Evidence for Coupled Biogenesis of Yeast Gap1 Permease and Sphingolipids: Essential Role in Transport Activity and Normal Control by Ubiquitination

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Current models for plasma membrane organization integrate the emerging concepts that membrane proteins tightly associate with surrounding lipids and that biogenesis of surface proteins and lipids may be coupled. We show here that the yeast general amino acid permease Gap1 synthesized in the absence of sphingolipid (SL) biosynthesis is delivered to the cell surface but undergoes rapid and unregulated down-regulation. Furthermore, the permease produced under these conditions but blocked at the cell surface is inactive, soluble in detergent, and more sensitive to proteases. We also show that SL biogenesis is crucial during Gap1 production and secretion but that it is dispensable once Gap1 has reached the plasma membrane. Moreover, the defects displayed by cell surface Gap1 neosynthesized in the absence of SL biosynthesis are not compensated by subsequent restoration of SL production. Finally, we show that down-regulation of Gap1 caused by lack of SL biogenesis involves the ubiquitination of the protein on lysines normally not accessible to ubiquitination and close to the membrane. We propose that coupled biogenesis of Gap1 and SLs would create an SL microenvironment essential to the normal conformation, function, and control of ubiquitination of the permease.

INTRODUCTION

Biological membranes were long seen as a “fluid mosaic” of proteins simply dispersed in a homogeneous lipid bilayer (Singer and Nicholson, 1972). This view contrasts with current models of the plasma membrane that take into account the heterogeneity of lipids in length and structure and the very high density of intrinsic proteins, ~30,000 per μm^2 (Jacobson *et al.*, 2007). These models also integrate the important notion of lateral heterogeneity, i.e., that the plasma membrane contains domains of various lipid compositions and physical properties. Among these domains, lipid rafts have been the subject of many studies. Although the definition of rafts is still debated (Pike, 2006), they are generally viewed as small domains of various sizes (~10–200 nm in diameter) that are formed by the dynamic assembly of sphingolipids with sterols (Simons and Ikonen, 1997; Jacobson *et al.*, 2007). These domains selectively include or exclude proteins, and they are thought to exist in a liquid-ordered phase distinct from the bulk liquid-disordered phase of the membrane (Brown and London, 1998). Their tightly packed state makes them more resistant to solubilization by non-ionic detergents such as Triton X-100, so that they yield detergent-resistant membranes (DRMs) after detergent extraction in the cold (London and Brown, 2000). Despite the

limitations of detergent-dependent methods and the caution that must be exerted when interpreting the resulting data, the isolation of DRMs remains a useful tool for investigating protein–lipid interactions and membrane domains (Brown and London, 1998; Shogomori and Brown, 2003; Lichtenberg *et al.*, 2005). A complementary approach consists in visualizing raft components directly in the plasma membrane of living cells. In *Saccharomyces cerevisiae*, all the plasma membrane proteins whose detergent solubility has been examined so far have been found in DRMs (Malinska *et al.*, 2004; Lauwers and André, 2006). Yet, proteins recovered in the same DRM can occupy different membrane domains in intact cells (Malinska *et al.*, 2003), raising the debated question of the exact relationship between DRMs in vitro and lipid domains in vivo (London and Brown, 2000; Shogomori and Brown, 2003; Lichtenberg *et al.*, 2005). Tanner and collaborators have indeed shown that two Triton X-100-insoluble proteins, the proton-ATPase Pma1 and the arginine permease Can1, are located in mutually exclusive discrete domains in the plasma membrane of vegetative cells, whereas the hexose permease Hxt1 is homogeneously distributed at the cell surface (Malinska *et al.*, 2003). The compartment defined by the presence of Pma1 is called the *membrane compartment* occupied by Pma1 (MCP), whereas the *membrane compartment* occupied by Can1 (MCC) contains several other detergent-insoluble proteins, namely, the uracil permease Fur4 (Malinska *et al.*, 2004), the Sur7 protein (Malinska *et al.*, 2004), the tryptophan permease Tat2 (Grossmann *et al.*, 2007), and the heterologously expressed hexose transporter Hup1 of *Chlorella kessleri* (Grossmann *et al.*, 2006). Furthermore, plasma membrane structures named eisosomes and proposed to be active sites of endocytosis colocalize with MCC patches (Walther *et al.*, 2006). Both MCC and MCP compartments are stable over

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Abbreviations used: DHS, dihydrosphingosine; DRM, detergent-resistant membrane; PHS, phytosphingosine; SL, sphingolipid.

time (Malinska *et al.*, 2003), and they are independent of the actin or microtubule cytoskeleton and of the cell wall (Malinska *et al.*, 2004). In a recent study, it was found that the patchy distribution of Hup1, Can1, Fur4, and Tat2 (but not Sur7) is lost upon membrane depolarization (Grossmann *et al.*, 2007). From all these results, it seems that the mechanisms responsible for detergent insolubility and those controlling the lateral distribution of yeast plasma membrane proteins obey to rules that are still poorly understood. According to the lipid shell model (Anderson and Jacobson, 2002), some transmembrane proteins are encased during their biosynthesis into a preassembled lipid microenvironment made of sphingolipids and sterols and corresponding to that of their future host membrane domain. Such lipid shells might confer to these membrane proteins both their detergent insolubility and sorting to specific subdomains of the plasma membrane. Several data sets are consistent with the lipid shell model. For example, a subpopulation of immobilized and disordered lipids is observable by electron-spin resonance in native membranes or lipid-protein systems, but not in protein-free membranes (Lee, 2003). Furthermore, the high-resolution structure of some membrane proteins shows lipid molecules tightly packed or even distorted against the surface of the protein to form a shell (Gonen *et al.*, 2005; Lee, 2005).

The yeast general amino acid permease Gap1 (Grenson *et al.*, 1970) is an attractive system for investigating the mechanisms controlling the membrane trafficking of cell surface proteins. The fate of the Gap1 permease is regulated according to the nitrogen supply conditions of the medium (Magasanik and Kaiser, 2002; Haguenauer-Tsapis and André, 2004). When cells grow on a nitrogen-poor medium, the *GAP1* gene is highly expressed (Jauniaux and Grenson, 1990) and the Gap1 protein accumulates at the plasma membrane in a highly active and stable form (Grenson, 1983; De Craene *et al.*, 2001). Endocytosis of Gap1 is triggered when a good nitrogen source such as ammonium or an excess of amino acids is added to the medium (Hein *et al.*, 1995; Stanbrough and Magasanik, 1995; Springael and André, 1998). The presence of these nitrogenous compounds probably inactivates Npr1, a protein kinase essential to protection of Gap1 against ubiquitination (De Craene *et al.*, 2001) and under the control of the TOR signaling pathway (Schmidt *et al.*, 1998). Endocytosis of Gap1 requires prior ubiquitination on two lysine residues present in the N-terminal tail of the permease, at positions 9 and 16 (Springael and André, 1998; Soetens *et al.*, 2001), and closer analysis of this process has revealed that the protein undergoes polyubiquitination with K63-type chains (Springael *et al.*, 1999). The ubiquitination of Gap1 requires the Rsp5 (Npi1) ubiquitin ligase (Hein *et al.*, 1995; Springael and André, 1998) acting together with the Bul1 and Bul2 factors (Yashiroda *et al.*, 1996; Helliwell *et al.*, 2001; Soetens *et al.*, 2001). Delivery of internalized Gap1 into the lumen of the vacuole where the protein is degraded requires its prior sorting into the multivesicular body (MVB) pathway (Nikko *et al.*, 2003), and defects at this level lead either to accumulation of the permease at the late endosome or to effective recycling to the plasma membrane by passage through the Golgi.

In a previous article, we reported that Gap1 fractionates with DRMs when it is present at the plasma membrane (Lauwers and André, 2006), and we proposed that this may be true for all cell surface proteins. Newly synthesized Gap1 acquires detergent insolubility at the Golgi level, and this property is preserved until the protein present at the cell surface is internalized by endocytosis. Moreover, sphingolipid neosynthesis seems essential to the accumulation of newly synthesized Gap1 at the cell surface. When this bio-

synthesis is defective, neosynthesized Gap1 is targeted to the endosome/vacuolar degradation pathway (Lauwers and André, 2006), as for Pma1 (Bagnat *et al.*, 2001; Gaigg *et al.*, 2005). In the present study, we have further investigated the role of sphingolipids in controlling the intrinsic function, ubiquitination, and membrane trafficking of the yeast Gap1 protein. We have also tested a prediction of the lipid shell model, i.e., that ongoing sphingolipid biosynthesis should be mostly important during Gap1 biogenesis and secretion, and less after Gap1 has integrated the plasma membrane.

MATERIALS AND METHODS

Strains, Plasmids, and Media

Unless otherwise indicated, all the *S. cerevisiae* strains used in this study derive from RH1800 (Table 1). Cells were grown at 24°C in minimal buffered medium (Jacobs *et al.*, 1980) (pH 6.1) with 3% glucose as carbon source, excepted in the confocal microscopy experiment where cells were grown in YNB medium with 3% glucose. To repress *GAP1* expression, cells were grown with 0.1% glutamine and 100 mM (NH₄)₂SO₄ as nitrogen sources. A medium containing 0.1% proline as nitrogen source was used to induce Gap1 synthesis. Shifts were performed by filtering cells and resuspending them in medium preheated to the permissive or restrictive temperature. Uracil (0.025%) was added to the medium for uracil-auxotrophic strains. We added D-erythro-dihydrosphingosine (Sigma-Aldrich, Schnellendorf, Germany) solubilized in ethanol to a final concentration of 1 μM, and control cells were treated with ethanol alone. Cell transformation, gene deletions, and excision of the KanMX4 cassette were performed as described previously (Soetens *et al.*, 2001). The plasmids used in this study are listed in Table 1. The YCpJYS12 plasmid was used to complement the *his4* and *leu2* mutations. Gap1 plasmids were constructed by in vivo recombination between one or two polymerase chain reaction (PCR) fragments carrying either the green fluorescent protein (GFP) or the Gap1-GFP sequence, and, respectively, the YCpGap1 or the YCpFL38 vector. Mutations resulting in the replacement of lysines by arginines were introduced into oligonucleotides used to amplify Gap1 fragments. All plasmids were verified by sequencing. All oligonucleotide sequences are available on request.

Permease Assays

Gap1 activity was determined by measuring the initial rate of uptake of ¹⁴C-labeled citrulline (20 μM), a specific substrate of the permease. To avoid competitive inhibition of citrulline transport by glutamine, cells grown on glutamine-containing medium were filtered, washed, and transferred to proline medium just before the transport assay.

Yeast-Cell Extracts and Western Blotting

Total protein extracts were obtained as described previously (Hein *et al.*, 1995). Proteins were resuspended in sample buffer and incubated for 10 min at 37°C before SDS-polyacrylamide gel electrophoresis (PAGE). After transfer to a nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany), the proteins were probed with polyclonal antibodies raised against Gap1 or Pma1 (De Craene *et al.*, 2001). Primary antibodies were detected with horseradish-peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) followed by enhanced chemiluminescence (Roche Diagnostics, Mannheim, Germany).

Limited Proteolysis

The experiment was performed essentially as described previously (Gong and Chang, 2001). Cells were lysed with glass beads in 200 μl buffer (50 mM HEPES pH 7.5, NaCl 300 mM). A total membrane fraction was generated by centrifugation at 100,000 × g for 60 min in a SW55 Ti rotor (Beckman Coulter, Fullerton, CA). Membranes were resuspended in buffer and incubated at a protein concentration of 0.5 mg/ml at 30°C and at a trypsin:protein ratio of 1:5. Samples were collected after 0, 0.5, 2, 5, 10, and 20 min, and the reaction was stopped by adding trypsin inhibitor. A control reaction was run for 20 min in the absence of trypsin. Proteins were precipitated by adding 10% trichloroacetic acid (TCA) and Gap1 was analyzed by Western blotting with anti-Gap1 antibodies as described previously. *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin and trypsin inhibitor type I-S from soybean were purchased from Sigma-Aldrich (Schnellendorf, Germany).

Subcellular Fractionation

Fractionation was performed as described previously (Bagnat *et al.*, 2001; Lauwers and André, 2006). Cells were lysed with glass beads in buffer L (25 mM Tris, pH 8, and 2.5 mM EDTA) supplemented with a cocktail of protease inhibitors (Roche Diagnostics) and 25 mM *N*-ethylmaleimide (NEM). The cleared lysate was centrifuged at 20,000 × g for 30 min in a SW55 Ti rotor

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference
Strain		
RH1800	<i>his4 leu2 ura3 bar1</i>	Munn <i>et al.</i> (1999)
RH3809	<i>lcb1-100 his4 leu2 ura3 bar1</i>	Munn <i>et al.</i> (1999)
EL041	<i>gap1Δ his4 leu2 ura3 bar1</i>	This study
EL103	<i>gap1Δ lcb1-100 his4 leu2 ura3 bar1</i>	This study
EL114	<i>end3Δ his4 leu2 ura3 bar1</i>	This study
EL117	<i>end3Δ lcb1-100 his4 leu2 ura3 bar1</i>	This study
EL123	<i>npi1 his4 leu2 ura3 bar1</i>	This study
EL125	<i>npi1 lcb1-100 his4 leu2 ura3 bar1</i>	This study
EL152	<i>vps27Δ his4 leu2 ura3 bar1</i>	This study
EL153	<i>vps27Δ lcb1-100 his4 leu2 ura3 bar1</i>	This study
23 344c ^a	<i>ura3</i>	Laboratory collection
EL090 ^a	<i>lag1Δ lac1Δ ura3</i>	This study
34 288b ^a	<i>GAP1::GFP::KanMX4 ura3</i>	Laboratory collection
GYS180 ^a	<i>GAP1::GFP::KanMX4 SUR7::mRFP::URA3</i>	This study
Plasmid		
YCpFL38	CEN-ARS URA3	Bonneaud <i>et al.</i> (1991)
YCpJYS12	CEN-ARS HIS4 LEU2	Springael and André, unpublished
YCpGap1	CEN-ARS URA3 GAP1	Hein and André (1997)
YCpGap1 ^{K9,16R}	CEN-ARS URA3 GAP1 ^{K9,16R}	Soetens <i>et al.</i> (2001)
YCpGap1 ^{9KR}	CEN-ARS URA3 GAP1 ^{9KR}	This study
pEL003	CEN-ARS URA3 GAP1-GFP	This study
pEL005	CEN-ARS URA3 GAP1 ^{K9,16R} -GFP	This study
pEL007	CEN-ARS URA3 GAP1 ^{K51,56,60,63R} -GFP	This study
pEL025	CEN-ARS URA3 GAP1 ^{9KR} -GFP	This study
pEL034	CEN-ARS URA3 GAP1 ^{K87,91R} -GFP	This study
pEL038	CEN-ARS URA3 GAP1 ^{K9,16,87,91R} -GFP	This study
pEL053	CEN-ARS URA3 GAP1 ^{K76R} -GFP	This study
pEL057	CEN-ARS URA3 GAP1 ^{K9,16,76,87,91R} -GFP	This study
pEL058	CEN-ARS URA3 GAP1 ^{K9,16,76R} -GFP	This study
pEL060	CEN-ARS URA3 GAP1 ^{K76,87,91R} -GFP	This study
YpISUR7-mRFP	Integrative URA3 SUR7-mRFP	Grossmann <i>et al.</i> (2007)

^a These strains derive from Σ1278b.

(Beckman Coulter), and the pellet was then resuspended in 20% glycerol in buffer B (10 mM Tris, pH7.4, 0.2 mM EDTA, and 0.2 mM dithiothreitol). The samples (500 μl) were loaded on top of a sucrose step gradient (0.5 ml, 53%; 1 ml, 43%; in buffer B) and centrifuged at 100,000 × *g* for 2 h in a Beckman SW55 Ti rotor. After centrifugation, six fractions of equal volume were collected from the top of the gradient, and the distribution of Pma1 and Gap1 was analyzed by Western blotting.

Fluorescence Microscopy

Cells transformed with the pEL003 or pEL025 plasmid were laid down on a thin layer of 1% agarose and viewed with a Nikon Eclipse E600 microscope equipped with appropriate fluorescence light filter sets. Images were captured with a Nikon DXM1200 digital camera and processed with Adobe Photoshop CS (Adobe Systems, Mountain View, CA).

Detergent-resistant Membrane Isolation

DRMs were isolated as described previously (Lauwers and André, 2006). Cells were harvested by filtration and lysed in TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA) supplemented with a cocktail of protease inhibitors (complete protease inhibitors; Roche Diagnostics) and 25 mM NEM. The cleared lysate was incubated with 1% Triton X-100 for 30 min on ice. Lysate (250 μl) was adjusted to 40% iodixanol by adding 500 μl of Optiprep (Axis-Shield, Oslo, Norway), and it was loaded at the bottom of a two-step gradient (1200 μl of 30% iodixanol in TNXE [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100], and 200 μl TNXE). The gradient was centrifuged at 46,000 rpm for 120 min at 4°C in a Beckman Sw55Ti rotor. Six fractions of equal volume were collected from the top of the gradient, and the proteins were precipitated by incubation with 10% TCA for 30 min. The precipitates were dissolved in 40 μl of 1 M Tris base plus 40 μl of 2X sample buffer (100 mM Tris-HCl, pH 6.8, 4 mM EDTA, 4% SDS, 20% glycerol, and 0.02% bromophenol blue) supplemented with 2% 2-mercaptoethanol. The samples were heated at 37°C for 15 min, and then they were analyzed by Western blotting for the presence of Gap1 and Pma1. Fractions

1–3 were pooled (R, Triton X-100-resistant membranes), as were fractions 4–6 (S, Triton X-100-soluble membranes).

Confocal Microscopy

Confocal sections were scanned using a LSM510-Meta confocal microscope (Zeiss, Jena, Germany). Double-labeled cells (Gap1-green fluorescent protein [GFP] and Sur7-monomeric red fluorescent protein [mRFP]) were sequentially scanned to avoid any cross talk of fluorescence channels. Living cells were immobilized by a thin film (~0.5 mm) of 1% agarose and observed.

RESULTS

The Gap1 Permease Produced in the Absence of Sphingolipid Biosynthesis Is Delivered to the Plasma Membrane but Undergoes Deregulated Ubiquitin-dependent Down-Regulation

We have previously reported that the yeast Gap1 permease, when present at the plasma membrane, fractionates with DRMs enriched in sphingolipids and ergosterol (Lauwers and André, 2006), these being the proposed biochemical equivalent of lipid rafts (Bagnat *et al.*, 2000). Moreover, SL neosynthesis proved essential to accumulation of newly synthesized Gap1 proteins at the cell surface. In a thermosensitive *lcb1-100* mutant defective in the first step of SL biosynthesis at the restrictive temperature of 37°C (Zanolari *et al.*, 2000), neosynthesized Gap1 protein, like the Pma1 ATPase (Bagnat *et al.*, 2001), does not accumulate at the cell surface, and it is instead targeted for degradation into the lumen of

the vacuole, the lysosome of yeast cells (Lauwers and André, 2006). To determine whether the Gap1 permease synthesized under these conditions reaches the plasma membrane before being degraded, we examined its fate in *lcb1-100* cells lacking End3, a protein required for the internalization step of endocytosis (Bénédicti *et al.*, 1994). Cells were first grown at the permissive temperature of 24°C in a medium containing glutamine and ammonium to repress Gap1 synthesis. They were then transferred to proline medium to induce

Gap1 production, either at 24°C or at the restrictive temperature of 37°C. Immunoblot analysis showed that Gap1 failed to accumulate to a high level when neosynthesized in *lcb1-100* cells shifted to 37°C (Figure 1A), in keeping with previous observations (Lauwers and André, 2006). Yet, Gap1 did accumulate at this temperature in the *lcb1-100 end3Δ* double mutant cells (Figure 1A). Marked stabilization of Gap1 was also observed in *lcb1-100* cells additionally deficient in Rsp5 (Npi1), the ubiquitin ligase required for ubiquitination and

Figure 1. Degradation of Gap1 in *lcb1-100* mutant cells involves its ubiquitin-dependent endocytosis and MVB sorting. (A) Gap1 synthesis was induced at 24 or 37°C in wild-type (RH1800), *lcb1-100* (RH3809), *end3Δ* (EL114), *end3Δ lcb1-100* (EL117), *npi1* (EL123), *npi1 lcb1-100* (EL125), *vps27Δ* (EL152) and *vps27Δ lcb1-100* (EL153) cells. Cells were first grown at 24°C in glutamine- and ammonium-containing medium. Aliquots were collected before (t0) and 120 min after the cells were shifted to proline-containing medium, and the presence of Gap1 and Pma1 in total cell extracts was analyzed by Western blotting. The experiments have been carried out at least twice, and proved reproducible; in each case the result of one representative experiment is shown. (B) Wild-type, *lcb1-100*, *end3Δ*, and *end3Δ lcb1-100* cells (strains as mentioned above) transformed with YCpGap1-GFP plasmid (pEL003) were observed before (t0) and 120 min after the cells were shifted to proline-containing medium. Left, Nomarski images. Right, GFP fluorescence. Bar, 5 μ m. (C) Schematic representation of the sphingolipid biosynthesis pathway in *S. cerevisiae*. Gene names are shown in italics. DHS-1P, dihydrosphingosine-1-phosphate; PHS-1P, phytosphingosine-1-phosphate; IPC, inositol phosphoceramide; MIPC, mannose inositol phosphoceramide, M(IP)₂C, mannose (inositol phosphate)₂ ceramide. (D) Gap1 synthesis was induced at 29°C in wild-type (23344c) and *lag1Δ lac1Δ* (EL090) cells. Gap1 activity was measured before and at different times after the beginning of the induction as described in *Materials and Methods*.

endocytosis of Gap1 (Hein *et al.*, 1995; Springael and André, 1998) (Figure 1A). Because delivery of endocytic cargoes to the vacuolar lumen requires their prior sorting in the MVB pathway (Babst, 2005), we also analyzed the fate of Gap1 neosynthesized in *lcb1-100* cells lacking Vps27, a class E Vps factor required for the sorting of cargoes destined for the internal vesicles of MVBs (Bilodeau *et al.*, 2003). Marked stabilization of the permease was also observed in this *lcb1-100 vps27Δ* strain (Figure 1A). Hence, when SL biosynthesis is impaired, newly synthesized Gap1 protein seems to be delivered to the plasma membrane before undergoing rapid ubiquitin-dependent down-regulation. These observations were confirmed by examining the localization of a functional Gap1-GFP fusion protein (Figure 1B). When wild-type or *end3Δ* cells carrying a plasmid encoding this Gap1-GFP construct were grown in glutamine- and ammonium-containing medium and then transferred to proline-containing medium at either 24 or 37°C for 2 h, fluorescence was observed at the cell surface regardless of the temperature, reflecting accumulation of Gap1-GFP at the plasma membrane. In *lcb1-100* cells, in contrast, whereas Gap1-GFP localized correctly to the cell surface when produced at 24°C, only a weak, intracellular fluorescent signal was detected when the permease was induced at 37°C. In the *lcb1-100 end3Δ* strain, however, Gap1-GFP neosynthesized at 37°C clearly labeled the cell surface (Figure 1B). This observation corroborates the results of immunoblot analysis (Figure 1A), and it confirms that the permease produced in the absence of SL biosynthesis is delivered to the plasma membrane before being efficiently endocytosed. This conclusion is also supported by the results of a subcellular fractionation experiment, where after a 2-h shift of the *lcb1-100 end3Δ* double mutant to proline medium at 37°C, ~60% of the Gap1 protein was found with the plasma membrane marker Pma1 in the bottom fractions of a sucrose density gradient (data not shown).

SL precursors, including sphingoid bases, ceramide, and sphingoid base-1-phosphate (Figure 1C), have been recognized as signaling molecules involved in a variety of cellular processes, including control of the cell cycle, stress responses, and apoptosis (Dickson and Lester, 2002; Spiegel and Milstien, 2003). In yeast cells, sphingoid bases (dihydrosphingosine [DHS] and phytosphingosine [PHS]) are involved in heat stress and in signaling pathways controlling endocytosis, the cortical actin cytoskeleton, cell wall integrity, growth regulation, and longevity (Dickson and Lester, 2002). For example, *lcb1-100* cells (also isolated as *end8* mutants) grown in complete medium are impaired at 37°C in the internalization step of Ste2, the α -pheromone receptor, and this defect can be overcome by the addition of DHS or PHS. Because these precursors do not need to be further metabolized to restore endocytosis, it seems that sphingoid bases themselves are required for endocytic internalization (Zanolari *et al.*, 2000). We thus sought to determine whether the requirement of sphingoid base synthesis for the accumulation of Gap1 at the plasma membrane reflects a direct role of these precursors, or whether they are required only to allow the synthesis of more complex SLs that would be important for Gap1 trafficking. To address this question, we monitored Gap1 uptake activity after induction of permease synthesis in wild-type and *lag1Δ lac1Δ* mutant cells devoid of ceramide synthase activity (Schorling *et al.*, 2001). In wild-type cells grown in ammonium medium and then transferred to proline medium, Gap1 activity increased over time (Figure 1D), reflecting the progressive accumulation of the newly synthesized permease at the cell surface. In *lag1Δ lac1Δ* cells, however, Gap1 transport activity was dramati-

cally reduced (Figure 1D), indicating that SL biosynthesis at least up to the ceramide level is required for the accumulation of Gap1 at the plasma membrane, as it has been reported for the plasma membrane ATPase Pma1 (Gaigg *et al.*, 2005).

The Gap1 Permease Produced in the Absence of Sphingolipid Neosynthesis Does Not Acquire Detergent Insolubility

The accumulation of Gap1 at the plasma membrane in the *lcb1-100 end3Δ* strain enabled us to examine the properties of the permease that is synthesized and delivered to the plasma membrane in the absence of SL neosynthesis. We first examined whether this Gap1 fractionates with DRMs. When Gap1 synthesis was induced in wild-type cells by shifting them to a proline-containing medium at 37°C, the permease was found to fractionate with DRMs (Figure 2A). In contrast, when the permease was similarly induced at 37°C in *lcb1-100 end3Δ* double mutant cells, a strong Gap1 signal was detected in the fractions corresponding to detergent-soluble membranes. The residual amount of Gap1 protein induced under the same conditions in the *lcb1-100* cells was also found to be soluble in Triton X-100 (Figure 2A). Hence, Gap1 produced in the absence of SL biogenesis, although stabilized at the plasma membrane, fails to fractionate with DRMs. This is surprising, because SLs are very stable in yeast (Dickson and Lester, 2002) and should thus be abundant in the plasma membrane, even if their neosynthesis has been blocked for 2 h, as in the above-mentioned experiment. Accordingly, under the same conditions the proton ATPase Pma1 remained insoluble in Triton X-100 (Figure 2A), indicating that SL-enriched domains were still present at the cell surface but that neosynthesized Gap1 failed to integrate into them. We have previously reported that the ability of Gap1 to associate with DRMs in *lcb1-100* cells can be restored by addition of the SL precursor DHS during Gap1 synthesis and secretion (Lauwers and André, 2006). Accordingly, when DHS was present in the growth medium during induction of Gap1 synthesis at 37°C, Gap1 stability and insolubility in Triton X-100 was completely restored in *lcb1-100* cells, and the permease also regained full detergent insolubility in *lcb1-100 end3Δ* double-mutant cells (Figure 2A).

The Gap1 Protein Produced in the Absence of Sphingolipid Neosynthesis Lacks Transport Activity and Is More Susceptible to Proteolysis

We have previously observed that Gap1 is systematically associated with DRMs when located at the plasma membrane (Lauwers and André, 2006). The use of an *lcb1-100 end3Δ* double mutant enabled us for the first time to maintain the Gap1 permease at the plasma membrane but outside SL-enriched domains. We were thus able to test whether the association of Gap1 with these domains is important for the transport activity of the protein (Figure 2B). When Gap1 synthesis was induced for 2 h in wild-type or *end3Δ* cells at 24 or 37°C, citrulline uptake activity was found to develop regardless of the temperature, reflecting Gap1 accumulation at the plasma membrane. As expected, no Gap1 activity was observed in *lcb1-100* cells at the restrictive temperature. Strikingly, the detergent-soluble permease accumulated at the plasma membrane in *lcb1-100 end3Δ* cells at 37°C proved inactive, whereas high permease activity was restored in this double mutant by addition of DHS in the course of Gap1 synthesis (Figure 2B). These results reveal that Gap1 protein synthesized in the absence of SL biosynthesis and blocked at the plasma membrane not only fails to integrate into SL-enriched domains but also is intrinsically inactive. Interest-

Figure 2. The Gap1 permease synthesized and accumulated at the plasma membrane in the *lcb1-100* mutant is inactive and detergent-soluble. (A–C) Wild-type, *lcb1-100*, *end3Δ*, and *end3Δ lcb1-100* cells (strains as in Figure 1) were grown at 24°C in glutamine and ammonium medium. Cells were transferred to proline-containing medium at 24 or 37°C, in the presence or the absence of 1 μM DHS. (A) Cells were collected 2 h after the shift at the restrictive temperature and lysed. Gap1 detergent-solubility was examined as described in *Materials and Methods*. R, Triton X-100-resistant membranes; S, Triton X-100-soluble membranes. (B) Gap1 activity was measured before (t0) and 2 h after the shift to proline-containing medium. The experiments have been carried out at least twice and proved reproducible; in each case, the result of one representative experiment is shown. (C) Gap1-GFP sensitivity to limited proteolysis was examined in *end3Δ* and *end3Δ lcb1-100* cells after a 2-h induction at 37°C in the absence or the presence of DHS. Total membranes were prepared and incubated in the absence or presence of trypsin at 30°C for various times, as described in *Materials and Methods*. Gap1-GFP was analyzed by Western blotting with anti-GFP antibodies. (D) Gap1 sensitivity to limited proteolysis was examined as described above in *gap1Δ* and *gap1Δ lcb1-100* cells expressing a nonubiquitinable variant of the permease, Gap1^{9KR}, after a 2-h induction at 37°C. Gap1^{9KR} was analyzed by Western blotting with anti-Gap1 antibodies.

ingly, the detergent-soluble Gap1-GFP permease accumulated at the plasma membrane in *lcb1-100 end3Δ* cells at 37°C also showed a marked increase in trypsin sensitivity compared with *end3Δ* cells (Figure 2C), indicating that segregation of Gap1 outside SL-enriched domains leads to a greater accessibility of the protein to proteases. Similarly, a variant form of Gap1 that is resistant to ubiquitination and endocytosis (see below) was much more susceptible to limited trypsinolysis when synthesized at 37°C in the *lcb1-100* mutant than in wild-type cells (Figure 2D). Because the anti-Gap1 antibodies used in this experiment were raised against the amino-terminal part of Gap1 (residues 1–90) (De Craene *et al.*, 2001), the fact that we did not detect any proteolytic fragment below the full-length Gap1 signal (Figure 2D) strongly suggests that the N-terminal tail is more accessible to proteolysis when the permease is present outside SL-enriched domains.

Down-Regulation of Gap1 Produced in the Absence of Sphingolipid Biosynthesis Involves Its Ubiquitination on Nonclassical Membrane-proximal Lysines

As shown above, the inactive and detergent-soluble Gap1 protein having reached the plasma membrane under conditions preventing SL biosynthesis is rapidly down-regulated

in a ubiquitin-dependent manner (Figure 1A). It is noteworthy that this down-regulation occurs although the medium contains only proline as a nitrogen source, i.e., under nitrogen-supply conditions that normally promote stable accumulation of Gap1 at the cell surface, this being dependent on Npr1 kinase (De Craene *et al.*, 2001). Gap1 down-regulation typically occurs when a good nitrogen source such as ammonium is added to the culture medium, conditions proposed to inactivate Npr1 (Schmidt *et al.*, 1998). It thus seems that the control exerted by Npr1 and protecting Gap1 against down-regulation on poor nitrogen media is defective when Gap1 has been produced in the absence of SL biosynthesis.

In wild-type cells, down-regulation of Gap1 caused by the transfer of cells to optimal nitrogen supply conditions involves ubiquitination of the permease on two ubiquitin-acceptor lysines, K9 and K16, located in its amino-terminal tail. A variant permease Gap1^{K9,16R}, where both lysines have been replaced with arginines, is resistant to ubiquitination and endocytosis, and it is thus protected against degradation (Soetens *et al.*, 2001). We sought to determine whether this Gap1^{K9,16R} variant is also resistant to degradation in cells defective in SL biosynthesis. Surprisingly, Gap1^{K9,16R} synthesized in *lcb1-100* cells behaved like the native Gap1 pro-

Figure 3. Degradation of Gap1 permease in the *lcb1-100* mutant involves its ubiquitination on additional membrane-proximal lysine residues. Cells of the *gap1Δ* (EL041) and *gap1Δ lcb1-100* (EL103) strains were grown at 24°C in glutamine and ammonium-containing medium, and transferred at 24 or 37°C in medium containing proline as the sole nitrogen source to induce Gap1 expression. (A) Cells expressing

tein, i.e., a strong Gap1 signal was immunodetected when the permease was produced at 24°C, but the mutant permease failed to accumulate at the plasma membrane (data not shown) and in immunoblots at the restrictive temperature of 37°C (Figure 3A), reflecting its down-regulation. Because the Rsp5 ubiquitin ligase is nevertheless required for degradation of Gap1 in *lcb1-100* cells at 37°C (Figure 1A), we reasoned that other lysines of the permease are probably ubiquitinated. We thus constructed a mutant permease, Gap1^{9KR}, with all nine lysine residues of the N-terminal tail replaced with arginines. When produced in wild-type cells at 24 or 37°C, the Gap1^{9KR} protein was normally secreted to the plasma membrane, and it was active at both temperatures (Figure 3, B–D). In the *lcb1-100* mutant at the restrictive temperature of 37°C, in contrast to the native permease and the Gap1^{K9,16R} variant, the newly synthesized Gap1^{9KR} protein seemed stable (Figure 3E). Furthermore, Gap1^{9KR} produced in wild-type or *lcb1-100* cells was found at the plasma membrane regardless of the temperature, as indicated by visualization of a functional Gap1^{9KR}–GFP fusion protein at the cell surface (Figure 3B) and by cofractionation of Gap1^{9KR} with the plasma membrane marker Pma1 in a sucrose density gradient (Figure 3C). Yet the cell surface-located Gap1^{9KR} proved inactive in the *lcb1-100* mutant at 37°C (Figure 3D), like the native Gap1 permease trapped at the plasma membrane in the *lcb1-100 end3Δ* mutant (Figure 2B). This again argues for the association of Gap1 with SL-enriched domains being crucial to the transport activity of the permease.

The above-mentioned results indicate that the down-regulation of cell surface Gap1 occurring in the absence of SL neosynthesis involves ubiquitination of the permease on other lysines of the N-terminal tail in addition to K9 and K16. We then analyzed systematically the effect of mutating these lysines in different combinations, by examining the fate of the mutant permeases in *lcb1-100* cells grown at 24°C in glutamine- and ammonium-containing medium and then transferred to proline-containing medium at 24 or 37°C (Figure 3E). All variant permeases were detected on immunoblots when produced at the permissive temperature. When lysines K9 and K16 were both replaced with arginines, or

when K76 was mutated alone or along with K87 and K91, the mutant Gap1 was not protected against degradation at the restrictive temperature (Figure 3E). When all five lysines were simultaneously mutated, the permease was stabilized at the plasma membrane in *lcb1-100* cells at 37°C (Figure 3E; data not shown). Conversely, replacing lysines 51, 56, 60, and 63 with arginines (Gap1^{K51–63R}) did not lead to greater stability of the permease at the restrictive temperature (Figure 3E). Thus, Gap1 protein newly synthesized in the absence of SL biosynthesis reaches the plasma membrane but undergoes unregulated Rsp5-dependent down-regulation involving Gap1 lysines not only at positions 9 and 16 but also at positions 76, 87, and 91, predicted to lie close to the first transmembrane segment of Gap1 (Figure 3F).

Sphingolipid Neosynthesis Is Specifically Required during Gap1 Production

The above-mentioned data show that arrest of SL neosynthesis during Gap1 production and secretion results in lack of Gap1 activity, increased sensitivity of the permease to trypsinolysis, inability of Gap1 to integrate existing SL-enriched domains (containing other proteins such as Pma1), and unregulated Gap1 down-regulation. We next wondered what would be the effect of an arrest of SL neosynthesis occurring after Gap1 protein has accumulated at the plasma membrane. We started with *lcb1-100* cells expressing Gap1^{9KR}–GFP and grown at the permissive temperature of 24°C with proline as the sole nitrogen source. Under these conditions, the permease accumulated at the plasma membrane in an active (data not shown) and stable form, and it fractionated largely with DRMs (Figure 4A), as expected. A significant amount of Gap1^{9KR} was also detected in the fractions corresponding to detergent-soluble membranes, probably corresponding to permeases en route to the plasma membrane and having not yet integrated SL-enriched domains. Accordingly, this soluble pool of Gap1^{9KR} was completely resorbed after permease synthesis was arrested and the cells grown for two more hours (Figure 4A). Gap1^{9KR}–GFP production was then blocked, and SL neosynthesis was arrested by transferring the cells to glutamine- and ammonium-containing medium at 37°C. No major change in permease detergent-solubility or activity was observed even 2 h after the shift to the restrictive temperature (Figure 4A; data not shown). Thus, once Gap1 has established its location within SL-enriched domains at the plasma membrane, there is no need for sustained SL neosynthesis to maintain this association, at least during the first hours after Gap1 production. This resembles the behavior of the proton ATPase Pma1, which remains detergent-insoluble even 2 h after arrest of SL neosynthesis (e.g., Figure 2A). We next performed the reverse experiment, preaccumulating Gap1^{9KR} at the plasma membrane outside SL-enriched domains and then allowing SL neosynthesis to resume. For this, *lcb1-100* mutant cells grown at 24°C were first shifted to proline-containing medium at 37°C to induce synthesis of Gap1^{9KR}. As expected, Gap1^{9KR} accumulated at the plasma membrane but was inactive (data not shown) and totally soluble in Triton X-100 (Figure 4B). Gap1^{9KR} synthesis was then blocked by transferring the cells to glutamine- and ammonium-containing medium, and 30 min later the SL precursor DHS was added to the culture to restore SL biosynthesis. Remarkably, the permease remained excluded from DRMs (Figure 4B) and inactive (data not shown), even 2 h after DHS addition, indicating that a restored SL supply cannot reinstate either the activity of the permease or its association with SL-enriched domains. Together, these results suggest that SL biosynthesis is crucial specifically during synthesis

Figure 3 (cont). plasmid-borne Gap1 or Gap1^{K9,16R}. Aliquots were collected before (t0) and 120 min after the shift, and the presence of Gap1 and Pma1 in total cell extracts was analyzed by Western blotting. (B) Cells expressing plasmid-borne Gap1–GFP or Gap1^{9KR}–GFP were observed before (t0) and 120 min after the shift to proline-containing medium. Left, Nomarski images. Right, GFP fluorescence. Bar, 5 μm. (C) Cells expressing plasmid-borne Gap1 or Gap1^{9KR} were lysed and fractionated on a sucrose step gradient as described in *Materials and Methods*. The distribution of Pma1 and Gap1 was analyzed by Western blotting. Fractions 5 and 6 correspond to the plasma membrane, whereas fractions 2–4 correspond to the ER, Golgi, and vacuolar membranes. The intracellular pool of Gap1 is expected to represent newly synthesized proteins that are present in the secretory pathway. (D) Permease activity was quantified in cells expressing plasmid-borne Gap1 or Gap1^{9KR}, before (t0) and 120 min after the shift to proline medium. Activity measurements were carried out at least twice, and they proved reproducible; the result of one representative experiment is shown. (E) Cells expressing various plasmid-borne Gap1–GFP mutant forms. Aliquots were collected 120 min after the shift to proline-containing medium, and the presence of Gap1 and Pma1 in total cell extracts was analyzed by western blotting. (F) Schematic representation of the Gap1 N-terminal region. The first transmembrane segment of the permease (predicted by TMHMM; CBS, Lyngby, Denmark) is represented by a gray cylinder. Lysines are indicated in bold, and lysines that are targets for ubiquitination in *lcb1-100* cells are enlarged.

Figure 4. Sphingolipid neosynthesis is required during Gap1 production and secretion, but not once the permease has reached the plasma membrane. (A) *gap1Δ* and *gap1Δ lcb1-100* cells expressing plasmid-borne Gap1^{9KR}-GFP were grown at 24°C in proline medium and then transferred for a 30-min incubation in glutamine- and ammonium-containing medium at 24°C to repress Gap1 synthesis. Next, cells were transferred to glutamine- and ammonium-containing medium at 37°C. Gap1 detergent solubility was examined in proline-grown cells and 2 h after the shift to the restrictive temperature, as described in *Materials and Methods*. R, Triton X-100-resistant membranes; S, Triton X-100-soluble membranes. (B) *gap1Δ* and *gap1Δ lcb1-100* cells expressing plasmid-borne Gap1^{9KR} were grown at 24°C in glutamine and ammonium medium, and then they were transferred to proline medium at 37°C for 2 h. Gap1 synthesis was then repressed by shifting back to glutamine and ammonium medium, and DHS was added to the culture 30 min later. Gap1 detergent-solubility was examined 2 h after induction of permease synthesis and 1 or 2 h after addition of DHS, as described in *Materials and Methods*.

and secretion of the permease. Accordingly, maintenance of this biosynthesis is not required to preserve the properties of Gap1 having reached the plasma membrane, and the defects displayed by Gap1 produced in the absence of SL biosynthesis seem irreversible, i.e., they are not compensated by restoring normal SL biosynthesis. This suggests that the biogenesis of Gap1 is somehow coupled to that of SLs, this being crucial for Gap1 to acquire detergent insolubility, activity, and normal control of its ubiquitination.

The Gap1 Permease Is Homogeneously Distributed at the Plasma Membrane

The notion of lipid rafts in yeast is still unclear, because all plasma membrane proteins so far tested fractionate with DRMs, the proposed biochemical equivalents of lipid rafts (Bagnat *et al.*, 2000), at least when present at the cell surface (Malinska *et al.*, 2004; Lauwers and André, 2006). Furthermore, confocal microscopy analysis has revealed that plasma membrane proteins are distributed between two mutually exclusive domains, called the MCC and the MCP, which both include DRM-associated proteins, suggesting that at least two kinds of lipid rafts coexist in living cells (Malinska *et al.*, 2003; Malinska *et al.*, 2004; Grossmann *et al.*, 2007). Eisosomes, characterized by the presence of several proteins, including Sur7 (Walther *et al.*, 2006), colocalize with the MCC. We thus sought to determine in which domain Gap1 segregates. Wild-type cells coexpressing Gap1-GFP and the Sur7-RFP protein, which was recently reported to colocalize with eisosomes (Walther *et al.*, 2006), were

therefore grown on proline-containing medium, i.e., under conditions where both proteins are insoluble in Triton X-100 1% (data not shown) (Malinska *et al.*, 2004). The Sur7 protein shows a clear patchy distribution at the cell surface (Figure 5A), as observed previously (Young *et al.*, 2002; Malinska *et al.*, 2004; Walther *et al.*, 2006). In contrast, Gap1-GFP displays a rather homogeneous signal at the plasma membrane, and it does not specifically colocalize with the Sur7 compartment (Figure 5A). We also observed that the complementary membrane domains defined by the presence of the ATPase

Figure 5. Gap1-GFP is homogeneously distributed at the plasma membrane. Localization of the Gap1 permease by confocal microscopy. (A) Cross sections (top row) and surface (middle row) views of GYS180 cells expressing Gap1-GFP (green in merge) and the MCC marker Sur7-mRFP (red in merge). Cells were grown on proline-containing medium as a steady-state condition. The intensity profile along the dashed arrow (merge image) shows no significant correlation between the fluorescence signal of Gap1-GFP and the distinct intensity peaks of Sur7-mRFP. Bar, 5 μm. (B) Cross sections of cells expressing Gap1-GFP visualized after a 2-h induction in proline-containing medium. Wild-type (RH1800) cells were induced at 24 or 37°C, whereas *end3Δ* (EL114) and *end3Δ lcb1-100* (EL117) cells were induced at 37°C. For better comparability of the protein distribution at the plasma membrane the images were adjusted to approximately the same total intensity. Bar, 5 μm.

Pma1 are not particularly enriched in Gap1-GFP (data not shown).

We then tested whether Gap1 distribution at the cell surface is altered when the permease is produced in the absence of SL biosynthesis. We first verified that Gap1-GFP is homogeneously distributed at the plasma membrane when its synthesis is induced for 2 h in wild-type cells. This proved to be the case, regardless of the temperature of induction (Figure 5B). A similar fluorescence pattern was observed when *end3Δ* or *end3Δ lcb1-100* mutant cells expressing Gap1-GFP were visualized after a 2-h induction at the restrictive temperature of 37°C (Figure 5B). This result indicates that the homogenous repartition of Gap1 at the plasma membrane is not altered when the permease fails to associate with SL-enriched domains.

The homogeneous distribution of Gap1 at the cell surface is reminiscent of the situation described for the hexose permease Hxt1 (Malinska *et al.*, 2003). The Gap1 and Hxt1 permeases thus seem to define a third group of yeast plasma membrane proteins, i.e., those that do not specifically segregate into the MCC or MCP. Furthermore, Gap1 plasma membrane repartition is not altered if the permease is produced in the absence of SL biosynthesis.

DISCUSSION

The main conclusion of this work is that de novo SL biosynthesis is crucial during Gap1 synthesis and/or secretion for the permease to be functional and stable at the plasma membrane. The permease synthesized in the *lcb1-100* mutant at the restrictive temperature of 37°C, i.e., in the absence of SL neosynthesis, is delivered to the cell surface, but it is rapidly down-regulated. Using mutations stabilizing the permease at the cell surface, we have shown that Gap1 synthesized under these conditions displays several abnormalities, including a complete lack of activity, an increased sensitivity to proteolysis, and an inability to fractionate with detergent-resistant membranes. These defects can be prevented if SL biogenesis is restored in *lcb1-100* cells by providing the SL precursor DHS during Gap1 synthesis and secretion, but they cannot be compensated by adding DHS after Gap1 has reached the plasma membrane. Conversely, once Gap1 has integrated the plasma membrane, there is no need for sustained SL synthesis to preserve its functional properties, at least during the first 2 h. We propose that these observations are most easily interpretable in terms of a model where the Gap1 permease associates with neosynthesized SLs during its production and/or secretion, this association shaping the permease into a conformation essential to activity. Lack of this association would irreversibly distort neosynthesized Gap1 into a conformation incompatible with its function. This permease would also be unable to associate with the abundant and stable SLs present at the plasma membrane, or to integrate SL-enriched domains, this probably accounting for its inability to fractionate with DRMs. That intrinsic plasma membrane proteins tightly associate with lipids is at the heart of the "lipid shell" model proposed by Anderson and Jacobson (2002). This model suggests that some transmembrane proteins have an affinity for sterols and SLs and that they become surrounded by a shell of these lipids during their production. Because of the molecular compatibility between lipids of the shell and those present in rafts, these proteins would be inclined to associate with pre-existing rafts. This might explain why lipid rafts selectively include (or exclude) some proteins, thus acquiring specific functional properties (Anderson and Jacobson, 2002). Formation of an SL shell has also been proposed to

strongly influence the conformation of the encased protein (Fantini, 2003). More generally, the lipid composition of the bilayer is thought to be a determinant of the activity and structure of intrinsic membrane proteins (Lee, 2004; Opekareva *et al.*, 2005; Zhang *et al.*, 2005). Interestingly, lipids forming an annular shell around transmembrane proteins have been revealed in some high-resolution structural studies, e.g., in the case of the lens-specific aquaporin-0 (Gonen *et al.*, 2005). In this case, tight packing of lipid fatty acyl chains against the rough surface of the protein was apparent, some of these chains being distorted to correctly wrap around the bulky side chain of the protein. This tight association probably contributes to the permeability barrier properties of the lipid bilayer in direct contact with the protein surface (Lee, 2005).

As mentioned above, the proposed association of proteins with a lipid shell might also account for their lateral distribution into domains of the plasma membrane (Anderson and Jacobson, 2002). In yeast, multiple permeases (Can1, Fur4, Tat2) and other membrane proteins (e.g., Sur7) colocalize in discrete patches constituting the MCC, whereas Pma1 is excluded from this compartment and apparently occupies the remaining space of the cell surface, called the MCP (Malinska *et al.*, 2003; Malinska *et al.*, 2004; Grossmann *et al.*, 2006). We have shown in this work that the Gap1 permease is instead homogeneously distributed at the plasma membrane and thus displays a lateral segregation profile very similar to that of the Hxt1 hexose transporter (Malinska *et al.*, 2003). Because all the above-cited proteins fractionate with DRMs (Dupré and Haguenaue-Tsapais, 2003; Malinska *et al.*, 2003, 2004; Umehayashi and Nakano, 2003; Lauwers and André, 2006), the question arises of why they segregate differently in the plasma membrane. One possibility is that all these proteins are detergent-insoluble because they are encased in a lipid shell during their biosynthesis and secretion. The lipid composition and structure of these shells, however, could differ substantially according to the protein, conferring specific aggregation properties that would determine protein self-distribution between distinct membrane domains (Figure 6). In support of this view, it is noteworthy that the protein components of the MCP and MCC show distinct lipid requirements. For example, biosynthesis of SLs but not ergosterol is essential to cell surface accumulation of Pma1 (Gaigg *et al.*, 2005), whereas ergosterol, which is more abundant in the MCC than in the MCP (Grossmann *et al.*, 2007), is necessary for plasma membrane targeting of Tat2

Figure 6. A model for lateral distribution of yeast plasma membrane proteins. Left, confocal surface view a yeast cell expressing Sur7-GFP to reveal the MCC. Middle, schematic representation of a single MCC patch surrounded by MCP. Right, hypothetical representation of proteins distributed in the MCC and/or MCP. Proteins (white circles) are encased in a lipid shell (ring around white circle) of various composition and structure and that determines the segregation properties of proteins. Some proteins self-aggregate via their lipid shell to form MCC patches (proteins with white rings). These MCC proteins cannot aggregate with Pma1 nor (possibly) with other proteins (marked with black rings) that self-assemble to form the MCP. Other proteins such as Gap1 and Hxt1 (marked with gray rings) are surrounded by a lipid shell compatible with those encasing both MCC and MCP proteins, and thus they do not specifically segregate in MCC or MCP.

(Umebayashi and Nakano, 2003). It has also recently been reported that the patchy distribution of some proteins normally present in the MCC is lost upon membrane depolarization (Grossmann *et al.*, 2007). This depolarization might affect the conformation of the intrinsic membrane proteins (Patzlaff *et al.*, 2002) and consequently their association with surrounding lipids and their ability to aggregate preferentially with other proteins.

Although the model of coupled biogenesis of Gap1 and associated lipids provides an attractive frame for interpreting our data and several previous observations, several questions remain open, e.g., which lipids associate with Gap1 and where in the secretory pathway does this interaction occur? We have previously reported that newly synthesized Gap1 protein acquires detergent insolubility at the level of the Golgi complex (Lauwers and André, 2006). In contrast, the early steps of SL biosynthesis, up to the formation of ceramide, occur in the endoplasmic reticulum (ER) (Futerman and Riezman, 2005). Ceramide is then transported to the Golgi where the polar head inositol phosphate is added to form inositol-phospho-ceramide (IPC). The further successive addition of mannose and a second inositol phosphate leads to formation of the complex SLs MIPC and $M(IP)_2C$, i.e., mannose-(inositol-P)₁ and ₂-ceramide, respectively (Figure 1C). A conceivable view is thus that neosynthesized Gap1 first associates with ceramide concomitantly with permease synthesis and folding at ER level, this being insufficient to confer to the permease its insolubility in DRMs. Subsequent maturation at Golgi level of the ceramide into complex SLs could reinforce the interaction between Gap1 and surrounding lipids, e.g., through noncovalent bonds between lipid headgroups and parts of Gap1 able to contact the lipid polar region (Lee, 2004), thus conferring to Gap1 insolubility in detergents. Alternatively, Gap1 might be able to associate only with mature SLs and not with ceramide itself.

Our data also suggest an important role of SL biogenesis during Gap1 production and secretion for proper control of the permease by ubiquitination. Under normal conditions, Gap1 undergoes ubiquitination on N-terminal lysines at positions 9 and 16 only once the control exerted by the Npr1 kinase has been released, i.e., when a favored nitrogen is added to the medium (De Craene *et al.*, 2001; Soetens *et al.*, 2001). In contrast, Gap1 proteins produced in the absence of SL neosynthesis escape this Npr1-dependent control and undergo efficient Rsp5-mediated down-regulation even in cells growing on a poor nitrogen source. Although this down-regulation can rely solely on the classical ubiquitin acceptor lysines 9 and 16 in the N-terminal tail, our data show that three additional lysines of this tail, at positions 76, 87, and 91, can also promote the unregulated down-regulation of Gap1. Under normal conditions of SL biogenesis, these three residues, located close to the membrane (Figure 3F), have so far not been implicated in nitrogen-regulated control of Gap1 ubiquitination. Yet in two global analyses of ubiquitin conjugates in yeast, Gap1 was found to be ubiquitinated at lysine 76 (Hitchcock *et al.*, 2003; Peng *et al.*, 2003). In both studies, yeast cells were grown on a standard rich medium, conditions under which *GAP1* gene transcription is largely repressed and Gap1 permease synthesized to a limited level is directly sorted from the Golgi to the endosome/vacuolar degradation pathway without passing through the plasma membrane (Magasanik and Kaiser, 2002). It is thus possible that ubiquitination of Gap1 at position 76 might also occur under particular environmental conditions, even when SL biogenesis occurs normally. In one of the above-cited proteomics studies, the analyzed cells contained a mu-

tation impairing degradation of protein substrates of the ER-associated degradation pathway (Hitchcock *et al.*, 2003), raising the possibility of a role of lysine 76 (and possibly lysines 87 and 91) in ubiquitination of Gap1 at ER level. Whatever the membrane compartment where ubiquitination of neosynthesized Gap1 takes place when SL biogenesis is defective, our results suggest that this modification is involved in a quality-control mechanism that ensures removal of misfolded permeases from the plasma membrane. Accordingly, Gap1 produced in the absence of SL biosynthesis is more susceptible to trypsinolysis, suggesting that the permease conformation is altered (Figure 2, C and D). SLs might thus act as chaperones to shape newly synthesized Gap1 into its correct conformation, similarly to the role of phosphatidylethanolamine in proper folding of the γ -aminobutyric acid permease GabP of *Escherichia coli* (Zhang *et al.*, 2005). Alternatively, a more direct role of SLs in protecting Gap1 against ubiquitination could also be considered. Although mammalian SLs are known to reside mainly in the exoplasmic leaflet of the plasma membrane (Holthuis *et al.*, 2001), ~20% of them are found in the cytoplasmic leaflet (Boon and Smith, 2002). Even if no data are currently available about the distribution of SLs over the two leaflets of the yeast plasma membrane (van Meer and Holthuis, 2000), it is thus conceivable that at least a small fraction of these lipids face the cytoplasm, in particular if they are forming a shell around transmembrane proteins. Because the cytosolic lysines 76, 87, and 91 of Gap1 are close to the membrane (Figure 3F), they might be part of the region containing the SL head groups and thus be inaccessible to posttranslational modifications. When Gap1 is not associated with SLs, these lysines would in contrast be exposed and targeted by the Rsp5 ubiquitin ligase, promoting degradation of the permease. Although this unregulated process might be considered a fortuitous consequence of impaired SL biogenesis, it might also be regarded as an actual quality-control mechanism ensuring that only Gap1 proteins correctly associated with SLs (and thus in an active conformation) can be stabilized at the cell surface.

SL biogenesis is also important for plasma membrane accumulation of several other proteins, including the Pma1 H⁺ ATPase (Bagnat *et al.*, 2001; Eisenkolb *et al.*, 2002; Wang and Chang, 2002; Gaigg *et al.*, 2005). In this case, a role of SLs in protein oligomerization has also been proposed (Wang and Chang, 2002). Furthermore, a mutant form of Pma1 (Pma1-10) that fails to associate with DRMs is normally targeted to the cell surface, but it undergoes rapid and ubiquitin-dependent down-regulation (Wang and Chang, 2002; Liu and Chang, 2006). Cell surface delivery of several other plasma membrane proteins is also defective in *lcb1-100* cells (Sutterlin *et al.*, 1997; Hearn *et al.*, 2003; Dupré and Haguénauer-Tsapis, 2003), although sphingoid base synthesis is not a general requirement for trafficking along the secretory pathway (Sutterlin *et al.*, 1997). In the Tat2 tryptophan permease, ergosterol is essential to targeting and accumulation at the plasma membrane (Umebayashi and Nakano, 2003). The picture emerging from these studies and the present work points to a crucial role of the lipid microenvironment of plasma membrane proteins in determining their intrinsic conformation and function, controlling their ubiquitination, and probably in determining their lateral distribution between distinct membrane domains.

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