The High Osmolarity Glycerol Response (HOG) MAP Kinase Pathway Controls Localization of a Yeast Golgi Glycosyltransferase

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Abstract. The yeast α-1,3-mannosyltransferase (Mnn1p) is localized to the Golgi by independent transmembrane and lumenal domain signals. The lumenal domain is localized to the Golgi complex when expressed as a soluble form (Mnn1-s) by exchange of its transmembrane domain for a cleavable signal sequence (Graham, T. R., and V. A. Krasnov. 1995. *Mol. Biol. Cell*. 6:809–824). Mutants that failed to retain the lumenal domain in the Golgi complex, called lumenal domain retention (*ldr*) mutants, were isolated by screening mutagenized yeast colonies for those that secreted Mnn1-s. Two genes were identified by this screen, *HOG1*, a gene encoding a mitogen-activated protein kinase (MAPK) that functions in the high osmolarity glycerol (HOG) pathway, and *LDR1*. We have found that basal signaling through the HOG pathway is required to localize Mnn1-s to the Golgi in standard osmotic conditions. Mutations in *HOG1* and *LDR1* also perturb localization of intact Mnn1p, resulting in its loss from early Golgi compartments and a concomitant increase of Mnn1p in later Golgi compartments.

Key words: *HOG1* • MAP kinase • Golgi localization • glycosyltransferase • *MNN1*

THE Golgi complex is at the center of the eukaryotic secretory pathway. It is responsible for sorting proteins that are to be secreted from the cell, transported forward (anterograde) to the plasma membrane, endosome, or lysosome/vacuole and backward (retrograde) to the ER. In addition to its central role in protein sorting, the Golgi complex contains an array of glycosyltransferases that elongate the core O- and N-linked carbohydrate structures added to glycoproteins in the ER. It also possesses proteases that cleave specific proteins as they pass through this organelle. These different Golgi enzymes are enriched in distinct subcompartments, referred to as the *cis*, *medial*, *trans*, and TGN that together compose the Golgi complex (Farquhar and Palade, 1998). Although the cisternae that compose the Golgi complex of *S. cerevisiae* are not arranged in an ordered stack as seen in electron micrographs of the Golgi complex in higher eukaryotes, the yeast Golgi is functionally ordered in a similar manner. Like the mammalian Golgi, transport of proteins through the yeast Golgi can be followed by observing the posttranslational modification of cargo proteins, which include N- and O-linked carbohydrate additions as well as proteolytic cleavage events. A model for the compartmental organization of the yeast Golgi complex based on a functional analysis of posttranslational modification events is shown in Fig. 1 *A* (Gaynor et al., 1994; Graham and Emr, 1991).

All known Golgi glycosyltransferases are type II integral membrane proteins (Kleene and Berger, 1993). Whereas no conserved Golgi localization signal has been identified for these enzymes, the transmembrane domains of several mammalian Golgi glycosyltransferases have been shown to localize reporter proteins to the Golgi complex (reviewed in Munro, 1998). More recently, the lumenal domains of a few of these proteins have been implicated in localization (Munro, 1998). However, the mechanism by which these sorting signals mediate retention is not clear, and very little information exists as to the identity of *trans*-acting factors that recognize these signals and localize glycosyltransferases to the Golgi. In addition, mechanisms by which the cell might regulate the distribution of Golgi glycosyltransferases within this organelle, perhaps to modulate the composition of carbohydrates on glycoproteins, glycolipids, or other carbohydrate polymers have not been described.

The yeast Golgi enzyme, α-1,3-mannosyltransferase (Mnn1p)[1] catalyzes the addition of terminal α-1,3-man-

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1. *Abbreviations used in this paper:* CPY, carboxypeptidase Y; ECL, enhanced chemiluminescent; EMS, ethylmethanesulfonate; HOG, high osmolarity glycerol response; *ldr*, lumenal domain retention; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinases; Mnn1p, α-1,3-mannosyltransferase; PGK, phosphoglycerate kinase; PKC, protein kinase C; SD, synthetic defined.

nose residues to N- and O-linked oligosaccharides (Graham et al., 1992; Yip et al., 1994), an event that marks the yeast medial or *trans*-Golgi (Graham and Emr, 1991; Fig. 1 *A*). Mnn1p has two Golgi localization domains that function independently of one another. The signal anchor domain of this type II protein serves as one localization signal. A fusion protein made with the Mnn1p transmembrane domain fused to the secreted enzyme invertase (see Fig. 1 *B*, *M39I*) is localized to the Golgi complex. Like Mnn1p, localization of M39I is clathrin dependent, as M39I is mislocalized to the plasma membrane of cells carrying a deletion of the clathrin heavy chain gene (*chc1*Δ; Graham and Krasnov, 1995; Graham et al., 1994). The Mnn1p lumenal domain also contains an independent localization signal. The Mnn1p signal-anchor and cytoplasmic tail domains were replaced with a cleavable signal peptide such that a truncated protein (see Fig. 1 *B*, *Mnn1-s*) that contained no amino acids in common with M39I was produced. Mnn1-s localized to the Golgi in a soluble, catalytically active form. Mnn1-s localization is saturable as the lumenal domain was secreted when overexpressed from a multi-copy plasmid. This differed from the transmembrane domain signal in that overexpression of M39I did not result in mislocalization. These observations suggested that these two domains mediate Mnn1p localization to the Golgi by separate mechanisms (Graham and Krasnov, 1995).

A screen for mutants that fail to correctly localize Mnn1-s to the Golgi and subsequently secrete it has resulted in the isolation of six lumenal domain retention (*ldr*) mutants. A genomic clone that complemented one of the *ldr* mutants was isolated and was found to be identical to *HOG1*, a mitogen-activated protein kinase (MAPK) family member involved in a signal transduction cascade that responds to high osmotic stress known as the HOG (high osmolarity glycerol response) pathway (Brewster et al., 1993; Wurgler-Murphy and Saito, 1997). We have further found that the other members of the HOG signal transduction cascade are involved in localizing Mnn1-s to the Golgi in nonosmotically stressed cells, implicating a role for this pathway in transducing signals that regulate the localization of *trans*-Golgi cisternal proteins in standard osmotic conditions used to culture yeast.

Materials and Methods

Strain Construction and Media

Strains used in this report are either shown in Table I or are described below. TRY1 through TRY6 are isolates from a mutagenized population of SEY6210 pMNN1-s and SEY6211 pMNN1-s. TRY1 and TRY6 are in the SEY6211 background and TRY2 through TRY5 are in the SEY6210 background. TRYxxx derivatives of the original mutants were made by backcrossing the parental mutant strain with SEY6210 or SEY6211 (Table I). The *vps1*^{ts}, *vps18*Δ, *vps21*Δ, *vps24*Δ, *vps33*Δ, and *vps35*Δ strains are in the SEY6210 genetic background (S. Emr).

SEY6210 *pmr1*::*LEU2* was constructed as described (Rudolph et al., 1989) by transforming SEY6210 with the 8.1-kb AatII fragment from pL119-3 containing the *LEU2* marked disruption of the *PMR1* gene. SEY6210 *hog1*::*LEU2* strain was constructed as described (Hall et al., 1996) by transforming SEY6210 with the SalI- and BamHI-digested pDHG14 containing the *LEU2* marked *hog1*Δ allele. SEY6210 *sho1*::*LEU2* was constructed by transforming SEY6210 with the BamHI- and XbaI-digested pSHO1::LEU2 plasmid (M. Gustin, Rice University, Houston, TX).

To perform linkage analysis of the mutations carried in derivatives of strains TRY2 through TRY6, we integrated the *MNN1-s* gene into the

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
SEY6210	*MAT* α *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson et al., 1988
SEY6211	*MAT* **a** *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9*	Robinson et al., 1988
TGY122	*MAT* α *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mnn1*::*LEU2*	This work
TGY381	*MAT* α *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 suc2-Δ9 ret1-1*	This work
TGY383	*MAT* α *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 suc2-Δ9 RET1*	This work
FY250	*mal GAL2+ ura3-52 his3Δ200 leu2Δ1 trp1Δ63 hog1*::*URA3*	Roeder, 1998
AMY36	*mal GAL2+ ura3-52 his3Δ200 leu2Δ1 trp1Δ63 HOG1*	Roeder, 1998
W303 α	*MAT* α *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 SUC2 GAL mal0*	Albertyn et al., 1994
W303 *ssk2*Δ *ssk22*Δ *sho1*Δ	*MAT* α *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 SUC2 GAL mal0 ssk2*::*LEU2 ssk22*::*LEU2 sho1*::*LEU2*	M. Gustin
W303 *ssk1*Δ	*MAT* **a** *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 SUC2 GAL mal0 ssk1*::*LEU2*	M. Gustin
L3865	*MAT* α *ade2 his3Δ200 leu2-3, 112 lys2Δ201 ura3-52 pmr1-1*::*HIS3*	Antebi and Fink, 1992
GPY1103	*MAT* **a** *leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-Δ8*::*LEU2*	G. Payne
LB1-16A	*MAT* α *mnn2 SUC2 mal gal2 CUP1*	Sipos et al., 1995
LB65-5D	*MAT* **a** *mnn5 SUC2 mal gal2 CUP1*	Sipos et al., 1995
JSY2092	*MAT* α *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pbs2*::*KANMX2*	Roeder, 1998
6210 *arf1*Δ	*MAT* α *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 arf1*::*HIS3*	Gaynor et al., 1998
TRY120	*MAT* **a** *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 his1-11*	This work
TRY121	*MAT* α *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 hog1-11*	This work
TRY220	*MAT* **a** *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 ldr1-2*	This work
TRY320	*MAT* **a** *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 ldr1-3*	This work
TRY321	*MAT* α *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ldr1-3*	This work
TRY410	*MAT* **a** *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 ldr1-4*	This work
TRY520	*MAT* **a** *ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 ldr1-5*	This work

chromosome of SEY6210 (to produce MLY1) by transforming SEY6210 with pRS304-MNN1-s linearized at the SmaI site. Derivatives of TRY2 through TRY6 were then crossed to MLY1 and segregants which carried *MNN1-s* were isolated by standard yeast techniques (Guthrie and Fink, 1991).

Standard rich (YPD) and synthetic-defined (SD) minimal media were used for culturing yeast (Sherman, 1991). Supplements were added to SD media in plates or liquid as needed to support yeast growth. Where stated in the text or figure legends the media for liquid and/or plates was supplemented with Na Hepes, pH 7.0, to a final concentration of 50 mM. Standard rich media was used to grow *Escherichia coli* (Miller, 1972).

DNA Manipulations

pMNN1-s, pRS426 MNN1-s, and pM39I were described previously (Graham and Krasnov, 1995). pRS315 MNN1-s was constructed by subcloning the 4.3-kb NotI-XhoI fragment containing the MNN1-s construct released from pRS426 MNN1-s into pRS315 (*CEN LEU2*; Sikorski and Hieter, 1989). pRS304-MNN1-s was constructed by subcloning the 4.7-kb SacI-XhoI fragment from pMNN1-s into pRS304 (*TRP1*; Sikorski and Hieter, 1989). To prepare pCM39S, site directed mutagenesis was performed on pTG104 (Graham and Krasnov, 1995) to introduce a SacI site 6 bp 5′ of the start codon and a BamHI site after codon 39 of the *MNN1* gene. A 135-bp SacI-BamHI fragment from pTG104 was then subcloned into SacI-BamHI treated pCM166S in order to replace the *MNN1* sequences encoding amino acids 1–166 with a smaller fragment encoding amino acids 1–39. pCM166S is a pSEYC308 construct containing the *PRC1* promoter (from EcoRI to SacI introduced 6 bp 5′ to the start codon), and the *MNN1* gene from the −6 bp relative to the start codon to a BamHI site after codon 166 of *MNN1*. Deletion analysis of pTR-26 was performed by generating six deletion constructs. Constructs pTR-26Δ1 through pTR-26Δ6 were generated by deleting a SnaBI fragment, a SacI-BssHII fragment, a NsiI-BssHII fragment, a NruI-BssHII fragment, a NsiI-SalI fragment, and a PvuII fragment, respectively. pTR-26Δ1, 3, and 5 failed to complement the Ldr$^-$ phenotype, whereas pTR-26Δ2, 4, and 6 all complemented the Ldr$^-$ phenotype, which implicated *HOG1*. Based on these results, pHOG1 was constructed by subcloning the 2.6-kb ClaI-ScaI fragment containing *HOG1* from pTR-26 into the ClaI- and SmaI-digested pRS313 (*CEN* and *HIS3*; Sikorski and Hieter, 1989) vector.

The standard lithium acetate method (Guthrie and Fink, 1991) was used to transform yeast in most cases. A high efficiency electroporation method (Meilhoc et al., 1990) was used to transform yeast with a single-copy yeast genomic library (Horazdovsky et al., 1994) using a BIO-RAD GENE PULSER II electroporation apparatus and 2-mm electroporation cuvettes from Bio-Rad Laboratories (Hercules, CA) or BTX (Genetronics, San Diego, CA). *E. coli* were transformed by the standard calcium chloride method (Maniatis et al., 1982) or electroporation using the apparatus described above and the protocol provided with the apparatus.

Colony Blot Assay

Yeast expressing Mnn1-s from a plasmid (pMNN1-s, pRS315 MNN1-s, or pRS426 MNN1-s) were grown for 2 d on selective minimal media at 30°C and replica plated onto YPD plates with 50 mM Na Hepes, pH 7.0, plates and incubated one additional day at 30°C. Each plate was then overlaid with a nitrocellulose filter and incubated 16–20 h at 30°C. The filters were then removed and rubbed with gloved hands under running deionized water to thoroughly remove residual yeast. The filters were then probed overnight with an affinity-purified anti-Mnn1p primary antibody (1:2,000), then for 1.5 h with a goat anti–rabbit horseradish peroxidase-conjugated secondary antibody (1:2,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and immune complexes were visualized using an enhanced chemiluminescent (ECL) assay (Nycomed Amersham Inc., Princeton, NJ) following the manufacturer's protocol. All antibody incubations and wash steps were performed at 4°C with gentle rocking.

Screen for ldr Mutants and Cloning of HOG1

The *ldr* mutants were isolated by spreading ethylmethanesulfonate (EMS; Fluka AG, Buchs, Switzerland) mutagenized (Guthrie and Fink, 1991) SEY6210 or SEY6211 cells carrying pMNN1-s on plates at a density of ~500 colonies per plate and screening using the colony blot assay. Viability was ~40%. The *HOG1* gene was cloned as follows. TRY120 pMNN1-s was transformed with a single-copy *LEU2* marked yeast genomic library (Horazdovsky et al., 1994). Plates containing transformed colonies at the same density described for the *ldr* mutant screen were screened for a Ldr$^+$ phenotype against a background of colonies with a Ldr$^-$ phenotype by colony blotting. This was done as described above with the exception that library transformants were selected on SD −ura −leu plates buffered with 50 mM Na Hepes, pH 7.0, and nitrocellulose was overlaid directly onto the transformation plates without replica plating. Colonies which gave a Ldr$^+$ phenotype were picked and retested in the presence of controls that included SEY6210 pMNN1-s pRS315 as a negative control and TRY120 pMNN1-s pRS315 as a positive control.

Differential Centrifugation and Sucrose Gradient Fractionation

Differential centrifugation analysis and sucrose gradient fractionation of Golgi membranes were performed essentially as described previously (Graham and Krasnov, 1995) with the following modifications. Cell lysis and differential centrifugations were performed in the presence of PMSF at 1 mM and the following protease inhibitors at a concentration of 1 μg/ml: pepstatin, leupeptin, aprotinin, antipain, and chymostatin. Fractions were not processed further after being removed from the gradient, but were assayed directly from the sucrose fractions collected. Typically, 13 fractions were collected from each sample, and fractions from different gradients were normalized to one another based on the sucrose density of each fraction as determined by the refractive index. Kex2p and GDPase assays, protein concentration determinations, and Western blots of the fractions were performed as described previously (Graham and Krasnov, 1995). Quantitation of Western blots from the sucrose gradients and for the section below was essentially done as previously described (Gaynor et al., 1998).

Quantitation of Mnn1-s Secretion

Cells (10 OD_{600}) were collected at logarithmic phase of growth and converted to spheroplasts as previously described (Graham and Krasnov, 1995) with addition of 25 μl of 1 mg/ml BSA to the spheroplasting buffer. The spheroplasts were collected by centrifugation for 3 min at room temperature at 10,000 rpm. The supernatant was removed to a fresh tube and 50% TCA was added to a final concentration of 10%, then centrifuged to pellet the proteins, and washed twice with acetone. The pellets were dried down and resuspended in 100 μl 1× sample buffer. The spheroplast pellets were resuspended in 1× sample buffer and boiled 5 min. They were then broken up by vortexing in the presence of glass beads, centrifuging at room temperature for 2 min, and then boiled again. 20 μl (2 OD equivalents) of both the spheroplasts and the periplasmic space fraction were fractionated by SDS-PAGE and Western blotted as described previously (Graham and Krasnov, 1995). Cell labeling and separation of cells from periplasmic space contents were done essentially as described (Seaman et al., 1997) except that protease inhibitors (same as listed above without PMSF) were included in the spheroplasting buffer. Immunoprecipitations were performed as described previously (Graham and Krasnov, 1995).

Results

The pmr1Δ Mutant Mislocalizes the Soluble Mnn1p Lumenal Domain

The lumenal domain of Mnn1p (Fig. 1 *B*, *Mnn1-s*) is efficiently localized to the Golgi complex independently of the Mnn1p transmembrane domain. However, Mnn1-s is secreted from the cell when overexpressed from a multicopy plasmid (Graham and Krasnov, 1995). This suggested that Mnn1-s may be retained by a saturable, receptor-mediated mechanism and that mutants that failed to properly sort Mnn1-s would secrete it. To detect such mutants, we used a colony blotting technique (Wilsbach and Payne, 1993) where yeast colonies growing on agar plates were overlaid with nitrocellulose discs for 16–20 h that were then washed free of yeast and probed with antibodies to Mnn1p. The colony blotting technique was tested using wild-type yeast expressing Mnn1-s on a single-copy plasmid as a negative control (Fig. 2 *A*, *WT*), and wild-type

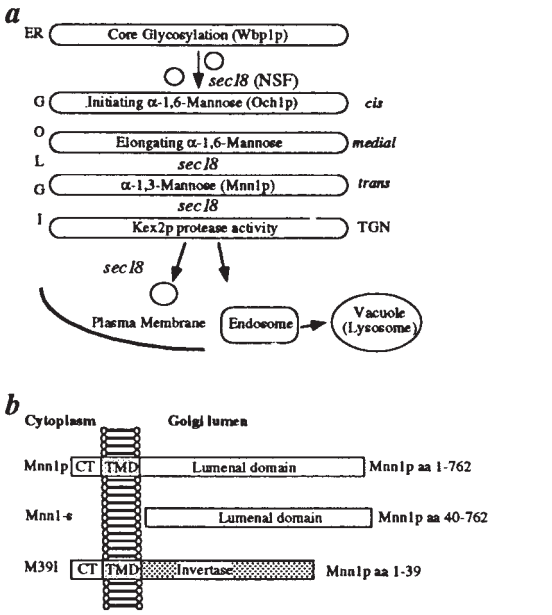


Figure 1. (*a*) Functional model for the compartmental organization of the yeast Golgi complex (Graham and Emr, 1991; Gaynor et al., 1994). (*b*) Schematic of the yeast α-1,3-mannosyltransferase (*Mnn1p*) and the fusion proteins used in this report. Wild-type Mnn1p, a type II integral membrane protein, is shown at the top. The lumenal domain is expressed in the secretory pathway as a soluble protein (*Mnn1-s*) by exchanging the transmembrane domain (*TMD*) and cytoplasmic tail (*CT*) for the cleavable signal peptide of CPY. The Mnn1p TMD and CT are fused to the secreted enzyme invertase and the resulting fusion protein (*M39I*) is used to examine TMD mediated localization; previous studies have indicated that the CT does not contribute to the localization of Mnn1p (Graham and Krasnov, 1995).

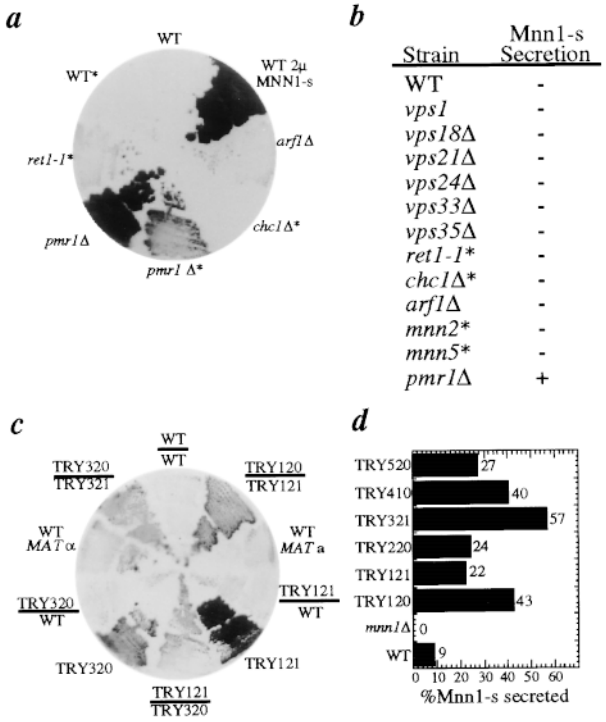


Figure 2. (*a*) Strains carrying null mutations in the *PMR1* gene mislocalize and secrete Mnn1-s. Strains were streaked out and then analyzed by the colony blot assay as described in Materials and Methods. All of the strains except WT 2μ MNN1-s are carrying pMNN1-s. Strains marked with an * indicate that it is a strain with a genetic background other than SEY6210. The strain abbreviations in the figure are as follows: WT 2μ MNN1-s (SEY6210 pRS426 MNN1-s); WT (SEY6210); *arf1*Δ (6210 *arf1*Δ); *chc1*Δ* (GPY1103); *pmr1*Δ* (L3865); *pmr1*Δ (SEY6210 *pmr1*Δ); *ret1-1* (TGY381); WT* (TGY383). (*b*) Summary of results from colony blot experiments with different secretory pathway mutants tested. All strains carry pMNN1-s. All *vps* strains are in the SEY6210 background. *mnn2** and *mnn5** are the LB1-16A and LB65-5D strains respectively. (*c*) The results of a complementation test between two *ldr* mutants (TRY121 and TRY320) is shown. All strains carry the pMNN1-s plasmid. (*d*) The percent Mnn1-s secreted from each strain was determined by Western blot as described in Materials and Methods. Mnn1-s is expressed at a slightly higher level than Mnn1p and has a different mobility by SDS-PAGE due to differences in N-glycosylation. This allowed us to distinguish Mnn1-s from endogenous Mnn1p in these experiments.

yeast overexpressing Mnn1-s from a multi-copy plasmid as a positive control (Fig. 2 *A*, *WT 2μ MNN1-s*). Colonies harboring the single-copy Mnn1-s plasmid produced a weak background signal and yeast overexpressing Mnn1-s (therefore secreting this protein) were easily detected by the colony blot assay, suggesting that it would be possible to screen for mutants by this method.

However, there was a concern that we would not be able to detect mutants mislocalizing Mnn1-s expressed from a single-copy plasmid due to the lower expression level. Specificity was also a concern as it was possible that any mutation that perturbed the secretory pathway would cause secretion of Mnn1-s. These two concerns were addressed by using the colony blot assay to examine known mutants expressing Mnn1-s from a single-copy plasmid for an Mnn1-s secretion phenotype.

Mutants containing null alleles of *PMR1*, which encodes a Golgi-localized, Ca^{++} ATPase (Rudolph et al., 1989) exhibit a defect in Golgi-specific carbohydrate modifications on secreted glycoproteins. These glycoproteins lack outer chain α-1,3-mannose on N-linked oligosaccharides, even though the cells express normal levels of α-1,3-mannosyltransferase activity in lysates (Verostek and Trimble, 1995). Two strains with different genetic backgrounds carrying a disruption of the *PMR1* gene and a single-copy plasmid expressing Mnn1-s were examined using the colony blot assay. Both *pmr1*Δ mutants secreted Mnn1-s and produced a signal on the filter that was substantially darker than the wild-type controls (Fig. 2 *A*). Secretion of Mnn1-s from *pmr1*Δ cells was specific since a number of other strains carrying mutations that perturb the Golgi were also tested by colony blot, but none of the other strains secreted Mnn1-s (Fig. 2, *A* and *B*). This result suggested that the glycosylation defect exhibited by the *pmr1*Δ mutant was at least partially attributable to mislocalization of Mnn1p.

Isolation of ldr-defective Mutants

All of the mutants tested perturb the Golgi complex in

various ways, but only the *pmr1*Δ mutant secreted Mnn1-s. The clear difference in signal between the *pmr1*Δ and wild-type cells expressing Mnn1-s from a single-copy plasmid indicated that the colony blotting assay could be used to identify mutations that would specifically perturb Mnn1-s localization. Approximately 15,500 EMS mutagenized colonies were screened by colony blotting for *ldr* mutants that secreted Mnn1-s. 30 mutants were isolated and crossed to the parental wild-type strains to make heterozygous diploids and tested by colony blotting to determine if the mutant phenotype was dominant or recessive (an example is shown in Fig. 2 *C*; *TRY320/WT*, *TRY121/WT*). Three of the strains carried dominant mutations and were not characterized further. We backcrossed each of the 27 recessive mutants with the parental strains and followed the mutations through two backcrosses. Only 6 of the 27 original mutants (TRY1 through TRY6) contained mutations which gave a clear 2:2 segregation of the phenotype in each backcross, which suggested a single gene mutation. The six mutants were intercrossed to one another and the resulting diploids assayed by colony blotting for the mutant phenotype in order to place the mutants into complementation groups. Members of a complementation group are defined by those mutants that when crossed to one another form a diploid that has a mutant phenotype. This suggests that both original haploid mutant strains used in the cross carried mutations in the same gene and the resulting diploid is now homozygous for mutant alleles at that locus. In most cases, a diploid heterozygous for mutations in two different genes would have a wild-type phenotype.

Fig. 2 *C* shows a representative complementation test between two *ldr* mutants. TRY120 and TRY121 are haploid segregants derived from the mutant strain TRY1 (0 or 1 at the end of the strain name indicates mating type a or α, respectively) and TRY320 and TRY321 are derived from strain TRY3. A diploid made by crossing strains carrying two independently isolated mutations (TRY121/TRY320) was compared with homozygous diploids that were made from strains carrying the same mutation (TRY120/TRY121 and TRY320/TRY321) which act as positive controls for the mutant phenotype. A wild-type diploid (WT/WT) was used as a negative control and the parental haploid strains are shown as well. In each case, the intercrossed diploids produced a signal on the colony blot that was similar to that produced by the homozygous mutant diploids. Therefore, it appeared that all six mutations fell into one complementation group.

To more quantitatively determine the level of Mnn1-s secreted from the different mutants compared with the wild-type strain, cells were separated from the contents of the periplasmic space where most secreted yeast proteins including Mnn1-s accumulate. The cell and periplasmic space fractions were analyzed by Western blotting with antibodies to Mnn1p. The signals for the two fractions were added together and the amount in the periplasmic space taken as a percent of the total. As shown in Fig. 2 *D*, the mutants secreted between 22 and 57% of the total Mnn1-s compared with an average of 9% for the wild-type strain. An *mnn1*Δ strain not carrying the plasmid was included as a negative control for the Western blot. All results were normalized after reprobing the blots with an antibody to the cytoplasmic protein phosphoglycerate kinase (PGK) to control for cell lysis. No significant difference was observed between the mutants and the wild-type with regard to the levels of PGK found in the two fractions indicating the above results were not due to cell lysis. We have also examined the medium from these cultures by Western blot and could not detect the intact Mnn1-s protein (data not shown). These data indicate that the dark signal observed for mutant strains by colony blot correlate to an increase in secretion of Mnn1-s to the periplasmic space. It is likely that the colony blot signal arises from Mnn1-s proteolytic fragments that are small enough to diffuse through the cell wall.

Comparable results were obtained when the wild-type strain and TRY120 were metabolically labeled with ^{35}S methionine/cysteine for 10 min and chased for 0, 15, 30, and 60 min. Mnn1-s was immunoprecipitated from the periplasmic space and cell fractions and the results quantitated by PhosphorImaging. At 30 min of chase there was a threefold increase in the level of Mnn1-s found in the periplasmic space of the mutant compared with the wild-type. In addition, carboxypeptidase Y (CPY) was immunoprecipitated from the same samples and we observed that transport to the vacuole was normal, indicating that these mutants did not impinge on the *vps* pathway (data not shown). In addition, CPY is a substrate for Mnn1p and the glycosylation pattern of CPY was similar to that of wild-type (data not shown). Mnn1-s did not appear to be produced in greater quantities in *ldr* mutants based on Western blots and on cell labeling experiments. Therefore, the secretion phenotype seen in the mutants was not likely caused by overexpression of the lumenal domain. Mnn1-s transcription is driven from the *PRC1* (CPY) promoter (Graham and Krasnov, 1995) and we also did not observe an increase in CPY expression in the mutants compared with the wild-type, again suggesting that the Mnn1-s transcriptional levels were similar between mutant and wild-type strains (data not shown).

The HOG1 Gene Complements the Ldr1⁻ Phenotype of TRY120

The fact that the *pmr1*Δ strain secretes Mnn1-s raised the possibility that we had isolated several new *pmr1* mutant alleles. To test this, we crossed the *pmr1*Δ strain with each *ldr* mutant and found the Ldr⁻ phenotype was complemented in each diploid. In addition, TRY120 and TRY220 were transformed with a multi-copy plasmid expressing a HA-tagged copy of the *PMR1* gene, which is known to be functional (Antebi and Fink, 1992). This plasmid did not complement the Ldr⁻ mutant phenotype. These data indicated that these mutants were not a collection of *pmr1* mutant alleles (data not shown).

The wild-type gene represented by the *ldr* mutation in TRY120 was cloned by screening a single-copy yeast genomic library for plasmids which complemented the Ldr⁻ mutant phenotype of TRY120. This was done by analyzing transformed colonies for those that produced a lighter signal on colony blots relative to neighboring noncomplemented colonies. TRY120 was used because the mutation it carried gave the strongest Ldr⁻ phenotype in both mating types after two backcrosses. A single clone was iso-

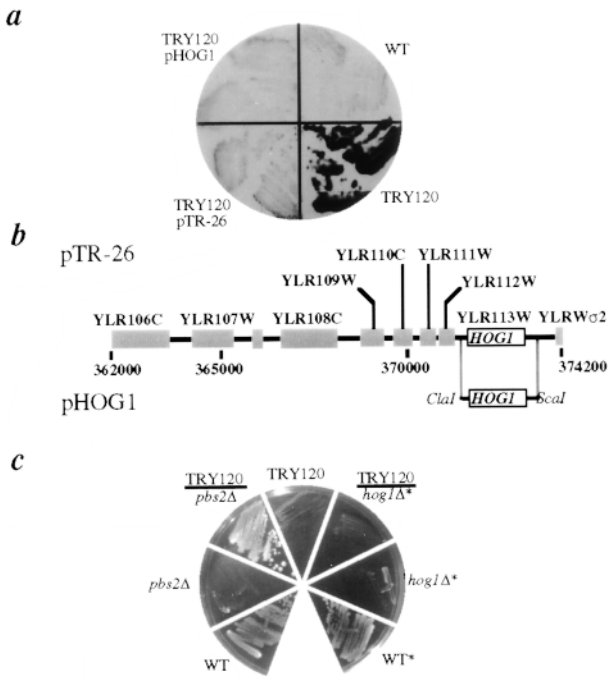
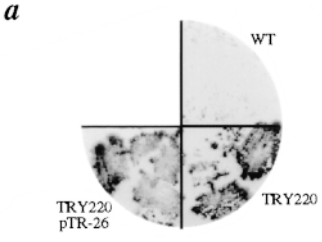


Figure 3. Strain TRY120 carries a mutant allele of *HOG1*. (*a*) Colony blot showing complementation of the TRY120 Ldr⁻ phenotype by pTR-26 and pHOG1. TRY120 pHOG1, TRY120 pTR-26, TRY120 pRS315, and WT (SEY6210 pRS315) were streaked out in duplicate and examined by colony blot. All strains carry pMNN1-s. (*b*) Primers that annealed to vector sequences on pTR-26 that flanked the yeast genomic insert were used to sequence the ends of the insert. These sequences were used in a BLAST search of the complete yeast genome database to reveal the genomic insert represented diagrammatically. The *HOG1* gene is depicted as an open box and sites used to subclone it into the pRS313 vector to make pHOG1 are shown as well. (*c*) A diploid made from TRY120 crossed to AMY36 (*hog1*Δ) fails to grow on 1.0 M sorbitol plates. Wild-type strains SEY6210 and FY250 (WT*) were streaked onto a YPD plate containing 1.0 M sorbitol along with haploid strains JSY2092 (SEY6210 *pbs*Δ), AMY36 (*hog1*Δ*), and TRY120. Diploids were made by crossing TRY120 with JSY2092 (TRY120/JSY2092) and AMY36 (TRY120/AMY36) and these diploids were also streaked on the 1.0 M sorbitol to test for complementation of the *pbs2*Δ- and *hog1*Δ-associated growth defects, respectively.

lated from 15,000 transformants that could complement the Ldr⁻ phenotype in a plasmid-linked manner (Fig. 3 *A*). The ends of the insert were sequenced and compared with the yeast genome sequence to identify the chromosome XII fragment shown in Fig. 3 *B*. Deletion analysis and subcloning revealed that the *HOG1* gene was responsible for complementing the Ldr⁻ phenotype (Fig. 3 *A* and Materials and Methods).

The *HOG1* gene encodes a well-characterized member of the MAPK family involved in the high osmolarity glycerol response (HOG) pathway (diagrammed in Fig. 8), a signal transduction cascade that responds to increases in extracellular osmolarity. Hog1p is activated through two redundant signal transduction pathways. The Sln1p-Ypd1p histidine kinase phosphorelay osmosensor complex actively represses Ssk1p through phosphorylation in normal osmotic conditions. In the presence of hyperosmotic stress the Sln1p-Ypd1p osmosensor complex becomes inactive. This relieves repression of Ssk1p, which in turn activates the HOG pathway through phosphorylation of the redundant MAPK kinase kinases (MAPKKKs) Ssk2p and Ssk22p. These MAPKKKs activate the MAPK kinase (MAPKK) Pbs2p, which then activates Hog1p through phosphorylation. Activated Hog1p in turn upregulates the transcription of osmotic stress response genes such as *GPD1*, which encodes glycerol-3-phosphate dehydrogenase and *HSP12*, which encodes one of the major small heat shock proteins in yeast. Alternatively, a second input into this pathway, the Sho1p osmosensor, activates the Ste11p MAPKKK, which in turn activates the Pbs2p MAPKK, which activates Hog1p (Wurgler-Murphy and Saito, 1997).

hog1 mutants are unable to grow in conditions of osmotic stress such as agar plates containing 1.0 M sorbitol. We found that TRY120 was unable to grow on 1 M sorbitol plates (Figs. 3 *C* and 4 *B*) and the *HOG1* gene expressed from a single-copy plasmid was able to complement the growth defect of TRY120 on 1 M sorbitol (Fig. 4 *B*). In addition, a diploid made by crossing TRY120 with a *hog1*Δ strain was also unable to grow on 1 M sorbitol (Fig. 3 *C*), and when this diploid was sporulated and the segregants examined by random spore analysis, 0 out of 209 segregants were able to grow on 1.5 M sorbitol plates confirming that TRY120 carries a mutant allele of *HOG1*. We now refer to this allele as *hog1-11*. Surprisingly, when one of the other *ldr* mutants, TRY220, was transformed with a plasmid containing the *HOG1* gene, the Ldr⁻ phenotype was not complemented (Fig. 4, *A* and *B*). In addition, TRY220 did not show a growth defect on 1 M sorbitol plates. These data indicated that the Ldr⁻ phenotype exhibited by TRY220 was caused by a mutation in a gene other than *HOG1*. The other four *ldr* mutants isolated in this screen (TRY3 through TRY6 or their derivatives) were also found to grow on 1 M sorbitol (Fig. 4 *B*) suggesting that these strains also did not carry mutant alleles of the *HOG1* gene. To clearly determine the number of



Strain	Plasmid	Growth on 1M Sorbitol	Mnn1-s secretion (s)
TRY120	-	-	s
TRY120	pTR-26	+	ns
TRY120	pHOG1	+	ns
TRY220	-	+	s
TRY220	pTR-26	+	s
TRY3-6	-	+	s

Figure 4. The *HOG1* gene does not complement the Ldr⁻ phenotype in TRY220. (*a*) WT (SEY6210 pMNN1-s), TRY220 pMNN1-s, and TRY220 pMNN1-s pTR-26 were streaked out in duplicate and tested by colony blot. (*b*) Summary table of results from testing TRY120 (*hog1-11*) and TRY220 (with or without) a plasmid borne copy of the *HOG1* gene for growth on 1 M sorbitol plates and for Mnn1-s secretion by colony blot. Other *ldr* mutants were tested for growth on 1 M sorbitol. *s* indicates Mnn1-s was secreted and *ns* indicates Mnn1-s was not secreted.

genes represented by the remaining five *ldr* mutants, we analyzed tetrads generated from intercrossing these mutant strains. The results from this linkage analysis showed that all five mutants carried mutant alleles of the same gene, *LDR1*, which we refer to as *ldr1-2* (TRY2) through *ldr1-6* (TRY6; data not shown). The failure to produce diploids that were complemented for the Ldr^- phenotype when derivatives of TRY1 (*hog1-11*) are crossed to any of the remaining five *ldr* mutants (Fig. 2 *C*, TRY121/TRY320) appears to result from nonallelic noncomplementation, a genetic interaction that suggests a functional interaction between the encoded proteins. This is a specific genetic interaction between *ldr1* and *hog1*, since a *pmr1*Δ strain complemented all of the mutants.

Effect of hog1-11 and ldr1-2 Mutations on Transmembrane Domain-mediated Localization Mechanism

Wild-type, *hog1*Δ, *hog1-11*, and *ldr1-2* cells were transformed with a single-copy plasmid expressing the M39I fusion protein (Fig. 1 *B*) and the level of invertase localized to the cell surface was assayed. Wild-type, *hog1*Δ, and *hog1-11* cells expressed the same low percentage of invertase activity at the cell surface (~6%) indicating that the transmembrane domain mediated localization mechanism was not defective in cells lacking functional Hog1p (Fig. 5). The same was found to be true for cells carrying a null allele of *PMR1* (Fig. 5). However, *ldr1-2* cells mislocalized ~15% of the invertase activity to the cell surface (Fig. 5). Although a modest increase relative to the wild-type strain, this effect was reproducible, and suggests that the *ldr1-2* mutation leads to defects in both the lumenal and transmembrane domain mediated localization mechanisms.

Golgi Compartment-specific Mislocalization of Intact Mnn1p in the hog1-11 and ldr1-2 Mutants

Although the *hog1-11* (TRY120) and *ldr1-2* (TRY220) mutations perturbed Mnn1-s localization, it was not clear if these mutations would perturb the localization of intact

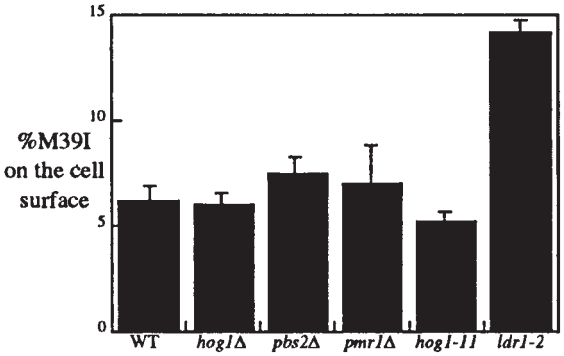


Figure 5. Mutations in the *HOG1* gene do not perturb the transmembrane domain-mediated localization mechanism. Wild-type, *hog1*Δ, *pbs2*Δ, *pmr1*Δ, *hog1-11*, and *ldr1-2* cells expressing M39I on a single-copy plasmid (pCM39S) were subjected to a liquid invertase assay (Bankaitis et al., 1986) to quantitate the level of M39I mislocalized to the cell surface.

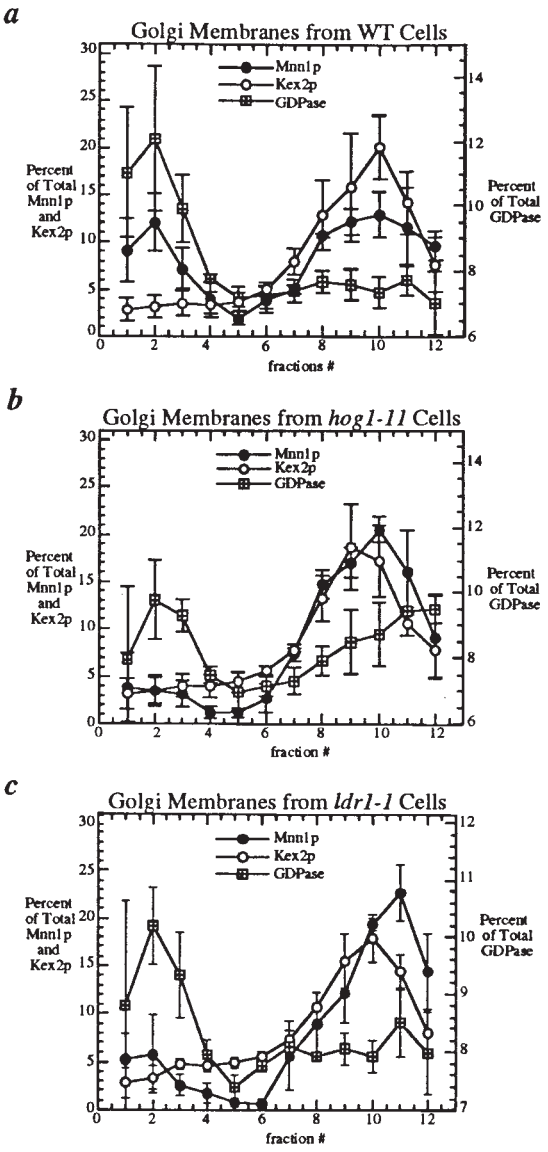


Figure 6. Sucrose gradient fractionation profiles of Golgi membranes from wild-type, *hog1-11*, and *ldr1-2* cells. Differential centrifugation, sucrose gradient fractionation, and enzyme assays were performed as described in Materials and Methods. Fractionation profiles of Kex2p, GDPase, and Mnn1p from (*a*) WT (SEY6210), (*b*) TRY120 (*hog1-11*), and (*c*) TRY220 (*ldr1-2*) cells were generated from the average of three experiments performed for each strain. The amount of each marker in a particular fraction was plotted as percentage of the total found in all the fractions. Quantitation of Western blots was done as described in Materials and Methods.

Mnn1p. To test this, Golgi membranes from *hog1-11*, *ldr1-2*, and wild-type strains expressing only wild-type Mnn1p were enriched by differential centrifugation and sucrose gradient fractionation. In brief, the strains were osmotically lysed and centrifuged at 1,000 *g* to pellet unlysed cells. Then the supernatants were centrifuged at 13,000 *g* to pellet the nuclear, vacuolar, ER, and plasma membranes (P13). Membranes within the supernatants from

the 13,000 *g* spin (S13) were then centrifuged through 20% sucrose at 100,000 *g* to pellet the Golgi membranes onto a 66% sucrose cushion (P100). The P100 was loaded onto the bottom of a sucrose density step gradient and centrifuged to equilibrium at 100,000 *g*. The gradient was then separated into fractions which were assayed for GDPase, a marker for the early Golgi compartments (Vowles and Payne, 1998) and Kex2p, a marker for the TGN (Cunningham and Wickner, 1989; Graham and Emr, 1991). The amount of Mnn1p in the P13, P100, and each gradient fraction was determined by Western blotting.

A significant alteration in the percentage of Mnn1p in the P13 fraction from the mutants was not observed, suggesting that Mnn1p was not accumulating in the ER, vacuolar membranes, or plasma membrane of the mutants. Mnn1p was enriched in the P100 from the wild-type cells as reported previously (Graham et al., 1994) and was also enriched to a similar extent in the P100 derived from the mutant strains (data not shown). However, the distribution of Mnn1p in the sucrose gradient fractions from the mutants differed significantly from the wild-type profile (Fig. 6, *A–C*). A peak containing 30–70% of Mnn1p is typically found in the less dense sucrose fractions (1–4) and cofractionates with the major peak of GDPase in gradients from wild-type cells (Fig. 6 *A*). This first peak of Mnn1p is nearly absent in gradients containing Golgi membranes from the mutants (Fig. 6, *B* and *C*). The Mnn1p lost from the less dense Golgi fractions in the mutants appears to have shifted to the more dense fractions that contain the TGN marker Kex2p. A similar profile to that shown in Fig. 6 *B* was observed for Golgi membranes isolated from *hog1*Δ cells.

As previously reported for Golgi preparations from wild-type cells, most of the GDPase activity was recovered in the less dense fractions of the sucrose gradient (Fig. 6 *A*, fractions 1–3) and most of the Kex2p activity peaked in the denser fractions (Fig. 6 *A*, fractions 8–11). GDPase activity was observed to have undergone a shift toward the denser fractions in the mutant strains relative to the wild-type, that is similar, but not as dramatic as that observed for Mnn1p. The gradient profile of Kex2p activity from the mutants was not significantly different than that observed for the wild-type (Fig. 6, *B* and *C*). These strains also carried an HA-epitope tagged version of Och1p which is commonly used as a marker for the Golgi. The distribution of Och1-HA among Golgi fractions was similar between mutant and wild-type strains (data not shown). The fact that the localization of Mnn1p was dramatically altered in the *hog1-11* and *ldr1-2* mutants, but the distribution of most other Golgi markers examined were relatively similar to wild-type suggests that the *hog1-11* and *ldr1-2* mutants specifically perturb protein localization within the Mnn1p compartment(s).

Immunofluorescence was used to examine the organization of the Golgi by analyzing the distribution of Golgi markers, Mnn1p, Kex2p, and Och1-HA as described previously (Graham et al., 1994). We found that Golgi morphology does not appear to be affected by the *hog1*Δ mutation. In addition, examination of wild-type and *hog1*Δ cells by transmission electron microscopy (Rieder et al., 1996) did not reveal any apparent aberrations in intracellular membranes or ultrastructure.

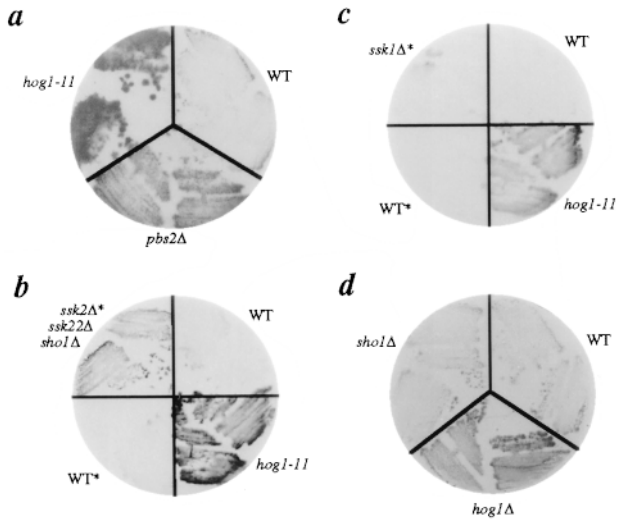


Figure 7. Examination of other mutants in the HOG pathway for the Ldr⁻ phenotype. Strains harboring null mutations in *PBS2* (*a*) *SSK2 SSK22 SHO1* (*b*) *SSK1* (*c*) and *SHO1* (*d*) were tested for Mnn1-s secretion by the colony blot assay. A *hog1* mutant and isogenic wild-type strains were included in each plate. Strains marked with an asterisk (*) are in the W303 background and all other strains are in the SEY6210 background. All strains assayed are carrying pMNN1-s.

Signal Transduction Through the HOG Pathway Is Required for Mnn1-s Localization

The fact that the Hog1p MAPK is required for proper localization of Mnn1p in the Golgi complex in the absence of hyperosmotic stress raised the question as to whether signal transduction through the HOG pathway may have a function in retaining Golgi proteins in nonstressed cells. To test this, a *pbs2*Δ (MAPKKΔ) mutant was analyzed for the Ldr⁻ phenotype by the colony blotting assay and, like *hog1-11* mutants, was found to secrete Mnn1-s (Fig. 7 *A*), but *pbs2*Δ did not perturb localization of M39I (Fig. 5). These results implied that the rest of the HOG pathway may be involved in localization of the Mnn1p lumenal domain. To block the HOG pathway response to hyperosmotic stress upstream of Pbs2p, both of the redundant receptor pathways, Sho1p and Sln1p-Ypd1p, must be blocked (Maeda et al., 1995; Wurgler-Murphy and Saito, 1997). A triple mutant was tested in which the genes encoding the redundant MAPKKKs Ssk2p and Ssk22p that function in the Sln1p-Ypd1p receptor pathway and the gene encoding the Sho1p receptor were all three knocked out and therefore has a block in both input branches of the HOG pathway (see Fig. 8). This triple mutant, *ssk2*Δ *ssk22*Δ *sho1*Δ, also mislocalized Mnn1-s as seen in Fig. 7 *B*. These results strongly suggest that a basal level of signaling through the entire HOG pathway in the absence of osmotic stress is required for Mnn1p localization. However, these results do not rule out the possibility that only one input branch and not both are involved. To test this, each branch was individually knocked out. An *ssk1*Δ null mutation knocks out the Sln1p-Ypd1p osmosensor pathway (Posas et al., 1996) and the *sho1*Δ null mutation blocks the

alternative pathway (Maeda et al., 1995). Neither the *ssk1*Δ mutation alone nor the *sho1*Δ mutation alone resulted in Mnn1-s secretion (Fig. 7, *C* and *D*). Therefore, a basal level of Hog1p activation through either branch of the pathway is sufficient to maintain proper localization of Mnn1-s.

Discussion

A screen for mutants that fail to properly localize the soluble lumenal domain of Mnn1p has uncovered six *ldr* mutants. Although these six mutants were all assigned to a single complementation group, they have been shown to carry mutations in two different genetic loci, *HOG1* and *LDR1*. The *HOG1* gene encodes a MAPK that is part of a well-characterized signal transduction cascade that responds to hyperosmotic stress (Fig. 8; Brewster et al., 1993; Wurgler-Murphy and Saito, 1997). We have found that mutations that inactivate upstream components of the HOG pathway (Figs. 7 and 8) also result in a Ldr$^-$ phenotype.

We had previously proposed that the Mnn1p transmembrane domain and lumenal domain localization signals mediate protein targeting through different mechanisms. This proposal is strongly supported by the current work as mutations in *HOG1*, *PMR1*, and *PBS2* that cause secretion of Mnn1-s do not cause mislocalization of M39I to the plasma membrane. Conversely, a mutation in the clathrin heavy chain causes mislocalization of M39I, but not Mnn1-s (Graham et al., 1994 and Fig. 2 *A*). Intact Mnn1p does not appear to be mislocalized to the plasma membrane in *hog1-11* or *ldr1-2* cells, apparently because the transmembrane domain–mediated localization mechanism is still intact. Instead, Mnn1p is lost from the early (less dense) Golgi compartments and accumulates in dense membranes containing Kex2p as seen in sucrose gradient fractionation experiments (Fig. 6, *B* and *C*). This phenotype is specific to Mnn1p as the Golgi marker Och1p-HA and the TGN marker Kex2p were both localized correctly (data not shown and Fig. 6, respectively). Golgi GDPase partially cofractionates with Mnn1p in gradients from wild-type cells; and a portion of GDPase was also shifted to denser fractions from both the *hog1-11* and *ldr1-2* mutants, but not to the same extent as Mnn1p.

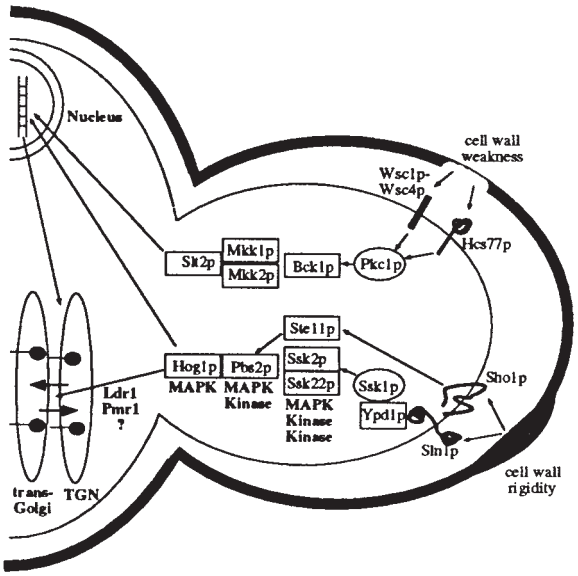


Figure 8. Model for the potential role of the HOG and PKC-MPK pathways in regulating cell wall biosynthesis. The HOG pathway is required for Golgi localization of Mnn1-s, controls the distribution of Mnn1p between early and late Golgi compartments, and upregulates the activity of an exoglucanase, possibly in response to excess rigidity in the cell wall. Conversely, the PKC-MPK pathway is thought to sense a weakened cell wall and respond by upregulating the transcription of cell wall biosynthetic enzymes. See Discussion for additional details.

The Nature of the Localization Signal within the Mnn1p Lumenal Domain

There are two models that could explain the mechanism by which the Mnn1p lumenal domain localization signal functions. The first model consists of a retrieval mechanism in which the lumenal domain is recognized by a receptor in the TGN when it leaves the *trans*-Golgi compartment. This receptor then facilitates the retrograde transport of the Mnn1p lumenal domain from the TGN to the *trans*-Golgi compartment from which it escaped. Disruption of the HOG pathway or of the *LDR1* gene would interrupt some aspect of the retrieval pathway which would then result in secretion of Mnn1-s. However, the localization signal within the Mnn1p transmembrane domain is thought to act through a retrieval mechanism (Graham and Krasnov, 1995) and in strains carrying mutations in the *HOG1* gene, M39I is not lost to the cell surface. In addition, Och1p localization does not appear to be perturbed in *hog1*Δ cells (data not shown), even though this protein is also thought to be retrieved from the Kex2p compartment (Harris and Waters, 1996). Mnn1-s is also not secreted in mutants carrying *ret1-1* or *vps1*Δ mutations, genetic lesions that are both thought to cause defects in Golgi-associated retrograde membrane trafficking. These observations lead us to suggest that a retrograde pathway from the Kex2p compartment to earlier Golgi compartments is not perturbed in the *hog1*Δ mutant. However, it is possible that inactivation of Hog1p perturbs the recruitment of Mnn1-s and its receptor into the retrograde pathway. The *ldr1-2* mutant does exhibit a partial defect in M39I localization and could therefore perturb the retrieval mechanism directly.

The second model suggests a retention mechanism, in which retention machinery in both the *trans*-Golgi compartment and TGN recognize the lumenal domain of Mnn1p and retain a population within each compartment. In this model, mutations in the *HOG1* and *LDR1* genes result in a loss of retention from the *trans*-Golgi compartment. If retention machinery is operating within the TGN alone, then its association with the Mnn1p lumenal domain may be sufficient to retard transmembrane domain–mediated retrieval to the *trans*-Golgi compartment that would result in a steady-state accumulation of Mnn1p in the TGN. Conversely, Mnn1-s would be secreted from the cell since only half of the retention machinery (that found in the TGN) would be operating and Mnn1-s lacks the transmembrane domain signal to prevent it from being

exocytosed. The fact that there is a slight accumulation of M39I on the plasma membrane in a *ldr1-2* mutant may suggest that these two models are not mutually exclusive.

HOG Pathway Control of Golgi Protein Localization and Cell Wall Biogenesis

In this report, we provide the first demonstration that Hog1p and a functional HOG pathway are required for the proper localization of a glycosyltransferase to the Golgi complex. This requirement was found in the absence of the hyperosmotic stress response and suggests a role for a basal level of signaling through the HOG pathway in regulating the distribution of proteins within the Golgi. Changing the compartmental distribution of glycosyltransferases within the Golgi may provide a novel mechanism for regulating the composition of the yeast cell wall, which consists of mannan (mannoproteins), β-1,3-glucan, β-1,6-glucan, and a small amount of chitin. Golgi glycosyltransferases are essential for biosynthesis of mannan (Cid et al., 1995) and β-1,6-glucan (Roemer et al., 1993; Roemer et al., 1994). In fact, proteins of the HOG pathway have previously been implicated in cell wall biosynthesis. For example, *SLN1*, *SSK2*, and *PTC1*, which encode the Sln1p osmosensor, the Ssk2p MAPKKK, and the protein serine/threonine phosphatase Ptc1p, respectively, were recovered in a screen for cell wall–defective yeast mutants based on calcofluor white hypersensitivity (Lussier et al., 1997).

In another screen, the MAPKK *PBS2* was identified as a gene that, when overexpressed on a multi-copy plasmid, would impart a killer toxin resistant phenotype, which indicates a defect in cell wall biosynthesis (Jiang et al., 1995). The β-1,6-glucan polymers appear to serve as a receptor for yeast killer toxin and a decrease in β-1,6-glucan content correlates with killer toxin resistance (Cid et al., 1995). Conversely, a *pbs*Δ strain was hypersensitive to killer toxin and exhibited an increase in β-1,6-glucan. Strains carrying a *ptc1*Δ mutation exhibited the same phenotype as strains overexpressing *PBS2*. In these experiments, *pbs2*Δ was found to be epistatic to *ptc1*Δ, suggesting that the *PTC1* encoded phosphatase was acting to suppress the HOG pathway function in cell wall biosynthesis (Jiang et al., 1995). In each case, these screens were carried out in standard osmotic conditions and suggest that the basally activated HOG pathway is required for normal Golgi function in cell wall biosynthesis. Further support for a role of the HOG pathway in Golgi function is that Pbs2p was shown to act as a multicopy suppressor of the growth defect of an *arf1*Δ mutant on sodium fluoride plates (Kahn et al., 1995).

How Does the HOG Pathway Influence Golgi Protein Localization?

Is Hog1p basal activity effecting Mnn1p localization by transcriptionally regulating genes involved in the localization process, or does Hog1p control localization more directly through posttranslational phosphorylation of Golgi proteins? Jiang et al. (1995) have presented evidence that Pbs2p and Hog1p participate in upregulating the transcription of the *EXG1* gene, which encodes an exo-β-glucanase that is involved in remodeling the yeast cell wall. This lends support to the idea that Hog1p is controlling Golgi organization through transcriptional regulation of unidentified secretory pathway genes. However, it is clear from the experiments described in this report that if Hog1p is acting through transcriptional regulation, that it is not significantly influencing the amount of Mnn1-s, M39I, or Mnn1p in the cells. In fact, another MAP kinase pathway, initiated by PKC (see Fig. 8), acts in concert with the cell cycle–regulated transcription factor SBF (Mbp1p-Swi6p) to modulate the transcription of at least six different cell wall biosynthetic genes including *MNN1* (Igual et al., 1996).

On the other hand, several examples of posttranslational signaling events that control events within the Golgi have been reported. An unidentified MAPK in the ERK1 family is necessary for fragmenting the Golgi in permeabilized mammalian cells when mitotic extracts are added (Acharya et al., 1998), and $G_{\beta\gamma}$ subunits of a trimeric G protein are also capable of inducing vesiculation of mammalian Golgi membranes in vitro (Jamora et al., 1997). Additionally, phosphorylation of furin, a protease in the mammalian TGN, modulates the trafficking of this protein through association with PACS-1 (Wan et al., 1998). In yeast, the cytoplasmic Vps15p protein kinase controls sorting of soluble vacuolar proteins from the lumen of the Kex2p compartment to an endosomal intermediate by phosphorylation of a cytoplasmic PI-3 kinase (Vps34p; Stack et al., 1995). The Vps15p and Vps34p kinases probably act through integral membrane protein receptors, such as the CPY receptor, Vps10p, to influence the sorting of soluble vacuolar proteins within the lumen of the Golgi complex. Likewise, if Hog1p acts through a posttranslational mechanism, we would expect that phosphorylation of Golgi-associated membrane protein(s) would influence Mnn1p localization.

Identification of the downstream targets of Hog1p will help determine how Hog1p controls Mnn1p localization. The *LDR1* gene product may be one such target, as strains containing the six *ldr* mutations isolated in this screen fail to complement one another despite the fact that two different genes are represented, *HOG1* and *LDR1*. This genetic interaction, termed nonallelic noncomplementation, often predicts an interaction between the products of the two genes. Thus, Ldr1p may be a substrate for the Hog1p kinase. In fact, the Ldr^- phenotype of two strains carrying different mutant alleles of *LDR1* (*ldr1-2* and *ldr1-6*) is mildly suppressed when the HOG pathway is activated by growth on agar plates containing 0.45 M NaCl while *pmr1*Δ strains and other *ldr* strains are unaffected (our unpublished observation). This suggests that Ldr1p may be a target of Hog1p. In addition, Pmr1p is a possible target for Hog1p since deletion of *PMR1* also causes secretion of Mnn1-s.

The HOG signaling cascade is initiated from two integral membrane proteins, Sln1p and Sho1p, that appear to sense an osmotic imbalance (Wurgler-Murphy and Saito, 1997). However, it is not understood how these proteins sense hyperosmotic conditions. The Mnn1p mislocalization phenotype was observed in the absence of osmotic stress, but correct localization requires a functional HOG pathway. What are the Sho1p/Sln1p osmosensors detecting that triggers signaling through the HOG pathway that

is necessary for optimal Mnn1p localization? Perhaps the two HOG receptors act to sense osmotic perturbations caused by changes in the cell wall during normal growth. The mitotic process of budding and the process of mating both require remodeling of the cell wall at particular points. It is thought that weaknesses in the cell wall induced by this remodeling process activate the protein kinase C MAPK (PKC-MPK) pathway to increase the expression of several cell wall biosynthetic enzymes (Cid et al., 1995; Igual et al., 1996; Fig. 8). Null mutations in the *PKC1* gene result in a weakened cell wall and a cell lysis phenotype that is remedied by the presence of high concentrations of solutes in the growth media.

Interestingly, the PKC-MPK pathway is also activated by hypotonic stress and is repressed by activation of the HOG pathway (Davenport et al., 1995). These observations suggest that the HOG and PKC-MPK pathway play opposing roles in the cell. We propose that one of these roles is to balance the plasticity of the cell wall during growth. As a yeast cell buds, shmoos, or undergoes any change in shape associated with growth, the cell wall must concomitantly expand to fit the expanding plasma membrane. In order for the rigid cell wall to undergo a compensatory increase in size it must be weakened at the point of new growth before the new cell wall material is added. As depicted diagrammatically in Fig. 8, the HOG osmosensors could act to detect an area in the cell wall at the point of growth that needs to be softened in order for expansion to occur. The transduction of signals through Hog1p results in upregulation of the exo-β-glucanase Exg1p, suppression of the PKC-MPK pathway, and rearrangements of specific cell wall biosynthetic enzymes within the Golgi (such as Mnn1p) that could alter the composition of the carbohydrate content of newly synthesized mannoproteins and β-glucans added to the cell wall. However, once the wall has been sufficiently weakened, the PKC-MPK pathway would be activated to increase cell wall biosynthesis at this point. This form of give and take regulation by these two complementary pathways would allow for constant sensing of the condition of the cell wall at the point of growth and has put in place two pathways which can also respond to the extreme conditions of hyper- or hypotonic stress. Thus, both the HOG pathway and the PKC-MPK pathway could be acting to coordinate organization and synthesis of cell wall biosynthetic enzymes in order to create a dynamic cell wall that can undergo rapid change, yet still remain a strong protective barrier.

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