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The UDPase activity of the *Kluyveromyces lactis* Golgi GDPase has a role in uridine nucleotide sugar transport into Golgi vesicles

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In *Saccharomyces cerevisiae* a Golgi lumenal GDPase (ScGda1p) generates GMP, the antiporter required for entry of GDP-mannose, from the cytosol, into the Golgi lumen. *Scgda1* deletion strains have severe defects in N- and O-mannosylation of proteins and glycosphingolipids. ScGda1p has also significant UDPase activity even though *S. cerevisiae* does not utilize uridine nucleotide sugars in its Golgi lumen. *Kluyveromyces lactis*, a species closely related to *S. cerevisiae*, transports UDP-N-acetylglucosamine into its Golgi lumen, where it is the sugar donor for terminal N-acetylglucosamine of the mannan chains. We have identified and cloned a *K. lactis* orthologue of ScGda1p. KlGda1p is 65% identical to ScGda1p and shares four apyrase conserved regions with other nucleoside diphosphatases. KlGda1p has UDPase activity as ScGda1p. Transport of both GDP-mannose, and UDP-GlcNAc was decreased into Golgi vesicles from *Klgda1* null mutants, demonstrating that KlGda1p generates both GMP and UMP required as antiporters for guanosine and uridine nucleotide sugar transport into the Golgi lumen. Membranes from *Klgda1* null mutants showed inhibition of glycosyltransferases utilizing uridine- and guanosine-nucleotide sugars, presumably due to accumulation of nucleoside diphosphates because the inhibition could be relieved by addition of apyrase to the incubations. *KlGDA1* and *ScGDA1* restore the wild-type phenotype of the other yeast *gda1* deletion mutant. Surprisingly, *KlGDA1* has only a role in O-glycosylation in *K. lactis* but also complements N-glycosylation defects in *S. cerevisiae*. Deletion mutants of both genes have altered cell wall stability and composition, demonstrating a broader role for the above enzymes.

Key words: yeast/Golgi/glycosylation/nucleoside diphosphatases

Introduction

Terminal mannosylation of yeast proteins and lipids occurs in the lumen of the Golgi apparatus. GDP-mannose, the sugar donor of these reactions, must first be transported by a specific transporter from the cytosol, its site of synthesis, into the Golgi lumen, where mannose is transferred to mannans by specific mannosyltransferases (Hong *et al.*, 1994; Berninsone and Hirschberg, 2000). The other reaction product, GDP, is then hydrolyzed by a GDPase to GMP, which then exits the Golgi lumen in a coupled, equimolar exchange with cytosolic GDP-mannose (Hong *et al.*, 1994; Berninsone and Hirschberg, 2000). This antiport cycle was originally described *in vitro* with Golgi vesicles of mammalian cells. Evidence of its physiological relevance has been obtained *in vivo* and *in vitro* in *Saccharomyces cerevisiae* (Abeijon *et al.*, 1993; Berninsone *et al.*, 1994). Following identification and purification of a Golgi lumenal, membrane-bound GDPase, the gene encoding this type II membrane protein (*GDA1*) was cloned and disrupted (Abeijon *et al.*, 1993). *gda1* null mutants have a block in O- and N- mannosylation of proteins, such as chitinase and carboxypeptidase Y, undermannosylation of invertase, and drastically reduced levels of mannosylphosphorylceramides (Abeijon *et al.*, 1993). These pleiotropic defects in mannosylation are most likely the result of reduced availability of GDP mannose in the Golgi lumen; indeed, transport of GDP-mannose into Golgi vesicles is impaired in *gda1* null mutants due to reduced levels of GMP in the Golgi lumen (Berninsone *et al.*, 1994). Ectoapyrases (E-ATPases) are enzymes that hydrolyze nucleoside tri- or diphosphates. An E-ATPase was recently cloned from *S. cerevisiae*, Ynd1p (Guillen *et al.*, 1999). This protein also plays a role in Golgi mannosylation, and *gda1ynd1* double mutants have a more severe phenotype than the single ones, suggesting functional redundance. Considered together, these results are consistent with a model in which nucleotide sugar transporter activities are regulated by the availability of the antiporter molecule in the Golgi lumen (Berninsone *et al.*, 1994). The antiport generation cycle by nucleoside diphosphatases constitutes a novel mechanism of regulation of post-translational modifications that occur in the Golgi lumen and affect most secreted and membrane proteins (Hong *et al.*, 1994; Berninsone and Hirschberg, 2000).

Nucleoside diphosphatase activities have also been described in mammalian membranes of the Golgi apparatus (Brandan and Fleischer, 1982; Wang and Guidotti, 1998) and endoplasmic reticulum (ER; Trombetta and Helenius, 1999; Ohkubo *et al.*, 1980). Two related nucleoside diphosphatases, hypothesized to have a role in generating nucleoside monophosphates in the lumen of the ER and Golgi apparatus, have recently been cloned. An ecto (E)-ATPase from human brain was shown to localize to the Golgi apparatus of transfected COS 7 cells (Wang and Guidotti, 1998). This protein has high activity toward UDP; lower toward GDP, CDP, and TDP; and lowest toward the corresponding nucleoside triphosphates. Trombetta and Helenius (1999) recently purified and cloned a rat liver ER nucleoside diphosphatase that hydrolyzes UDP,

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GDP, and IDP but no other nucleoside phosphates. These mammalian nucleoside diphosphatases share high sequence similarity in four conserved apyrase regions with Gdalp and other proteins of still unidentified function. A Golgi GDPase has also been detected in plants and hypothesized to yield GMP, the putative antiporter for GDP-fucose transport into the Golgi lumen (Wulff *et al.*, 2000).

We showed previously that the *S. cerevisiae* Golgi Gda1p could hydrolyze UDP, in addition to GDP, albeit at considerably slower rate. In the presence of Mn^{2+} ions, the UDPase activity was 33% of GDPase activity, whereas in the presence of Ca^{2+} ions it was 11% (Yanagisawa *et al.*, 1990). The role of this UDPase activity is not clear because *S. cerevisiae* solely utilizes GDP-mannose as sugar donor in the Golgi lumen and no reaction utilizing uridine nucleotide sugars has been described in the Golgi apparatus of this organism.

Kluyveromyces lactis is a yeast species closely related to *S. cerevisiae*; its outer mannan chains differ from those of *S. cerevisiae* by having terminal N-acetylglucosamine and no mannose phosphate (Raschke and Ballou, 1972). Previous studies from our laboratory characterized a mutant of *K. lactis* that lacked terminal N-acetylglucosamine as a consequence of a deficiency in the Golgi UDP-N-acetylglucosamine transporter (Abeijon *et al.*, 1996a). Phenotypic correction of this mutant allowed the molecular cloning of the first nucleotide sugar transporter gene (Abeijon *et al.*, 1996b) which was later found to be contiguous to the N- acetylglucosaminyttransferase that catalyzes the addition of the terminal N- acetylglucosamine to the *K. lactis* outer mannan chains (Hirschberg *et al.*, 1998).

K. lactis uses both guanosine and uridine nucleotide sugars for synthesis of its sugar polymers in the lumen of its Golgi apparatus. We were therefore interested in determining whether it has a nucleoside diphosphatase with both GDPase and UDPase activities in the Golgi lumen, and if so, its role in transport of GDP-mannose and UDP-GlcNAc into Golgi vesicles.

Results

The K. lactis *Golgi GDPase gene (KlGDA1) is a new member of the apyrase family*

We cloned the *K. lactis* counterpart of the *S. cerevisiae GDA1* gene using the latter as a probe in a low-stringency screen of a *K. lactis* genomic library. The *K. lactis* GDPase gene (*KlGDA1*; GenBank accession no. AJ401304) encodes a protein of 522 amino acids that is 65% identical to the *S. cerevisiae GDA1*. The Kyte-Doolittle algorithm revealed a single hydrophobic domain close to the amino terminus (not shown), consistent with a type II membrane protein like the *S. cerevisiae* counterpart. Alignment of the KlGda1p with other proteins in the DataBank shows its homology to apyrases. All these proteins share four apyrase conserved motifs as shown in Figure 1. Sucrose gradient fractionation of wild-type cells showed that the GDPase activity comigrated with Golgi membrane markers and was separated from an ER marker (Figure 2). The catalytic domain appears to be luminal because low concentrations of Triton X-100 are required for detecting its activity (not shown).

We then constructed a null mutant of the *KlGDA1* gene to study the physiological function of the KlGda1p. The disrupted strain lost most (95%) of its ability to hydrolyze both GDP and UDP *in vitro*; hydrolysis of both GDP and UDP was restored on transformation of the deletion strain with a plasmid carrying either the *K. lactis* (not shown) or the *S. cerevisiae* gene (Table I). Golgi membrane extracts from *K. lactis* wild type showed preferential hydrolytic activity towards GDP compared to UDP in the presence of either Ca^{2+} or Mn^{2+} (Table I), although the latter ion stimulated the UDPase activity by approximately fivefold compared to calcium ions. Membranes from disrupted strains showed virtually no UDPase activity *in vitro* (Table I).

Translocation of uridine and guanosine nucleotide sugars into Golgi-enriched vesicles is reduced in Klgda1Δ

We had previously shown that *S. cerevisiae* Gda1p can hydrolize UDP *in vitro*, in addition to its principal substrate, GDP. No other nucleoside phosphates were substrates. To determine the function of this UDPase activity, we studied transport of both GDP-mannose- and uridine-containing nucleotide sugars into Golgi vesicles from wild-type and *Klgda1* null mutants. *K. lactis*, in contrast to *S. cerevisiae*, has N-acetylglucosamine bound to its mannan chains and UDP-GlcNAc must first be transported into the *K. lactis* Golgi lumen before the sugar can be transferred to the mannans, yielding also UDP as reaction product. This UDP must be converted to UMP before it can exit the Golgi lumen in a coupled exchange with the entry of (additional) UDP-GlcNAc.

If KlGda1p is also a UDPase *in vivo*, one would predict that the null mutant would have a reduction in transport of UDP-GlcNAc into *K. lactis* Golgi vesicles, in addition to that of GDP-mannose. As can be seen in Figure 3, transport of UDP-GlcNAc into the lumen of Golgi-enriched vesicles from *K. lactis Klgda1* null mutants was approximately half compared to that into wild-type vesicles. This reduction was seen in both solutes within vesicles as well as in sugars bound to macromolecules within the vesicles. Transport of GDP-mannose and UDP-glucose was also reduced (Figure 3). Reduction of UDP-glucose transport had not been observed in *S. cerevisiae gda1* null mutants (not shown), suggesting that *K. lactis* may have a UDP-glucose transport activity in both the Golgi apparatus and endoplasmic reticulum. In *S. cerevisiae* UDP-glucose enters primarily into the endoplasmic reticulum (Castro *et al.*, 1999), whereas Gda1p resides in the Golgi.

Inhibition of α1,2 mannosyltransferase and α1,2 N-acetylglucosaminyltransferase activities in Klgda1Δ protein extracts containing permeabilized Golgi vesicles

Once nucleotide sugars enter the lumen of the Golgi apparatus, glycosyltransferases catalyze the transfer of the sugars to endogenous acceptors; the other reaction products, nucleoside diphosphates, are converted by the Golgi GDPase/UDPase to the corresponding nucleoside monophosphates. These then exit the lumen in an antiport with uridine- and guanosine-nucleotide sugars. GDP and UDP are inhibitors of glycosyltransferases that use guanosine- and uridine-diphosphate sugars as substrates. One would therefore predict that in *Klgda1Δ permeabilized* Golgi vesicles, glycosyltransferases are inhibited compared to the corresponding activities in wild-type

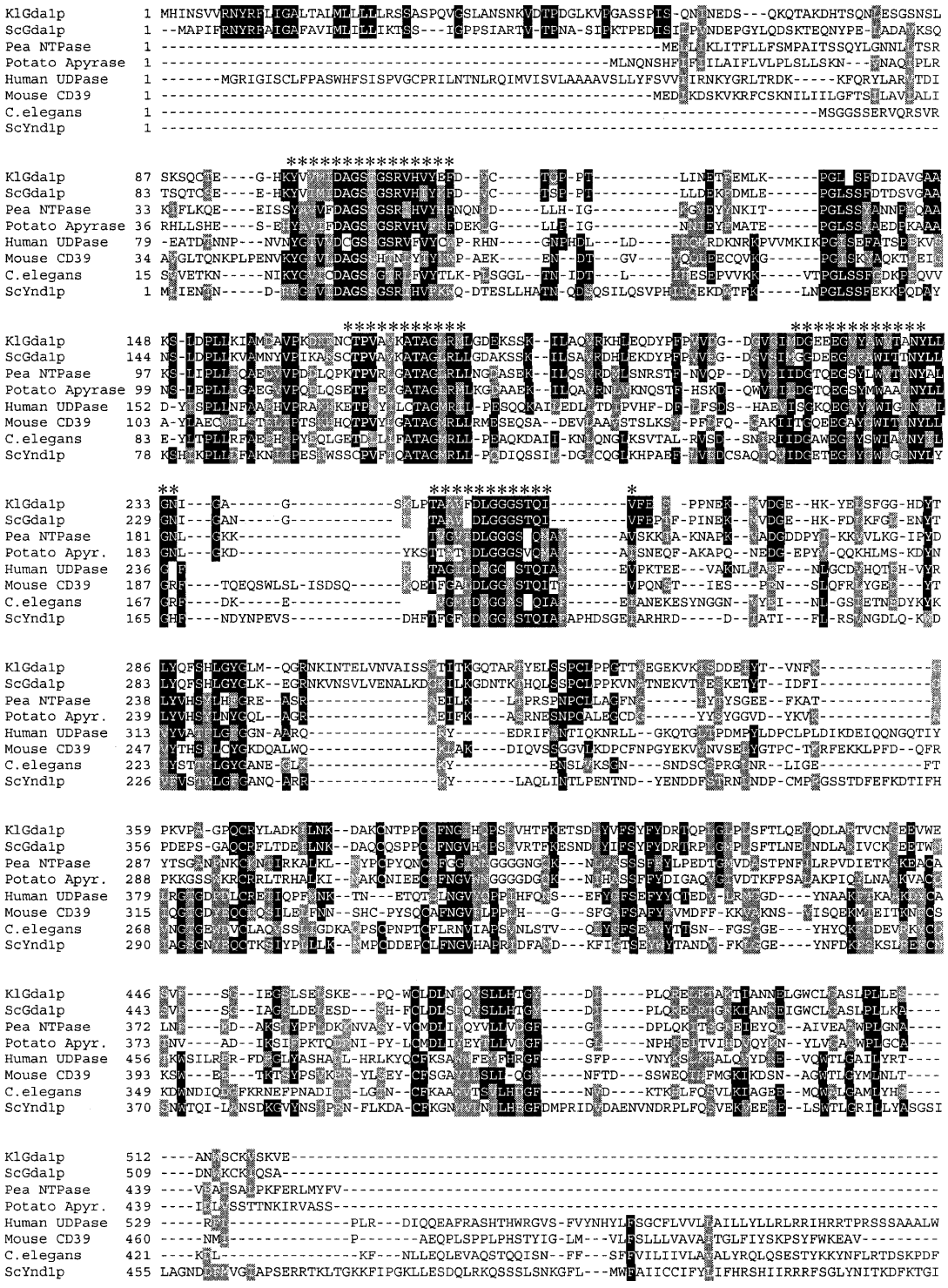

Fig. 1. Multiple alignment of proteins related to Klgda1p. The BOXSHADE program was used to compare sequences of proteins, from fungi to mammals. Black background shows identical residues and gray background shows conservative substitutions. Asterisk indicates conserved domains with putative apyrase activity. Accession numbers for the sequences used in the alignment are: *K. lactis* GDPase, AJ401304; *S. cerevisiae* GDPase, NP_010872; pea NTPase, BAA89275; potato ATPase, P80595; human CD39, NP_001767; mouse CD39, NP_033978; *C. elegans,* Q18411; *S. cerevisiae YND1,* NP_010920; human Golgi UDPase AF016032.

permeabilized vesicles. This is because the GDP generated as by-product following the transfer of mannose (from GDP-mannose) to the exogenous acceptor is accumulated in the incubation medium. Inhibition would not be seen in the wild-type extracts because there Gda1p hydrolyzes GDP, which is inhibitory, to GMP, which is not. As shown in Figure 4A, α1,2 mannosyltransferse activity was indeed reduced in Golgi membranes from null mutants compared to those from wild-type cells (column 1

Table I. Guanosine and uridine-diphosphatase activities in membranes of *K. lactis*

	KlGDA1		*Klgda1Δ*		*Klgda1Δ + [pScGDA1]*	
Substrate	Ca^{2+}	Mn^{2+}	Ca^{2+}	Mn^{2+}	Ca^{2+}	Mn^{2+}
GDP	100 ± 0.7	97 ± 0.6	4 ± 1.5	1 ± 0.7	100 ± 0.5	87 ± 1.9
UDP	4 ± 0.0	21 ± 0.2	0 ± 1.8	0 ± 0.9	2 ± 0.0	9 ± 1.1

Membranes of *K. lactis* wild type, *gda1Δ* and null mutants transformed with an S. cerevisiae GDA1 multicopy plasmid were assayed for hydrolysis of GDP and UDP in the presence of Ca^{2+} and Mn^{2+} as described in Materials and methods. Results are expressed as percentage of GDP hydrolysis of wild-type membranes in the presence of Ca^{2+} and are the mean of three independent experiments ± SE. The value for hydrolysis of GDP/Ca^{2+} in the wild type was 3.4 nmol phosphate/μg prot/20 min and 23.7 nmol phosphate/μg prot/20 min for *Klgda1Δ + [pScGDA1]*.

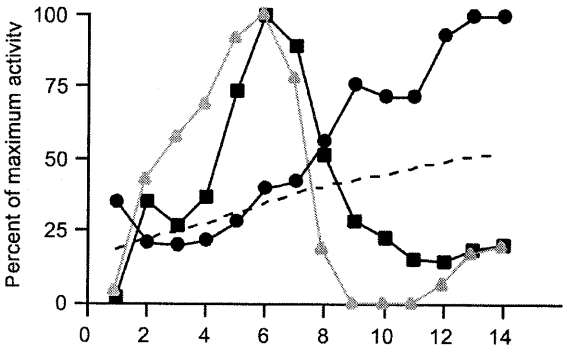

Fig. 2. The GDPase and UDPase Activities are in the Golgi apparatus. Enzyme activities were assayed after velocity sucrose gradient centrifugation. Cells were converted to spheroplasts and the 10,000 × *g* supernatant was loaded on to a 25–50% sucrose gradient. Fractions were collected from the top, diluted, and centrifuged at 100,000 × *g* for 40 min. Pellets were resuspended and used for the assays indicated. Black circles: endoplasmic reticulum marker: NADPH cytochrome C reductase. Black squares: Golgi marker: α1,2-mannosyltransferase. Gray triangles: GDPase activity. Dashed line: sucrose concentration.

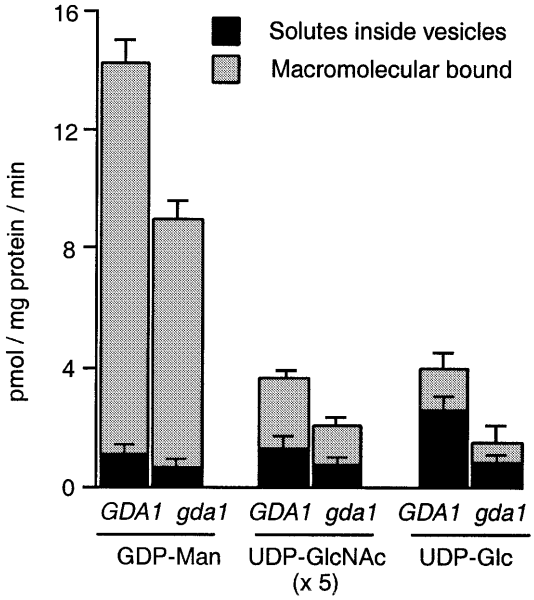

Fig. 3. Translocation of nucleotide sugars into Golgi vesicles. Vesicles from wild-type and *Klgda1* mutant cells were assayed for transport of GDP-Man, UDP-GlcNAc, and UDP-Glc at 30°C for 3 min. Black bars: solutes within vesicles; gray bars: sugars bound to macromolecules. Results are mean ± SE of three independent experiments.

versus 5). Moreover, this inhibition could be specifically overcome by addition of apyrase to the assay of extracts from *Klgda1Δ* (columns 6–8). As expected, addition of apyrase had no effect on wild type extracts (columns 2–4). These results demonstrate that the inhibition was caused by accumulation of GDP in the incubation medium of extracts from the mutant. One would also postulate that the above inhibition of glycosyltransferases by nucleoside diphosphates in *Klgda1Δ* Golgi membranes would be higher when increasing the concentration of nucleotide sugar substrates in the assay, because this would result in higher concentrations of nucleoside diphosphate by-products. This inhibition should, in turn, be overcome by membranes from *Klgda1Δ* cells expressing a plasmid copy of the *ScGDA1* gene. Figures 4B and 5 show that indeed this occurred and that the mannosyltransferase activity appeared to be more sensitive to inhibition by GDP than the α1,2 N-acetylglucosaminyltransferase activity to inhibition by UDP. These results suggest that in the absence of Golgi GDPase/UDPase activities, GDP and UDP generated from the glycosyltransferase reactions inhibit such enzymes and probably impair the correct glycosylation of macromolecules.

KlGDA1 has a role in O-glycosylation

To determine whether *KIGDA1* is the functional homolog of *GDA1* from *S. cerevisiae*, we compared the glycosylation phenotypes of *Klgda1Δ* cells with those already reported for *S. cerevisiae gda1* mutants. Using N-glycosylation reporters, such as invertase and carboxypeptidase Y, we found, to our surprise, no differences between the *K. lactis* wild type and null mutant, even though drastic differences in the behavior of the above proteins had been previously observed between *S. cerevisiae* wild type and *gda1* null mutants (Abeijon *et al.*, 1993). No differences in binding of GS II lectin to cell surfaces of wild-type and *Klgda1* cells were detected. Although *Klgda1Δ* cells did not display any defect in N-glycosylation, the *KlGDA1* gene complemented all the alterations in N-glycosylation of *S. cerevisiae gda1* null mutants, including CPY glycosylation, synthesis of outer mannan chains of cell wall mannoproteins and also lipid mannosylation (not shown).

We then used chitinase, a heavily O-glycosylated protein that is secreted into the media, as a probe for changes in O-glycosylation. As can be seen in Figure 6 lanes 4 and 5, chitinase from the null mutant migrated faster than that of wild-type cells. We had previously shown that in *S. cerevisiae gda1* null mutants this faster migration of chitinase on SDS gels was the result of decreased O-mannosylation (Abeijon *et al.*, 1993).

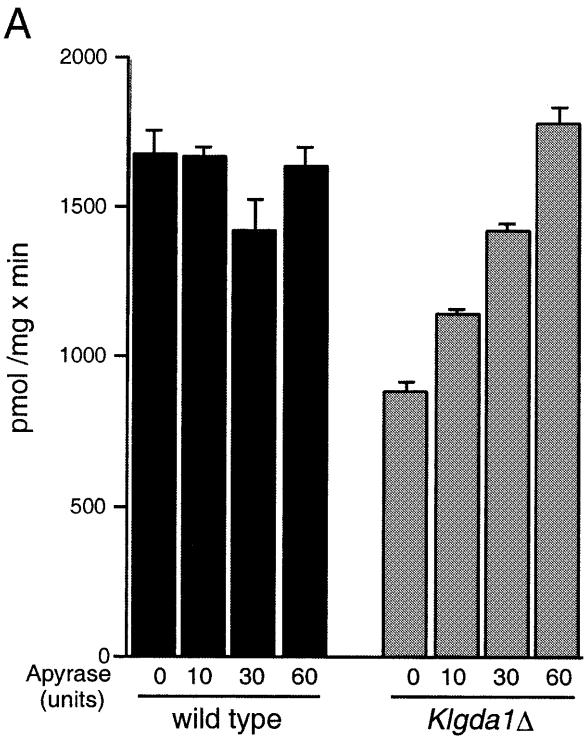

Fig. 4. αl, 2-mannosyltransferase activity is reduced in *Klgda1*Δ protein extracts containing permeabilized Golgi vesicles (**A**) Protein extracts containing wild-type or mutant permeabilized Golgi vesicles were assayed for mannose transfer to methyl αD- mannopyranoside in the presence of different concentrations of apyrase added to the incubation medium. (**B**) Effect of GDP-mannose concentration and cross-species gene complementation on α1,2 mannosyltransferase activity. Results are mean ± SE of three independent experiment.

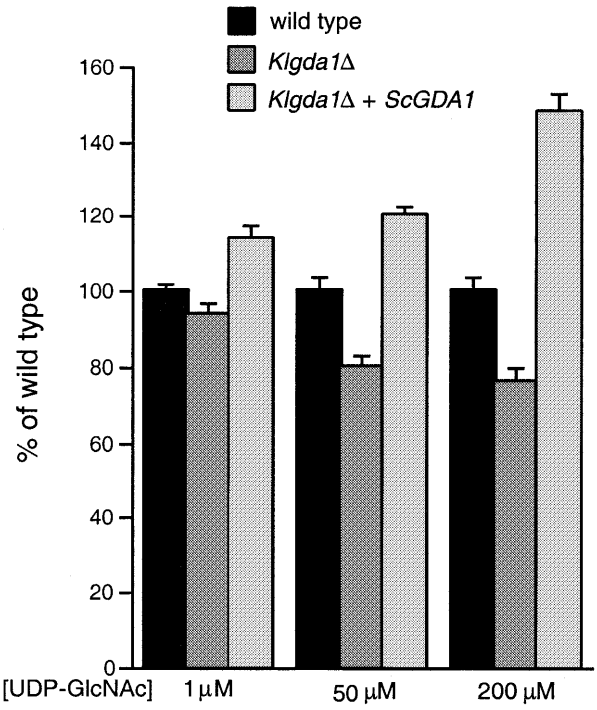

Fig. 5. Effect of UDP-GlcNAc concentration and cross-species complementation on α1,2-N-acetylglucosaminyltransferase activity. Transfer of GlcNac to the exogenous acceptor Man α1,3 Man was measured as described in Materials and methods. Results are mean ± SE of three independent experiments.

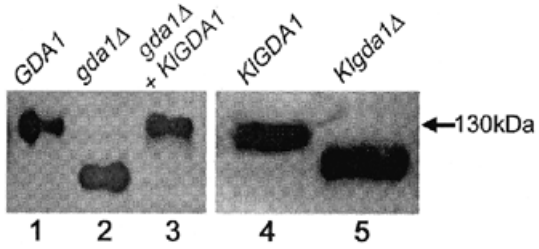

Fig. 6. Chitinase analysis of *K. lactis* and *S. cerevisiae* cells. Secreted chitinase was isolated by binding to chitin, separated by SDS–PAGE, and visualized by Western blots with antibodies against *S. cerevisiae* chitinase.

The same behavior was observed here (Figure 6 lanes 1 and 2). We also observed a slight increase of shorter mannose chains on β-elimination of total glycoproteins from the null mutant compared to wild-type cells (not shown). Finally, we tested the ability of a plasmid containing *KlGDA1* to correct the mannosylation defect of chitinase expressed by the *S. cerevisiae* null mutants. As can be seen in Figure 6, lane 3, *S. cerevisiae* chitinase regained its wild-type mobility, demonstrating functional homology between the *S. cerevisiae* and *K. lactis GDA1* genes.

KlGda1p has a role in cell wall morphogenesis

The yeast cell wall contains glucans and mannoproteins as major components, and chitin, a polymer of N-acetyl-glucosamine, is a minor one. The enzymes involved in the synthesis of these polymers, as those of mannoproteins, either

travel to the plasma membrane via secretory vesicles or are localized in organelles of the secretory pathway, such as the Golgi apparatus. We therefore determined whether deletion of *KlGDA1* had an effect of cell wall strength by measuring sensitivity toward cell lysis following β-1,3 glucanase (Zymolyase™) treatment. As can be seen in Figure 7A and B, null mutant cells were more sensitive toward lysis following Zymolyase™ compared to wild-type cells; this sensitivity was even more pronounced in cultures grown at 37°C than those grown at 23°C. When cells were grown at 37°C the sensitivity toward lysis of the mutant increased slightly, whereas that of the wild-type cells decreased. Indeed, after 2 min of incubation with Zymolyase™ only 60% of the mutant cells grown at 37°C were resistant to lysis compared to 80% of those grown at 23°C. Cell walls from wild-type cells were considerably more resistant to Zymolyase™ treatment than mutants grown at either temperature (Figure 7A,B).

The above-described cell wall fragility had not been examined in the *S. cerevisiae gda1* null mutants. We therefore compared the strength of the cell wall in *S. cerevisiae* wild-type and *gda1* cells. Surprisingly, we found a behavior opposite to that of *K. lactis*. The cell wall of *S. cerevisiae gda1* mutants was more resistant to lysis following Zymolyase™ treatment than that of wild-type cells grown at either 23°C or 37°C. Mutant cells grown at 37°C showed an even higher resistance toward lysis than those grown at 23°C (not shown).

The different lysis behavior of cells following Zymolyase™ treatment suggested differences in cell wall composition. To test this hypothesis, alkali-insoluble glucans were prepared from cell walls of wild-type and *gda1* null mutants of *S. cerevisiae* and *K. lactis*. Deletion of *GDA1* in *K. lactis* resulted in an increase of cell wall β-1,3 glucans, relative to wild type, in cells grown at both 23°C and 37°C. A more robust change was seen at the latter temperature (Figure 8, top). An increase of β-1,6 glucans was also observed in *K. lactis gda1* mutants, but only when grown at 37°C. In *S. cerevisiae*, contrary to *K. lactis*, null mutants did not show an increase of β-1,3 glucans but a decrease; this was more pronounced in cells grown at 23°C than 37°C and was also observed to a lesser extent for β-1,6 glucans. Chitin, another cell wall component, was not changed in the *K. lactis gda1* null mutant but showed a twofold increase in the corresponding mutant of *S. cerevisiae* (Figure 9).

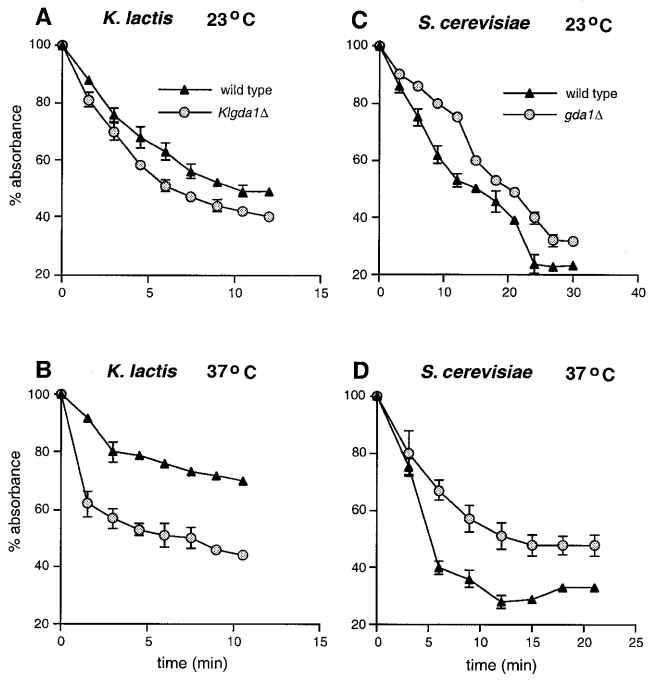

Fig. 7. *K. lactis* and *S. cerevisiae* cell lysis sensitivity following treatment with Zymolyase™. Cells were treated with zymolyase for different times and lysis was measured by the decrease in OD_{660} after osmotic shock. Results are mean ± SE of three independent experiments.

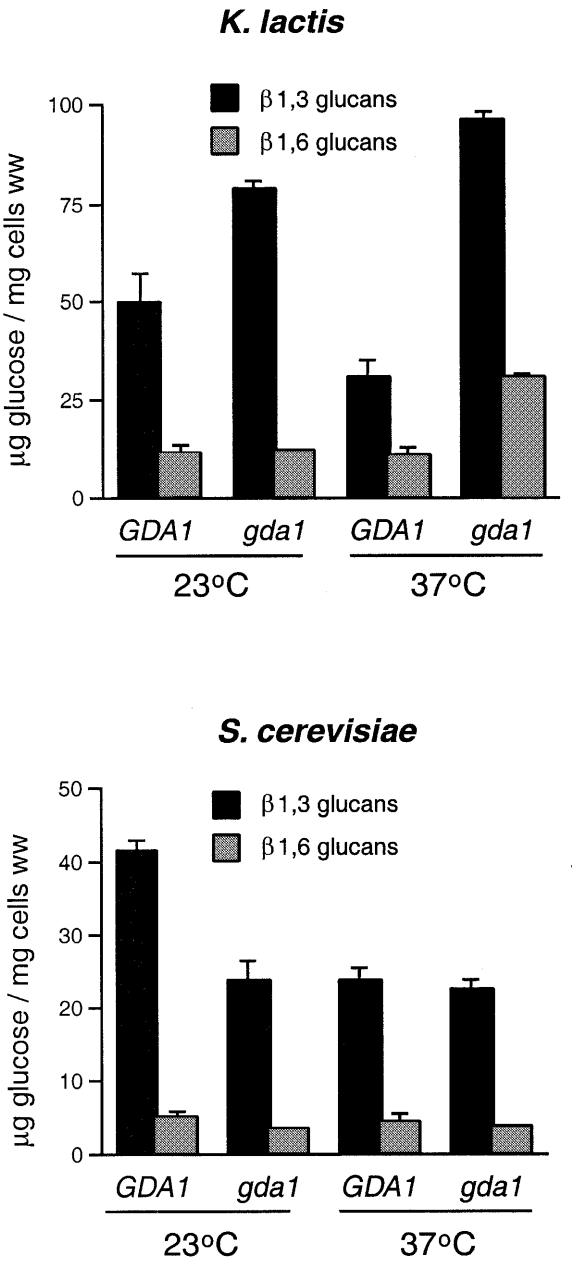

Fig. 8. Cell wall glucans of *K. lactis* and *S. cerevisiae.* Cell wall β1,3- and β1,6-glucans were prepared and measured as described in Materials and methods. Results are mean ± SE of three independent experiments.

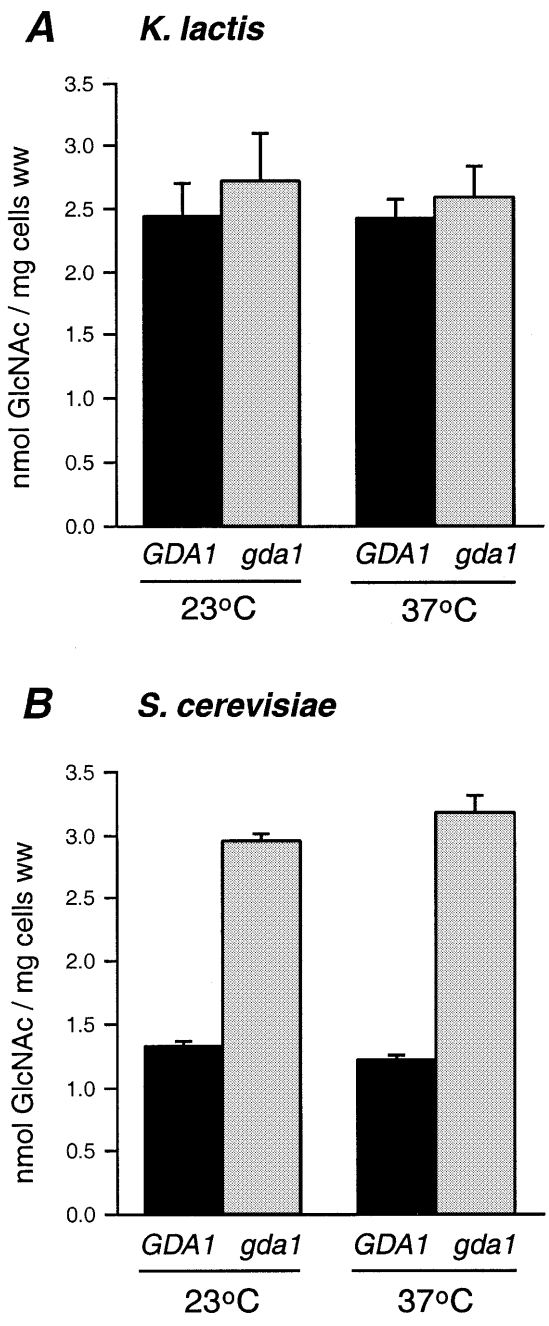

Fig. 9. Chitin content of *K. lactis* and *S. cerevisiae.* Chitin content of cells was measured as described in Materials and methods. Results are mean ± SE of three independent experiments.

Discussion

The previous studies of the *S. cerevisiae* Golgi GDPase have provided strong evidence for the role of this enzyme in the nucleotide sugar transporter/antiport cycle. Evidence that this cycle has a pivotal function in regulating those postranslational modifications that occur in the lumen of the Golgi apparatus and affects approximately 80% of all secreted and membrane proteins in eukaryotes is beginning to emerge. Several important and novel conclusions were obtained from the results presented here: (1) *K. lactis* has a GDPase/UDPase, KlGda1p, that corrects *S. cerevisiae* N-and O-mannosylation defects *in vivo*; in *K. lactis* it only affects O-mannosylation *in vivo*. (2) The UDPase activity of KlGda1p has a role in transport of uridine nucleotide sugars into Golgi vesicles *in vitro* and in generating UMP; it thereby overcomes the inhibition by UDP of those glycosyltransferases that use uridine nucleotide sugars as substrates. These studies could not be performed in *S. cerevisiae* because no reactions using uridine nucleotide sugar substrates are known to occur in its Golgi apparatus. (3) The GDPase activity of *K. lactis* Gda1p is required, as that from *S.cerevisiae*, for efficient transport of GDP-mannose into the Golgi lumen.

Deletion of *KlGDA1* resulted in some residual GDPase activity in the *K. lactis* membranes, while almost no remaining activity had been detected in membranes of *S. cerevisiae gda1* null mutants (Abeijon *et al.*, 1993). Even though a very low nucleoside diphosphatase activity can be measured *in vitro* in *S. cerevisiae gda1* null mutants, another apyrase, Ynd1p, has recently been identified and cloned in this organism (Guillen *et al.*, 1999). The substrate specificity of Ynd1p is broader than that of Gda1p, but their function is apparently partially redundant because the double mutant *gda1*Δ *ynd1*Δ has a more severe glycosylation phenotype than any of the individual mutants (Guillen *et al.*, 1999). Ynd1p apyrase activity in the Golgi lumen has been shown to have a very narrow optimal pH, between 7.5 and 8, and to be down-regulated by direct binding of its cytosolic domain to the activator subunit Vma13p of the vacuolar H^+ ATPase (Zhong *et al.*, 2000). Most likely, *K. lactis* has at least one other nucleoside diphosphatase or apyrase that generates nucleoside monophosphates in its Golgi lumen. This could explain why N-glycosylation was not affected in *KlGDA1* mutants.

The possibility of multiple proteins in the Golgi lumen giving rise to nucleoside monophosphates is particularly relevant to mammalian Golgi membranes where evidence already suggests that more that one protein with nucleoside diphosphatase activity may reside (Wang and Guidotti, 1998). These observations, in addition to results of this study, strongly suggest that there is regulation of glycosylation also at the level of the production of the antiporter molecules involved in the nucleotide sugar transport cycle in higher and lower eukaryotes.

These studies are the first to show a role for *GDA1* in yeast cell wall morphogenesis. Surprisingly, despite of the amino acid sequence similarity between *ScGDA1* and *KlGDA1* and their functional homology, deletion of each gene had very different effects on osmotic stability and cell wall polymer composition of both null mutant strains compared to each other and to their respective wild types. These differences were even more accentuated when the cells were grown at 37°C instead of 23°C. Thus, *K. lactis gda1* null mutants were more sensitive toward β-1,3 glucanase–induced cell lysis than their wild type possibly because they had increased cell wall β-1,3 glucans at both temperatures. β-1,6 glucans were increased only when mutants were grown at 37°C, and chitin was unchanged at both growth temperatures. In marked contrast, *S. cerevisiae* deletion mutants were more resistant toward β-1,3 glucanase–induced cell lysis than wild type at both temperatures. This behavior may be a consequence of increased cell wall chitin content found in *S. cerevisiae* mutants grown at both temperatures. Chitin fibers may give strength to the wall and make it more

resistant to osmotic shock, even in the presence of a decrease in β-1,3 glucans. The changes found in the cell wall of *S. cerevisiae gda1*Δ mutants are very similar to those reported on inactivation of the *KNR4* gene (Kapteyn *et al.*, 1999). This gene appears to encode a cytoplasmic protein that is part of a regulatory complex involved in the assembly of the cell wall (Martin *et al.*, 1999).

Although the mechanism underlying the above changes in cell wall polymers in *GDA1* null mutants from both yeast species are not clearly understood, they may be related to changes in the O-glycosylation pathway. Recently, a highly O-glycosylated cell wall protein family that is not anchored to the wall by the clasical glycosylphosphatidylinositol anchor PIR-CWP was characterized (Toh-e *et al.*, 1993; Mrsa and Tanner, 1999). These proteins are thought to play a compensatory role when cell walls are weakened; O-linked sugars may be necessary to link these proteins to β-1,3-glucans (Ketela *et al.*, 1999).Ccw11p, an abundant PIR-related protein, is absent in cell walls of a *pmt4*Δ strain, which is known to be defective in O-glycosylation (Mrsa *et al.*, 1997). Two potential sensors of cell wall stress, Hkr1p and Mid2p, are exclusively O-glycosylated (Yabe *et al.*, 1996; Kuranda and Robbins , 1991) and may also play a role in regulating the different compensatory changes occurring in the wall of *K. lactis* and *S. cerevisiae*. The direct targets of these modifications will be subject of further studies and may provide insights into the molecular basis of the organization and dynamics of the yeast cell wall.

Materials and methods

Strains, media, and reagents

K. lactis strain KL8 (MG 1/2, *MAT* α, *ura A*, *arg-*, *lys-*, K^+, $pKD1^+$) was obtained from C. Falcone. *S. cerevisiae* strains, G2-10 (*MAT* α, *ura3-52*, *lys2-801 am*, *ade2-101 oc,trp1-Δ1, his3-Δ200, leu2-Δ1*) and G2-11 (*MAT* α, *ura3-52*, *lys2-801 am*, *ade2-101 oc*, *trp1-Δ1*, *his3-Δ200*, *leu2-Δ1, gda1::LEU2*) were described previously (Abeijon *et al.*, 1993). Cells were grown in YPD or SD medium supplemented with 1% casamino acids. Solid media were made by adding 2% agar to the liquid stock. 5-Fluoro-orotic acid was added when indicated to a final concentration of 0.1%. Yeast cells were grown at 30°C. Transformations with plasmids were done by electroporation (Abeijon *et al.*, 1996b). *Escherichia coli* strain DH5α (Gibco BRL) was grown in LB medium with 50 μg/ml ampicillin when needed. Reagents for media were from Difco Laboratories (Detroit, MI). Unless otherwise stated, all other reagents were from Sigma-Aldrich (St. Louis, MO). GDP-[^{3}H]mannose (15 Ci/mmol), UDP-[^{3}H]glucose (11.5 Ci/mmol), and UDP- [^{3}H]N-acetyl-D-glucosamine (30 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA).

Isolation of KlGDA1 gene

A *K. lactis* genomic library previously made in our laboratory (Abeijon *et al.*, 1996a) was screened at low stringency (Maniatis *et al.*, 1997) using the open reading frame (ORF) of the *S. cerevisiae GDA1* gene as probe (Abeijon *et al.*, 1993). A positive clone, pDL-12, caused threefold overexpression of GDPase activity when transformed into *K. lactis*. Restriction analysis and sequencing of the 11.5-kb insert demonstrated a complete ORF of 1.5 kb that was homologous to the *S. cerevisiae GDA1* gene. The whole ORF was located within a 1.9-kb HindIII fragment that was subcloned into the HindIII site of Kep6 to obtain pDL-13. The Kep6 vector has a segment of pKD1 that carries the replication origin of the 1.6-μm plasmid (Bianchi *et al.*, 1987). Stable replication of this vector requires the presence of resident pKD1 in the recipient yeast cell. The copy number and stability of this plasmid is comparable to the 2-μm plasmid–derived vectors of *S. cerevisiae*. Kep6 also has the *URA3* gene of *S. cerevisiae* that complements the *uraA* mutation of *K. lactis*; it also contains pBR322 sequences to allow selection and amplification in *E. coli.* The complete sequence of the 1.9-kb genomic HindIII fragment containing the *KlGDA1* gene was deposited in the EMBL Nucleotide Sequence Database with the accession number AJ401304.

Construction of Klgda1 null mutant strain, KL8Δ

Inactivation of the *KlGDA1* gene was achieved by the *URA* blaster protocol (Alani *et al.*, 1987). To obtain the disruption plasmid, pBlueKlhUh, the 1.9-kb HindIII fragment containing the ORF was cloned into the HindIII site of pBluescript (Stratagene, La Jolla, CA) whose BamHI site had been previously removed. An internal 0.9-kb BamHI fragment from the ORF was replaced with a 3.8-kb BamHI–BamHl fragment from plasmid pNKY51 (kindly provided by N. Kleckner) that contained the *URA3* gene flanked by hisG direct repeats. Recombination between the flanking direct repeats results in the elimination of the *URA3* gene and recovery of uracil auxotrophy. Digestion of pBlueKlhUh with Xhol and EcoRl released a linear fragment containing the disruption cassette that was introduced in *K. lactis* strain K18 by electroporation. DNA from transformants was used to confirm the disruption of the *KlGDA1* gene by polymerase chain reaction (PCR) analysis. The following primers were generated for this purpose: Forward primer 5′-CAAAAGCAAACAGCCAAA-GACC-3′ (located at position + 198). Reverse primer 5′-CGTC-TTTGCTGTTCTCAACTCTC-3′ (located at position +1475). These primers amplified a 1.2-kb PCR product in wild-type cells; in mutant cells a product of 4.3 kb was obtained. PCR of genomic DNA from mutant cells after the pop-out of the hisG–URA–hisG sequence resulted in a product of 1.6 kb. Correct integration was found in 1% of the clones and was further confirmed by assaying for reduced GDPase/UDPase activity in membrane protein extracts.

Construction of plasmids pKEpSC and pYEpKl for cross-species complementation

To express *GDA1* and *KlGDA1* in *K. lactis* and *S. cerevisiae*, we constructed the following plasmids. A 2.2-kb HindIII–NheI fragment containing the *S. cerevisiae* gene was cloned into Kep6 previously opened with HindIII and NheI to generate the *K. lactis* plasmid pKEpSc. Similarly, a 1.9-kb HindIII fragment containing the *KlGDA1* gene was cloned into the HindIII site of the *S. cerevisiae* plasmid Yep 352 to generate plasmid pYEpKl. *K. lactis* and *S. cerevisiae* null mutants were then transformed with pKEpSc and pYEpKl, respectively.

Preparation of vesicle fractions and sucrose gradient fractionation

Vesicle fractions were prepared from cells (6 L), grown to a density of 0.8 OD_{600}. After washing with cold 10 mM sodium azide as previously described (Abeijon *et al.*, 1993), cells were

resuspended in spheroplast buffer (50 mM potassium phosphate, pH 7.5, 1.4 M sorbitol, 10 mM sodium azide, 0.3% β-mercaptoethanol) containing 0.5 mg/ml of Zymolyase 100T (Seikagaku Co., Tokyo) and incubated at 37°C for 40 min. The spheroplast suspension was centrifuged and the pellet was washed with membrane buffer (10 mM triethanolamine acetic acid, pH 7.2, 0.8 M sorbitol, 1 mM EDTA, 1 μg/ml leupeptin, 0.5 mM PMSF) and resuspended in the same buffer. Spheroplasts were then broken by passage through a narrow-bore 10-ml glass pipette, diluted in membrane buffer, and centrifuged at 1000 × *g* for 10 min to obtain P1. The supernatant was centrifuged to 10,000 × *g* for 20 min to obtain P2, and the resulting supernatant was again centrifuged at 100,000 × *g* for 30 min to obtain P3. This fraction is enriched in Golgi markers, such as GDPase and α1,2 mannosyltransferase, and was used for nucleotide sugar transport assays. Vesicles were kept frozen at –70°C. Protein was measured using the BCA method (Bio-Rad, Hercules, CA).

For sucrose velocity gradient fractionation experiments, 1-L cultures were used. Speroplasts were prepared as described above and suspended in membrane buffer. The P2 supernatant fraction was prepared and the final concentration of $MgCl_2$ was adjusted to 1 mM. Aliquots (9 ml) were placed on top of two 30-ml preformed, 25–50% continuous sucrose gradients in Beckman SW28 centrifuge tubes. The sucrose solutions contained 1 mM $MgCl_2$ and 10 mM triethanolamine acetic acid, pH 7.2. Gradients were centrifuged at 4°C for 90 min, at 25,000 r.p.m. in an L8-90 Beckman Ultracentrifuge as described previously (Abeijon *et al.*, 1993). Twelve 2.5-ml fractions were collected from the top of each gradient, diluted, and centrifuged at 100,000 × *g* for 30 min; pellets were resuspended in membrane buffer.

Nucleotide diphosphate hydrolysis assay

Hydrolysis of GDP, UDP, and ADP was measured in vesicle fractions as previously described (Yanagisawa *et al.*, 1990) in buffer containing 0.2 M imidazole, pH 7.5, 10 mM $CaC1_2$ or 10 mM $MnC1_2$, 0.1 % Triton X-100, and 2 mM GDP, UDP, or ADP. One hundred microliters of this solution, containing 5–10 μg of sample protein, were incubated at 30°C for 20–30 min. The reaction was stopped by transferring the tubes to ice and adding 10 μl of 10% SDS. To determine the amount of phosphate released during hydrolysis, 200 μl of water and 700 μl of AMES reagent (1:6 mixture of 10% ascorbic acid and 0.42% ammonium molybdate in 1 N sulfuric acid) were added to each tube; following incubation at 40°C for 20 min, absorbance was measured at 660 nm.

α1,2-N-acetylglucosaminyltransferase and α1,2-mannosyltransferase assays

Reactions were performed in a 50 μl final volume containing 30 μg of membrane protein, 50 mM HEPES, pH 7.2, 0.1% Triton X-100, 10 mM $MnCl_2$, and 10–200 μM GDP-[^{3}H]mannose (0.1 μCi) or UDP-[^{3}H]GlcNAc (0.1 μCi). As exogenous acceptors, 10 mM methyl αD-mannopyranoside or 0.5 mM 3-O-α-D-mannopyranosyl-D-mannopyranoside were added for mannosyltransferase and GlcNAc transferase reactions, respectively. After, incubations at 30°C for 25 min, reactions were stopped by adding 0.4 ml of 10 mM EDTA. Radioactive substrates were separated from acceptors by binding of the former to a 1-ml Dowex-1 column; the radioactivity in the eluate, containing the acceptor, was measured. Between 10 and 60 units of potato apyrase (Grade VII, Sigma) were added to the reaction mixture when needed.

Nucleotide sugar translocation assay

The theoretical basis for the translocation assay of nucleotide derivatives into vesicles has been described in detail (Perez and Hirschberg, 1986). Briefly, Golgi-enriched vesicles (P3 fraction, 0.25–0.5 mg protein) were incubated at 30°C or 0°C for 3 min with the corresponding radioactive nucleotide sugar in the following 1-ml reaction mixture: 10 mM Tris–HCl (pH 7.4), 0.25 M sucrose, 0.15 KCl, 1 mM $CaC1_2$, and 5 mM $MgC1_2$. Parallel incubations were done with the standard vesicle nonpenetrator [^{3}H] acetate. The reaction was stopped with 2 ml of ice-cold 0.25 M sucrose, 1 mM EDTA. Vesicles were separated from the incubation medium by centrifugation at 100,000 × *g* for 40 min. Total acid-soluble radioactivity, S_t, associated with the washed vesicle pellets was determined. The amount of radioactivity within vesicles in the pellet, S_i, was calculated by multiplying the concentration of solutes in the incubation medium times the volume outside the vesicles in the pellet, V_o, of the nonpenetrator [^{3}H] acetate (1.9 μl/mg protein). Transport activity is defined as S_i after incubations at 30°C minus S_i after incubations at 0°C. Latency of the vesicles was determined by measuring the lumenal Golgi GDPase activity, as described above, in the presence and absence of Triton X-100; it was 90% or higher in every instance. For *gda1*Δ strains, latency of N-actetylglucosamiyltransferase was measured.

Zymolyase sensitivity assay

Cells were grown to exponential phase at 23°C or 37°C. 5 × 10^8 cells were resuspended in 4 ml of buffered sorbitol (20 mM Tris–HCl, pH 7.2, 1.2 M sorbitol, 10 mM $MgCl_2$) with 3% 2-mercaptoethanol. After 10 min, 1 ml of 1 mg/ml Zymolyase 100T was added, and the cells were incubated at 23°C. Lysis was determined by measurements of A_{660} after 1:10 dilution in water of samples taken at 1.5-min intervals for *K. lactis* and 3-min intervals for *S. cerevisiae*, due to their different cell wall fragility.

Cell wall analysis

Cells were harvested and walls were isolated after mechanical disruption with glass beads (0.45–0.55 mm diameter) (as described in Perez and Hirschberg, 1986). Total alkali-insoluble fractions were obtained (Nguyen *et al.*, 1998) and β-1,6/β-1,3 glucan content was measured as described (Boone *et al.*, 1990). Hexose content was determined before and after dialysis using the borosulphuric acid method (Roemer and Bussey, 1991). Chitin was determined according to Bulawa *et al.* (1986).

Chitinase analysis

Native chitinase was purified from late exponential cultures of *K. lactis* and *S. cerevisiae* mutant and wild-type cells grown in YPD. The *S. cerevisiae* mutant strain carrying the *KlGDA1* gene was grown overnight in minimal medium and shifted to YPD for three generations. Chitinase was isolated by its binding to chitin as described (Maniatis, 1997). Proteins were then subjected to 6% SDS–PAGE and detected by Western blot using a rabbit antiserum against chitinase (kindly provided by Dr. Charles Specht) and anti-rabbit IgG conjugated to

peroxidase (Promega, Madison, WI). Final visualization was done with the Renaissance chemiluminescence detection kit (NEN Life Science, Boston, MA) according to the manufacturer's instructions.

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Abbreviations

E-ATPase, ectoapyrase; ER, endoplasmic reticulum; ORF, open reading frame; PCR, polymerase chain reaction.

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