Protein Sorting in the Late Golgi of *Saccharomyces cerevisiae* Does Not Require Mannosylated Sphingolipids*

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Glycosphingolipids are widely viewed as integral components of the Golgi-based machinery by which membrane proteins are targeted to compartments of the endosomal/lysosomal system and to the surface domains of polarized cells. The yeast *Saccharomyces cerevisiae* creates glycosphingolipids by transferring mannose to the head group of inositol phosphorylceramide (IPC), yielding mannosyl-IPC (MIPC). Addition of an extra phosphinositol group onto MIPC generates mannosylated IPC (MIPC). HA, hemagglutinin; ORF, open reading frame; GFP, green fluorescent protein; ER, endoplasmic reticulum; CPY, carboxypeptidase Y; ALP, alkaline phosphatase.

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1 The abbreviations used are: GPI, glycosylphosphatidylinositol; IPC, inositol phosphorylceramide; MIPC, mannosyl-IPC; HA, hemagglutinin; ORF, open reading frame; GFP, green fluorescent protein; ER, endoplasmic reticulum; CPY, carboxypeptidase Y; ALP, alkaline phosphatase.

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the yeast Golgi (23). IPC is then mannosylated to yield mannosyl-IPC (MIPC), which in turn can receive a second phospohoinositol group from phosphatidylinositol to generate the final and by far most abundant sphingolipid, M(IP)2C (22). MIPC and M(IP)2C synthesis occurs in the lumen of the Golgi (22, 24). Whereas IPC is highly enriched in Golgi and vacuolar membranes, the largest amounts of MIPC and M(IP)2C are found in the plasma membrane (25). Hence, the yeast Golgi seems to be a branching point in sphingolipid trafficking from where mannosylated sphingolipids selectively migrate to the cell surface and sphingolipids without the sugar moiety reach the vacuole. However, direct evidence that mannosylated sphingolipids play a role in cargo sorting to the cell surface is lacking.

Addressing the biological function of mannosylated sphingolipids in yeast is hampered by the fact that little is known about the enzyme(s) responsible for their synthesis. Three structurally unrelated genes have been implicated in the mannosylation of IPC. The Vrg4 gene encodes a nucleotide sugar transporter that mediates GDP-mannose import into the Golgi lumen (24). Besides being essential for IPC mannosylation, Vrg4 also affects N-linked and O-linked glycoprotein modifications (24). Null mutations in either the Csg1 or Csg2 gene cause a reduction in, but do not completely eliminate IPC synthesis (26, 27). Csg1p is predicted to have a catalytic function since it contains a region of 93 amino acids with homology to the yeast α-1,6-mannosyltransferase, Och1p (27). The function of Csg2p is less obvious. Csg2p contains an EF-Ca2+-binding domain and has been localized to the ER where it may play a role in Ca2+ homeostasis (28). The recent finding that Csg2p forms a complex with Csg1p raises the possibility that IPC mannosyltransferase activity in yeast is regulated by Ca2+ through Csg2p (29).

Yeast open reading frame YBR161w, recently renamed CSH1, encodes a protein exhibiting strong similarity to the putative sphingolipid mannosyltransferase, Csg1p (27, 29). Here we report that Csh1p is functionally homologous to Csg1p and provide evidence that Csg1p and Csh1p function as two putative sphingolipid mannosyltransferases. Loss of Csg1p and Csh1p had no effect on the delivery of vacuolar proteins or on the packaging of cell surface components into distinct classes of secretory vesicles. From these results, we conclude that the organization of the various post-Golgi delivery pathways in yeast does not depend on production of mannosylated sphingolipids.

Experimental Procedures

Strains and Plasmids—Unless indicated otherwise, yeast strains were grown at 28 °C to mid-logarithmic phase (0.5–1.0 OD600 in synthetic dextrose (S.D.) medium or in yeast extract-peptone-dextrose (YPD) medium. Yeast transformations were carried out as described (30). The yeast mutants Δeps12Δ,Δanm3,Δanp1,Δmnt100 and Δcan1 were all derived from the strain SEY6210 (MATa ura3-52 his3 Δsso2 leu2-3,112 trp1 Δsso1 suc2-29 lys2-801) and have been described elsewhere (31, 32). All other gene deletion phenotypes were characterized in the strain EHY227 (MATa sec6-4 TPI1:SUC2:TRP1 ura3-52 his3 Δsso2 leu2-3,112 trp1 Δsso1 suc2-29 lys2-801) and have been described elsewhere (31, 32). All other gene deletion phenotypes were characterized in the strain EHY227 (MATa sec6-4 TPI1:SUC2:TRP1 ura3-52 his3 Δsso2 leu2-3,112 trp1 Δsso1 suc2-29 lys2-801) and have been described elsewhere (31, 32). All other gene deletion phenotypes were characterized in the strain EHY227 (MATa sec6-4 TPI1:SUC2:TRP1 ura3-52 his3 Δsso2 leu2-3,112 trp1 Δsso1 suc2-29 lys2-801) and have been described elsewhere (31, 32). All other gene deletion phenotypes were characterized in the strain EHY227 (MATa sec6-4 TPI1:SUC2:TRP1 ura3-52 his3 Δsso2 leu2-3,112 trp1 Δsso1 suc2-29 lys2-801) and have been described elsewhere (31, 32). All other gene deletion phenotypes were characterized in the strain EHY227 (MATa sec6-4 TPI1:SUC2:TRP1 ura3-52 his3 Δsso2 leu2-3,112 trp1 Δsso1 suc2-29 lys2-801) and have been described elsewhere (31, 32). All other gene deletion phenotypes were characterized in the strain EHY227 (MATa sec6-4 TPI1:SUC2:TRP1 ura3-52 his3 Δsso2 leu2-3,112 trp1 Δsso1 suc2-29 lys2-801) and have been described elsewhere (31, 32).

Extraction and Analysis of Secretory Cargo Sorting in Glycosphingolipid-deficient Yeast

Lipid Analysis—Exponentially grown cells (0.5 OD600) were inoculated in 5 ml of S.D. medium containing 10 μCi myo-1H-inositol (16 Ci/mmol; ICN Biomedicals, Eschwege) and grown for 16 h at 30 °C. Cells were harvested by centrifugation, washed twice with 10 ml NaN3 and lipids extracted by bead shaking in H2O/methanol/chloroform (5:16:16). The organic extracts were dried and subjected to butyl alcohol/ water partitioning. Lipids recovered from the butyl alcohol phase were deacylated by mild base treatment using 0.2 n NaOH in methanol. After neutralizing with 1 n acetic acid, lipids were extracted with chloroform/methanol (2:1). The TLC plate was dipped in 0.4% 2,5-diphenyloxazole dissolved in 2-methylpropanol supplemented with 10% xylenol (37) and 1H-labeled lipids detected by fluorography using Kodak X-Omat S films exposed at ~ 80 °C. Alternatively, 1H-labeled lipids were detected by exposure to BAS-TR2040 imaging screens (Fuji, Japan) and read out on a BIO-RAD Personal Molecular Image Analyzer (BioRad, Hercules, CA).

Analysis of IPC Mannosyltransferase Activity in Cell Extracts—Exponentially grown Δcsg1Δcsh1 cells (2.5 OD600) were inoculated in 50 ml S.D. medium containing 100 μCi myo-1H-inositol and then grown for 16 h at 30 °C. Cells were harvested by centrifugation, washed twice with 10 ml NaN3, and lysed by bead shaking in lysis buffer (50 mM HEPES, pH 7.2, 10 mM MgCl2, 1 mM NEM) supplemented with fresh protease inhibitors. After removal of unbroken cells (500 × g, 10 min), membranes were collected (100,000 × g, 60 min) and solubilized in 1 ml of lysis buffer containing 1% and fresh protease inhibitors. After incubation for 60 min at room temperature, the extract was centrifuged (100,000 × g, 60 min), and 50-μl aliquots were stored at −80 °C. In addition, 400 OD600 of non-radio labeled, exponentially grown wild-type and Δcsg1Δcsh1 cells transformed with multicopy CSG1, CSH1, or control plasmids were lysed by bead shaking in 4 ml of ice-cold lysis buffer containing fresh protease inhibitors. Upon removal of unbroken cells, total membranes were collected, resuspended in 1 ml of ice-cold lysis buffer containing 1% Triton X-100, and rotated at 4 °C for 4 min. The assays, 50 μl of radiolabeled extract was mixed with 150 μl of unlabeled extract and then preincubated with 10 mM GDP-mannose (Sigma-Aldrich) for 10 min at 30 °C. Reactions were diluted 10-fold in lysis buffer and then incubated for 2 h at 30 °C. Reactions were stopped by adding 6.4 ml of chloroform:methanol (1:2.2). Lipids were extracted, deacylated, and separated by TLC as above.

Antibodies and Immunoblotting—Peptides corresponding to C-termi- nal domains of Csg1p and Csh1p (Fig. 1) were synthesized and then coupled to a carrier before immunization of rabbits. The resulting antisera were affinity-purified against peptides coupled to NHS-acti- vated Sepharose 4 Fast Flow according to instructions of the manufac- turer (Amersham Biosciences). Affinity-purified antibodies were used at a dilution of 1:1,000 for immunoprecipitation and immuno- microscopy. Rabbit polyclonal antibodies to CPY, Goa1p, Pep12p, Tlg1p, and Tlg2p were described previously (38). Rabbit polyclonal antibodies to Sso2p were provided by S. Keränen, (Biotecnology and Food Research, Espoo, Finland) and to Gas1p by H. Riezman (Sciences II, Geneve, Switzerland). The Myc epitope was detected with anti-Myc antibody 3F10, mouse monoclonal antibody 12CA5 (Roche Applied Science), and rabbit polyclonal antibodies to Gas1p (Santa Cruz Biotechnology) and the HA epitope with rat monoclonal antibody 3F10, mouse monoclonal antibody 12CA5 (Roche Applied Science) or rabbit polyclonal antibodies (Santa Cruz Biotechnology). For immunoblotting, all antibody incubations were carried out in phosphate-buffered saline containing 5% dried milk and 0.5% Tween-20. A chemiluminescent detection system with peroxor 1600 (BioRad) blots were developed using a chemiluminescent substrate kit (Pierce). Chemiluminescent bands were quantified using a GS-710 cal-ibrating imaging densitometer (BioRad) with QuantityOne software.

Immunofluorescence Microscopy—Exponentially grown cells were
beads slurry, 5 mg/ml bovine serum albumin, and 15

The reactions were performed using magnetic Dynabeads protein G (Dynal Biotech GmbH, Hamburg, Germany) loaded with mouse anti-HA (12CA5) or anti-Myc (9E10) monoclonal antibodies. Beads were incubated with antibodies for 40 min at room temperature and antibodies bound quantified by SDS-PAGE. Anti-HA beads contained 0.35 μg of 12CA5/μl of bead-sluurry and control beads contained 0.1 μg of 9E10/μl bead-sluurry. For immunoisolation of Pma1p-containing vesicles, a 300-μl reaction was prepared in lysis buffer containing 126 μl Dynabeads slurry, 5 mg/ml bovine serum albumin, and 15 μl membranes from Nycodenz gradient PM-ATPase peak fractions obtained by fractionating membranes derived from 1 g of cells. The reactions were rotated gently at 4 °C for 2 h. Supernatants were subjected to centrifugation (100,000 × g, 1 h, 4 °C), and membrane pellets were resuspended in 100 μl of SDS sample buffer. Beads were washed twice for 30 min in 1 ml of bovine serum albumin-containing lysis buffer, twice in lysis buffer, and resuspended in 75 μl of SDS sample buffer. Bound and unbound membranes were analyzed by immunoblottting.

**RESULTS**

**CSH1 Encodes a Novel Putative IPC Mannosyltransferase—** Comparative sequence analysis revealed that Csh1p is 67% identical to Csg1p and has the same predicted protein topology, namely a putative N-terminal signal sequence and two potential transmembrane segments localized to the C-terminal half of the protein (Fig. 1). Between the signal sequence and first transmembrane segment there is a region of 93 residues sharing 29% identity with the luminal portion (residues 96–197) of the yeast α,1,6-mannosyltransferase, Och1p (39). This region contains a conserved DXXD motif that occurs in a wide range of glycosyltransferase families and likely forms part of a catalytic site (40).

Csg1p is required for accumulation of mannosylated sphingolipids in yeast and its similarity to Csh1p raises the possibility that Csh1p represents an alternative IPC mannosyltransferase that functions independently of Csg1p. To investigate this possibility, we constructed yeast strains in which the ORFs of CSG1, CSH1, or both were removed. TLC analysis of alkaline-treated lipid extracts prepared from myo-[3H]inositol-labeled cells showed that, compared with the wild-type strain, the Δcsg1 mutant produced greatly reduced levels of the mannosylated sphingolipids MIPC and M(IP)2C, and accumulated IPC-C and IPC-D (Fig. 2A, lanes 1 and 2; note that IPC-C contains a monohydroxylated C26 fatty acid whereas the C26 fatty acid in IPC-D is dihy-
Fig. 2. Csg1p and Csh1p have redundant functions in sphingolipid mannosylation. A, deletion of CSG1 and CSH1 abolishes IPC mannosylation. Yeast cells were labeled overnight with myo-[3H]inositol and the lipid extracts either deacylated by mild alkaline hydrolysis with NaOH (+) or control incubated (−). Lipids were extracted, separated by TLC, and then visualized by autoradiography as described under Experimental Procedures. Lane 1, wild-type; lane 2, Δcsg1; lane 3, Δcsh1; lane 4, Δcsg1Δcsh1; lanes 5 and 6, Δip1. Note that IPT1 is known to have an essential function in M(IP)2C synthesis. B, analysis of IPC mannosyltransferase activity in Triton X-100 extracts derived from wild-type and Δcsg1Δcsh1 cells. Extracts prepared from myo-[3H]inositol-labeled Δcsg1Δcsh1 cells were either control-incubated (lane 1) or mixed with extracts of unlabeled wild-type cells (lanes 2 and 3), Δcsg1Δcsh1 cells (lane 4), or Δcsg1Δcsh1 cells transformed with a multicopy vector containing CSG1 (lane 5) or CSH1 (lane 6). Incubations were performed in the presence (+) or absence (−) of 1 mM GDP-mannose as described under Experimental Procedures. Lipids were extracted, deacylated, and then separated by TLC before autoradiography.

...dronylated; (41). Unlike Δcsg1 cells, the Δcsh1 mutant produced IPC and mannosylated IPC species at ratios similar to those in wild-type cells (Fig. 2A, lanes 1 and 3). In the Δcsg1Δcsh1 double mutant, however, production of MIPC and M(IP)2C was completely abolished (Fig. 2A, lane 4). These results are consistent with those reported in a recent study (29) and indicate that Csg1p and Csh1p have redundant functions in IPC mannosylation.

The block in MIPC and M(IP)2C synthesis observed in Δcsg1Δcsh1 cells can be explained by a complete loss of IPC mannosyltransferase activity, but may also be due to a defective delivery of IPC or GDP-mannose to the transferase-containing compartment. To explore these possibilities, we analyzed the IPC mannosyltransferase activity in detergent extracts derived from wild-type and Δcsg1Δcsh1 cells. To this end, Triton X-100 extracts prepared from myo-[3H]inositol-labeled Δcsg1Δcsh1 cells were mixed with extracts from unlabeled wild-type or mutant cells, and then incubated in the presence or absence of externally added GDP-mannose. When extracts from inositol-labeled Δcsg1Δcsh1 cells were incubated with unlabeled wild-type cell extracts, radioactive IPC was converted to MIPC and M(IP)2C in a GDP-mannose-dependent manner (Fig. 2B, lanes 2 and 3). In contrast, addition of GDP-mannose to Δcsg1Δcsh1 cell extracts was not sufficient to support MIPC and M(IP)2C synthesis (Fig. 2B, lanes 1 and 4). However, when inositol-labeled Δcsg1Δcsh1 extracts were incubated with extracts from unlabeled Δcsg1Δcsh1 cells transformed with the CSG1 or CSH1 gene on a multicopy plasmid, the GDP-mannose-dependent mannosylation of IPC was restored (Fig. 2B, lanes 5 and 6). These results indicate that Δcsg1Δcsh1 cells are defective in IPC mannosyltransferase activity rather than in IPC or GDP-mannose transport.

To investigate whether loss of Csg1p and Csh1p also affects protein mannosylation, we next examined the glycosylation state of invertase produced in wild-type and mutant strains. This periplasmic enzyme undergoes extensive outer chain mannan addition on 8–10 of its N-linked glycans while passing through the Golgi (42). Consequently, its electrophoretic mobility is increased when enzymes responsible for mannan synthesis are removed (32, 43). Immunoblot analysis of cells expressing Myc-tagged invertase showed that the gel mobility of the protein produced in the Δcsg1Δcsh1 mutant was indistinguishable from that in wild-type cells (Fig. 3, lanes 1 and 3). In contrast, loss of mannosyltransferases involved in the initiation (Van1p) or elongation (Anp1p, Mnn10p) of the mannan backbone caused a substantial increase in the gel mobility of invertase (Fig. 3, lanes 4–6). These results demonstrate that protein mannosylation occurs independently of Csg1p and Csh1p, and that the defect in sphingolipid mannosylation in Δcsg1Δcsh1 cells is specific.

Collectively, our results suggest that yeast contains two independent IPC mannosyltransferases: one encoded by CSG1 and likely responsible for producing the bulk of mannosylated IPC, and the second one encoded by CSH1 and corresponding to a minor IPC mannosyltransferase activity.

Q. Lisman and J. C. M. Holthuis, unpublished data.
Membrane Topology of Csg1p and Csh1p—Golgi-associated glycosyltransferases generally have a type II topology with a short cytoplasmic tail and a large catalytic domain in the lumen (e.g. Mnt1p). Csg1p and Csh1p, on the other hand, contain a putative N-terminal signal sequence and two potential membrane spans that predict a different membrane topology where both termini of the protein are situated in the lumen (Fig. 4A). To test this prediction, we introduced three copies of Csg1p-HA in wild-type and various mannosyltransferase mutant cells expressing Myc-tagged invertase, to control for equal loading. The positions of size markers (kDa) are indicated. Lane 1, wild-type; lane 2, Δcsg1; lane 3, Δcsg1Δcsh1; lane 4, Δanp1; lane 5, Δmnn10; lane 6, Δsan1.

Csg1p and Csh1p Co-localize with IPC Synthase to the medial-Golgi (23, 44). To verify co-localization of Csg1p/Csh1p and Aur1p by a complementary method, cells expressing Aur1p with three copies of the HA epitope inserted at its cytosolic C terminus were lysed and Aur1p-containing membranes immunoprecipitated using anti-HA antibodies to magnetic beads. This method allowed the isolation of nearly 20% of the Aur1p-HA containing membranes from a cell lysate (Fig. 8A). Strikingly, a similar fraction of Csg1p-containing membranes was bound to the beads. Binding of Aur1p-HA- and Csg1p-containing membranes was strictly dependent on the presence of anti-HA antibodies on beads. Membranes containing the ER marker Dpm1p did not bind. As additional control, the immunoprecipitation procedure was repeated on lysates of cells expressing the HA-tag on the cytosolic N terminus of the vacuolar t-SNARE, Vam3p. As shown in Fig. 8B, anti-HA beads brought down nearly half of the Vam3p-containing membranes. Under these conditions, neither Csg1p- nor Dpm1p-containing membranes did bind. Collectively, our results indicate that Csg1p and Csh1p primarily reside with the IPC synthase in a medial compartment of the yeast Golgi.

Golg-to-Vacuole Transport Pathways Are Unaffected in Mutants Deficient in Mannosylated Sphingolipids—in mammals, glycosphingolipids have been implicated in targeting membrane proteins from the Golgi to compartments of the endosomal/lysosomal system (20) and to the surface domains of polarized cells (12–15). To investigate whether mannosylated sphingolipids in yeast serve a similar role, the Δcsg1Δcsh1 mutant was analyzed for possible defects in post-Golgi delivery pathways.

In yeast, biosynthetic transport of proteins from the Golgi to the vacuole proceeds through two separate pathways, the carboxypeptidase Y (CPY) pathway and the alkaline phosphatase (ALP) pathway. Whereas the CPY pathway mediates a clathrin-dependent delivery of vacuolar proteins via late (prevacuolar) endosomes, the ALP pathway provides an alternative, clathrin-independent route that bypasses late endosomes and requires the AP-3 adaptor protein complex (2). The vacuolar protease CPY is synthesized as a p1 precursor in the ER, modified to a p2 precursor, and released from the AP-3 adaptor protein complex (2). The vacuolar protease CPY is synthesized as a p1 precursor in the ER, modified to a p2 precursor, and released from the AP-3 adaptor protein complex (2).
The vacuolar membrane protein ALP is synthesized as a precursor that undergoes proteolytic processing in the vacuole yielding a smaller mature form (46). As shown in Fig. 9, Δsgf1Δcsh1 cells displayed no significant delay in ALP maturation. In the Δpep12Δcsh1 mutant, on the other hand, ALP maturation was abolished. These results show that mannosylated sphingolipids in yeast do not serve a critical function in clathrin- or AP-3-mediated protein transport from the Golgi to the vacuole. Moreover, the efficient processing of newly synthesized CPY and ALP in Δsgf1Δcsh1 cells indicates that blocking sphingolipid mannosylation has no general effect on forward transport through the Golgi apparatus. Consistent with this notion, Δsgf1Δcsh1 and wild-type cells contain similar amounts of Golgi-modified invertase (Fig. 3).

**Mannosylated Sphingolipids Are Not Required for Sorting Cell Surface Proteins into Distinct Classes of Secretory Vesicles**

The characterization of secretory vesicles that accumulate in late exocytic yeast mutants (e.g. sec1, sec6) has identified two vesicle populations with different densities and distinct cargo proteins, indicating the existence of two parallel routes from the Golgi to the plasma membrane (7–9). The more abundant, lighter density vesicles contain the major plasma membrane ATPase Pma1p whereas the denser vesicles contain the periplasmic enzymes invertase and acidic phosphatase. Sorting invertase into the dense class of vesicles requires clathrin and an intact Golgi-to-late endosome transport pathway (9, 47). From these observations, it has been suggested that invertase is sorted from Pma1p at the late Golgi for delivery to late endosomes, from where high-density vesicles bud that carry invertase to the cell surface.

To investigate whether mannosylated sphingolipids play a role in the organization of membrane trafficking to the cell surface, we disrupted the CSG1 and CSH1 genes in the late secretory mutant sec6-4 and analyzed the strain for defects in secretory cargo sorting. The sec6-4 strain harbors a temperature-sensitive mutation in a component of the exocyst protein complex that is required for polarized fusion of exocytic vesicles with the plasma membrane (48). The sec6-4 mutant grows like wild-type cells at 25 °C, but growth ceases at 38 °C, and cells accumulate plasma membrane ATPase- and invertase-containing vesicles that can be separated by equilibrium isodensity centrifugation on Nycodenz gradients (7). To this end, sec6-4 cells were grown at 25 °C, shifted to 38 °C for 90 min, lysed and then subjected to a 13,000 × g spin to remove most of the ER, nuclei, vacuoles, mitochondria, and plasma membrane. Next, a high-speed (100,000 × g) membrane pellet enriched in secretory vesicles was collected and loaded at the bottom of a linear 16–26% Nycodenz gradient in 0.8 M sorbitol. As shown in Fig. 10, gradient fractionation of membranes from 38 °C-shifted cells resulted in two peaks of enzyme activities that were absent when fractionation was performed on 25 °C-grown cells: a low density peak (fractions 4–9) containing ATPase activity and a higher density peak (fractions 10–15) containing invertase activity (note that the invertase peak found near the bottom of the gradient (fractions 17–20) likely corresponds to the cytoplasmic, non-glycosylated form of the enzyme). Western blot analysis revealed that Pma1p co-fractionates with the lower density membranes, confirming that the detected ATPase activity is due to this protein. In contrast, markers for the ER (Dpm1p) and Golgi (Gos1p) did not peak with either vesicle population, and their levels in gradients of 25 °C grown and 38 °C shifted cells were very similar (Fig. 10 and data not shown). This indicates that the detected ATPase and invertase peaks are not due to the accumulation or fragmentation of the ER or Golgi apparatus.

The fractionation profiles of ATPase activity, Pma1p and invertase in gradients of 38 °C-shifted sec6-4Δcsg1Δcsh1 cells closely resembled those found for sec6-4 cells (Fig. 10). This shows that mannosylated sphingolipids are required neither for the biogenesis of the light or the dense class of secretory
vesicles, nor for segregating Pma1p and invertase into these different vesicle populations. Since glycosphingolipids have previously been implicated in the sorting of GPI-linked proteins (13–15), we wished to determine which of the two secretory vesicle classes in yeast mediates transport of the GPI-anchored cell surface protein Gas1p. Western blot analysis revealed that Gas1p co-fractionates with Pma1p and ATPase activity in gradients of 38°C-shifted sec6-4 cells, regardless of whether Csg1p and Csh1p were present (Fig. 10). A similar fractionation profile was observed for the GPI-linked protein Ysp1p (data not shown). The co-fractionation of GPI-linked proteins and Pma1p suggests that these proteins are packaged into a common carrier. However, it is also possible that GPI-linked proteins are sorted into a different class of vesicles with fractionation properties similar to that of Pma1p-transporting vesicles. To distinguish between these possibilities, we immunosolated Pma1p-containing vesicles from 38°C-shifted sec6-4 and sec6-4Δcsg1Δcsh1 cells, and assessed whether these vesicles contained Gas1p. Immunosolations were performed with membranes derived from 38°C-shifted cells expressing Pma1p with three copies of the HA epitope inserted at its cytosolic N terminus. Membranes were fractionated on a Nycodenz gradient as above and Pma1p-HA containing vesicles isolated from the ATPase peak fraction (fraction 7) using anti-HA monoclonal antibodies bound to magnetic beads. This allowed the isolation of about 70% of Pma1p-HA and 50% of Gas1p present in the sec6-4 ATPase peak fraction (Fig. 11). Immunolocalization of Pma1p-HA from the sec6-4Δcsg1Δcsh1-derived ATPase peak was less efficient (24% total), but brought down a similar portion of Gas1p (16%). In both cases, binding of Pma1p-HA and Gas1p containing membranes was strictly dependent on the presence of anti-HA antibodies on the beads. It therefore appears that Pma1p and Gas1p are packaged into a common transport carrier for delivery to the cell surface. Moreover, our findings demonstrate that sorting of GPI-linked proteins in the late secretory pathway of yeast essentially occurs independently of mannosylated sphingolipids.

**DISCUSSION**

The results presented in this study indicate that the yeast genes *CSG1* and *CSH1* encode proteins with a primary and redundant function in the mannosylation of phosphoinositol-containing sphingolipids. Our finding that Δcsg1Δcsh1 cells exhibit a specific and complete block in sphingolipid mannosylation offered an opportunity to explore the potential role of mannosylated sphingolipids in secretory cargo sorting in yeast.

A primary function of Csg1p and Csh1p as sphingolipid mannosyltransferases is supported by the following observations. First, removal of Csg1p and Csh1p suffices to abolish MIPC and M(IP)2C synthesis, resulting in accumulation of MIPC precursor, IPC. Second, biochemical characterization of the IPC mannosyltransferase activity in cell extracts revealed that the inability of Δcsg1Δcsh1 cells to generate MIPC and M(IP)2C cannot be attributed to a defective delivery of GDP-mannose or IPC to the transferase-containing compartment. Third, Csg1p and Csh1p share a region of homology with the yeast α-1,6-mannosyltransferase Och1p and contain a con-

**Fig. 5.** Subcellular fractionation of Csg1p and Csh1p. A, immunoblots containing equal amounts of total protein extracts prepared from Δcsg1 cells (lane 1), Δcsh1 cells (lane 2), or wild-type cells transformed with a multicopy vector containing *CSG1* (lane 3) or *CSH1* (lane 4). Blots were stained with polyclonal anti-Csg1p or anti-Csh1p antibodies that were raised against synthetic peptides corresponding to areas with the least sequence homology (see Fig. 1). B, sucrose gradient fractionation of membranes. A high-speed membrane pellet (100,000 × g) prepared from yeast cells expressing HA-tagged Aur1p was fractionated on a sucrose density gradient. Fractions were assayed for IPC synthase and Kex2p enzyme activities as described under “Experimental Procedures.” Fractions were also analyzed by immunoblotting using polyclonal antibodies against Csg1p, Csh1p and several organelar markers. A mouse monoclonal anti-HA antibody was used to detect Gas1p containing membranes was strictly dependent on the presence of anti-HA antibodies on the beads. It therefore appears that Pma1p and Gas1p are packaged into a common transport carrier for delivery to the cell surface. Moreover, our findings demonstrate that sorting of GPI-linked proteins in the late secretory pathway of yeast essentially occurs independently of mannosylated sphingolipids.

**Fig. 6.** Colocalization of Csg1p and Csh1p by immunofluorescence. A, immunofluorescence confocal micrographs of yeast cells stained with affinity-purified rabbit polyclonal antibodies directed against Csg1p (α-Csg1p) or Csh1p (α-Csh1p). Note that anti-Csg1p fluorescence is observed in wild-type (*CSH1*) cells, but not in Δcsg1 cells, whereas anti-Csh1p fluorescence occurs only in cells overexpressing Csh1p from a multicopy plasmid (*CSH1*-2µ). B, double-label immunofluorescence confocal micrographs comparing the localization of HA-tagged Csg1p (*CSH1*-HA) with Csh1p expressed from a multicopy plasmid (*CSH1*-2µ). Anti-HA staining was with rat monoclonal antibody 3F10. Most of the Csg1p-positive structures were also positive for Csh1p (arrows). Bars, 3 µm.
FIG. 7. Csg1p and Csh1p colocalize with Aur1p by immunofluorescence. Double-label immunofluorescence confocal micrographs comparing the localization of Csg1p and Csh1p with that of GFP-tagged Sed5p (cis Golgi) or HA-tagged Aur1p (medial–Golgi). Staining of Csg1p and Csh1p was performed as in Fig. 6 with Csh1p expressed from a multicopy plasmid (CSH1-2μ). Staining of HA-tagged Aur1p was with the rat mAb, 3F10. Csg1p and Csh1p positive structures were often positive for Aur1p, but not for Sed5p. Bar, 5 μm.

served DXD motif, which is part of a catalytic site found in many known glycosyltransferases (40). Fourth, Csg1p and Csh1p are localized to the yeast Golgi where sphingolipid mannosylation is known to occur (49). Fifth, protease protection analysis and the utilization of N-linked glycosylation sites in Csg1p predict a membrane topology with the Och1p-homology domain and DXD motif positioned in the Golgi lumen, hence in keeping with the fact that sphingolipid mannosylation takes place on the luminal aspect of the Golgi (24).

Whether Csg1p and Csh1p are IPC mannosyltransferases or represent catalytic subunits of two distinct IPC mannosyltransferase complexes remains to be established. Recent work revealed that Csg1p and Csh1p occur in a complex with Csg2p, a putative Ca\(^{2+}\)-binding membrane protein lacking homology to glycosyltransferases. Several lines of evidence suggest that the role of Csg2p in these complexes is regulatory rather than enzymatic (29). In any case, the latter study and our present findings point to the existence of two independent IPC mannosyltransferases in yeast. So why would yeast need two distinct sphingolipid mannosyltransferases? We found that Csg1p and Csh1p are co-localized with IPC synthase to a medial compartment of the Golgi. Hence, the expression of two sphingolipid mannosyltransferases unlikely serves to accommodate a need for synthesizing mannosylated sphingolipids at different cellular locations. It should be noted that yeast IPC is not a monomolecular lipid species, but represents a mixture of molecules that differ in the chain length and the extent of hydroxylation of both the sphingoid base and fatty acid (22). This raises the possibility that the two IPC mannosyltransferases differ in substrate specificity. Indeed, metabolic labeling of Δcsg1 and Δcsh1 cells with \(^{3}H\)dihydrosphingosine revealed some differences in activity between Csg1p and Csh1p toward particular molecular species of IPC (29). The biological implications of this finding remain to be established.

A key function attributed to sphingolipids is their ability to self-associate into membrane microdomains/rafts, especially when sterols are present. Formation of sphingolipid/sterol-rich microdomains is important for lateral sorting of membrane proteins, in particular those containing a GPI anchor (50, 51). Previous work in yeast has shown that sphingolipid depletion affects both raft association and cell surface delivery of Pma1p and GPI-anchored proteins, i.e., Gas1p (17–19). In these studies, sphingolipid synthesis was blocked using a conditional allele of serine palmitoyltransferase activity, which catalyzes
the first committed step of sphingolipid synthesis (22). Precisely what structural determinants on sphingolipids are critical for a correct delivery of cell surface components has remained an open issue. The availability of a yeast strain with a primary block in IPC mannosylation led us to investigate whether maturation of the sphingolipid head group serves a role in organizing membrane trafficking to the plasma membrane.

Yeast harbors two transport routes from the Golgi to the plasma membrane. One route mediates delivery of Pma1p and Gas1p and with polyclonal antibodies against the GPI-anchored protein, Gas1p, or the sec6-4/membrane pellets from temperature-shifted, Pma1p-HA-expressing sec6-4 cells (Fig. 10) were used to immunoisolate Pma1p-containing vesicles with anti-HA monoclonal antibodies (αHA) bound to Dynabeads protein G. Immunoisolations with Dynabeads containing anti-Myc monoclonal antibodies served as control. The percentage of immunoprecipitated Pma1p and Gas1p was determined by Western blot analysis. B, beads; S, supernatant.

While the other one carries the secretory enzyme invertase among its cargo (7). Our data show that the GPI-anchored protein, Gas1p, segregates from the invertase route and is packaged with Pma1p into a common transport carrier for delivery to the plasma membrane. Blocking sphingolipid mannosylation by disrupting CSG1 and CSH1 had no effect on the sorting of these cargo molecules and we observed that temperature-shifted sec6-4Δcsg1Δcsh1 cells accumulate population of secretory vesicles with characteristics indistinguishable from those generated in sec6-4 cells. Moreover, thin-section electron microscopy revealed that both cell types accumulate very similar amounts of secretory vesicles (data not shown). Hence, mannosylated sphingolipids appear fully dispensable for the biogenesis of the two classes of secretory vesicles that mediate cell surface transport in yeast.

Also transport through the Golgi seems unaffected by a block in sphingolipid mannosylation. This can be inferred from the fact that Δcsg1Δcsh1 cells deliver newly synthesized vacuolar proteins at wild-type kinetics and do not contain higher levels of Golgi-modified invertase than wild-type cells. Collectively, our data indicate that the plasma membrane trafficking defects previously reported for mutants blocked in the first committed step of sphingolipid synthesis cannot be ascribed to a deficiency in complex mannosylated sphingolipids (17–19). In fact, we found no evidence for a critical function of mannosylated sphingolipids in any of the known post-Golgi delivery pathways in yeast.

It has been shown that GDP-mannose transport into the Golgi lumen is essential for cell growth (24). Since mannosylation of proteins in the Golgi does not appear to be essential, it has been suggested that the strict requirement of GDP-mannose transport involves its effect on sphingolipid mannosylation (24). This idea is inconsistent with our present findings. Mannosylated sphingolipids are abundant components of the yeast plasma membrane, accounting for up to 8% of its total mass (25, 52). Therefore, it is somewhat surprising that a complete block in their synthesis has little if any effect on cell growth, at least under standard growth conditions (YEPD or synthetic medium at 30 °C). Strains deleted for CSG1 are hypersensitive for calcium (27) and we and others (29) found that this phenotype is aggravated upon additional loss of CSH1. This calcium sensitivity is likely due to accumulation and/or mislocalization of IPC (more specifically IPC-C) rather than depletion of MIPC or M(IP)2C (27). Interestingly, recent work suggests that M(IP)2C synthesis is controlled in coordination with multidrug resistance in yeast, and that this lipid serves a role in determining the activity of drug transporters in and/or the permeability properties of the plasma membrane (53). How mannosylated sphingolipids contribute to the functional orga-
zation of the plasma membrane poses an intriguing problem for future research.

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