phenotype of gdal null mutants and our current hypothesis of the Golgi localization and function of the gdalp? Current models of nucleotide sugar transport into the Golgi lumen predict a critical role for gdalp in those lumenal reactions requiring GDP-mannose as substrates: after mannosylation of proteins and lipids, GDP, a reaction product, is converted by gdalp to GMP which can exit the Golgi lumen in an exchange coupled to entry of additional GDP-mannose from the cytosol. In the absence of gdalp, transport of GDP-mannose into the Golgi lumen should be diminished, leading to reduced availability of the nucleotide sugar and inhibition of those mannosylation reactions occurring in this compartment.

Upon examining total O-glycosylation of proteins we found a threefold increase in the amount of base sensitive monosaccharide in the gdal strains compared to wild type, strongly suggesting that O-linked sugar chains were normally initiated by the addition of a single mannose residue. Gdalp is not expected to play a role in this initiation step, because it is well established that the biosynthesis of O-linked carbohydrate chains starts in the ER, with the first sugar being transferred to serine or threonine residues of the protein from dolichol-*P*-mannose (Haselbeck and Tanner, 1983, Orlean, 1990). Dolichol-*P*-mannose synthase has its active site towards the cytosol (Beck, P. J., C. Albright, P. Orlean, P. W. Robbins, M. J. Gething, and J. F. Sambrook. 1990. *J. Cell Biol.* 111:37*a*), and transport of GDP-mannose into a lumenal compartment is not required for this reaction. It has been proposed that dol-*P*-man synthase can catalyze the translocation of the mannosylated dol-*P*-man from the cytosolic to the luminal side of the ER (Haselbeck and Tanner, 1982), where the sugar becomes attached to the Ser/Thr of the proteins by a recently purified protein O-mannosyltransferase (Sharma et al., 1991).

GDP-mannose is probably the direct donor of the second to fifth O-mannoses which are added stepwise to complete the O-linked mannosyl chains (Haselbeck and Tanner, 1983). These reactions probably occur in the lumen of the Golgi apparatus. Analyses of bulk cell mannoproteins of gdal null mutants showed a virtual complete absence of O-linked mannose chains with 3, 4, or 5 sugars though, unexpectedly, mannobiose was similar to wild type. Because the second O-linked mannose is thought to be added in the Golgi lumen, we were initially surprised by the amount of O-linked mannobiose found in gdal mutants. However, Kuranda and Robbins (1991) reported that intracellular chitinase from sec18 labeled at the restrictive temperature contained more than half of the sugars as mannobiose, the remainder being mannose. Significant amounts of mannobiose, in addition to mannose, were also detected in cell wall mannans of sec18 (Haselbeck and Tanner, 1983). Recent studies have shown that sec18 (together with sec17 and 22) belongs to a subset of sec mutants blocked in ER to Golgi transport (class II), in which 50-nm vesicles and enlarged ER structures accumulate (Kaiser and Schekman, 1990). These mutants are believed to be blocked in fusion of these vesicles to the target compartment while class I mutants (sec12, 14, 16, and 23), which accumulate only ER structures appear to be blocked earlier in secretion, during the budding stage (Kaiser and Schekman, 1990). It will be important to determine the length of the O-linked mannose chains synthesized by these mutants.

The α1,2 mannosyltransferase (mntlp) responsible for the addition of the third O-linked mannose has been localized by subcellular fractionation to the Golgi, and colocalizes with the GDPase activity (Abeijon et al., 1989). This α1,2 mannosyltransferase activity is inhibited in the gdal mutant; thus the O-glycosylation phenotype of gdal is very similar to that of mntl null mutants (Häusler et al., 1992). This provides independent evidence for both activities occurring in the same subcellular compartment.

Bulk mannoproteins and secreted chitinase from pmrl strains contained almost normal amounts of O-linked M_3 species and thus appear to be blocked in O-mannosylation later than gdal mutants. Subcompartmentation may play a role in the differential effects of these mutations on Golgi processing, since gdalp and pmrlp would be thought to alter Golgi glycosylation in a general, non-specific fashion.

The vacuolar serine protease CPY has been used to detect

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alterations in Golgi N-glycosylation since conversion of the core glycosylated 61 kD, ER, form of the proenzyme (plCPY) to the 69 kD precursor 2 (p2CPY) is solely due to the addition in the Golgi of five mannoses to each of the four N-linked core units present in the proenzyme (Hasilik and Tanner 1978*a*). This precursor is converted by proteolytic cleavage to a mature, 61-kD form in the vacuole. Glycosylation is not required for correct targeting and processing of CPY (Hasilik and Tanner, 1978*b*). As predicted, gdal mutants could not synthesize p2CPY and the mature form migrated faster than wild-type on SDS-PAGE. These differences were solely due to glycosylation because mutant and wild-type CPY showed equal SDS-PAGE migration after endo H treatment.

External invertase synthesized by wild-type strains has long outer mannan chain added to core-N-linked oligosaccharides (Ballou, 1990), while that secreted by gdal mutants was underglycosylated but not completely lacking outer mannan chains. Invertase secreted by the mutants is more heterogeneous than that by wild type and partially lacks the terminal α1, 3 linked mannose units added in the Golgi apparatus. A substantial portion of it showed only core oligosaccharides which are added in the ER. Even though the glycosylation defect observed in invertase is less pronounced than that of CPY and chitinase, four different reasons can explain why a severe decrease in the availability of GDP-mannose in the Golgi lumen of gda 1 mutants may differentially affect individual glycosylation reactions: (*a*) Different mannosyltransferases have distinct Kms for GDP-mannose (Nakajima and Ballou, 1975). Thus, low concentrations of the nucleotide sugar may affect mannosylations of different substrates to a different extent. (*b*) Different mannosyltransferases most likely also have different Ki for GDP and thereby selectively affect the synthesis of different glycosylated products. (*c*) The Golgi apparatus of *S. cerevisiae* is a subcompartmentalized organelle based on structural (Preuss et al., 1992) and functional evidence (Franzusoff and Schekman, 1989). The various subcompartments most likely have different local substrate concentrations. (*d*) Invertase has mannose phosphate on the outer chains, the donor of which is also GDP mannose, although the reaction product in this case is GMP, and not GDP. The transfer of mannose phosphate is a lumenal reaction and can generate the putative antiporter, GMP, independently of gdalp. GMP present in the Golgi lumen could in turn facilitate the entry of additional cytosolic GDP-mannose, causing the glycosylation defects induced by the gdal mutation to be less severe in those subcompartments where addition of mannose phosphate occurs. This hypothesis can be tested in a double mutant gdal, mnn6 (defective in phosphomannan formation [Ballou, 1990]) where invertase may only be core glycosylated. In more general terms, we recognize that the antiporter activity required to sustain the level of mannosylation found in the deletion strain may be derived from GMP generated by enzymes other than gdalp or from weak antiporter activity of GDP. Mutations in peripheral Golgi proteins arf1 and ypt1 lead to a glycosylation phenotype of invertase very similar to gdal (Stearns et al., 1990). Although CPY also contains mannose-phosphate, the addition probably occurs at a different subcellular site than for invertase (Stevens et al., 1982). Sec18 showed almost no phosphate radiolabeled invertase while CPY phosphate content was normal. In addition, conversion of plCPY to p2CPY did not result in additional phosphate incorporation (Stevens et al., 1982). The gdal and pmr1 mutations, which have quantitatively different effects on N-glycosylation of CPY and invertase, provide another example of disparity between secretory and vacuolar proteins routed through the yeast Golgi.

Sphingolipids are ubiquitous eukaryotic plasma membrane constituents that contain a common hydrophobic portion (ceramide) which is anchored in the membrane, and a variable hydrophilic region which in *S. cerevisiae* has mannosylinositol phosphate exposed to the cell surface (Patton and Lester, 1992). In higher eukaryotes they may act as mediators of signal transduction and cellular regulation (Hakomori, 1990; Hannum and Bell, 1989). Our results with gdal strains provide direct evidence that the biosynthesis of the mannosyl-sphingolipids occurs in the lumen of the *S. cerevisiae* Golgi apparatus, supporting previous studies which suggest that GDP-mannose is the direct mannose donor in the formation of MIPC (Becker and Lester, 1980) and that these reactions probably occur in the Golgi apparatus (Puoti et al., 1991). The severe decrease of the above lipids in the gdal mutant strains opens the possibility of studying their influence on membrane activities in vivo. Studies in vitro have shown that H^+ ATPase, a major protein of the *S. cerevisiae* plasma membrane is stimulated by sphingolipids (Patton and Lester, 1992).

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