Cell growth–dependent coordination of lipid signaling and glycosylation is mediated by interactions between Sac1p and Dpm1p

Frank Faulhammer,[1] Gerlinde Konrad,[2] Ben Brankatschk,[1] Sabina Tahirovic,[2] Andreas Knödler,[1] and Peter Mayinger[1]

[1]Division of Nephrology and Hypertension, Oregon Health & Science University, Portland, OR 97239
[2]Zentrum für Molekulare Biologie der Universität Heidelberg, D-69120 Heidelberg, Germany

The integral membrane lipid phosphatase Sac1p regulates local pools of phosphatidylinositol-4-phosphate (PtdIns(4)P) at endoplasmic reticulum (ER) and Golgi membranes. PtdIns(4)P is important for Golgi trafficking, yet the significance of PtdIns(4)P for ER function is unknown. It also remains unknown how localization of Sac1p to distinct organellar membranes is mediated. Here, we show that a COOH-terminal region in yeast Sac1p is crucial for ER targeting by directly interacting with dolicholphosphate mannose synthase Dpm1p. The interaction with Dpm1p persists during exponential cell division but is rapidly abolished when cell growth slows because of nutrient limitation, causing translocation of Sac1p to Golgi membranes. Cell growth–dependent shuttling of Sac1p between the ER and the Golgi is important for reciprocal control of PtdIns(4)P levels at these organelles. The fraction of Sac1p resident at the ER is also required for efficient dolichol oligosaccharide biosynthesis. Thus, the lipid phosphatase Sac1p may be a key regulator, coordinating the secretory capacity of ER and Golgi membranes in response to growth conditions.

Introduction

Phosphorylated derivatives of phosphatidylinositol (PtdIns; phosphoinositides) play an essential role in regulating membrane trafficking (De Matteis et al., 2002). The maintenance of separate intracellular pools of these lipids is intimately connected with the coordination of the secretory pathway (Simonsen et al., 2001). The Sac1 lipid phosphatase functions in the local control of phosphoinositides at ER and Golgi membranes (Foti et al., 2001; Schorr et al., 2001; Konrad et al., 2002). We have shown recently that yeast Sac1p regulates a PtdIns(4)P pool that is important for Golgi trafficking (Schorr et al., 2001). The role of Sac1p in the ER is less well understood. There is evidence that an ER-resident portion of Sac1p controls PtdIns-3-phosphate (PtdIns(3)P) and PtdIns(4)P levels in this organelle (Foti et al., 2001; Konrad et al., 2002). Genetic and biochemical analyses suggested that Sac1p controls ATP uptake into the ER lumen (Mayinger et al., 1995; Kochendorfer et al., 1999). However, the precise function of Sac1p in the ER has not been determined.

Correspondence to Peter Mayinger: mayinger@ohsu.edu

Abbreviations used in this paper: BMH, 1,6-bis-maleimidohexane; CPY, carboxypeptidase Y; Dol-P-Man, dolichol phosphate mannose; PtdIns, phosphatidylinositol.

The online version of this article contains supplemental material.

The mammalian orthologues of Sac1 contain a dilysine ER localization signal and interact with the coatomer (COPI) complex (Rohde et al., 2003). Yeast Sac1p contains none of the known sequence motives that effect ER retention, and it is unknown how the localization of this protein to ER and Golgi membranes is achieved. Here, we show that the dolichol phosphate mannose (Dol-P-Man) synthase Dpm1p is responsible for ER localization of Sac1p during times of rapid cell proliferation. Starvation-induced shut down of cell growth is accompanied by fast and reversible translocation of Sac1p to Golgi membranes. The cell growth–dependent interaction between Sac1p and Dpm1p is important for efficient oligosaccharide biosynthesis at the ER.

Results and discussion

A GFP-tagged version of Sac1p mainly localizes to ER membranes (Foti et al., 2001; Konrad et al., 2002), as is indicated by colocalization with RFP-tagged Alg9p, an ER-resident mannosyl transferase (Burda and Aebi, 1999; Fig. 1 A). To define ER-targeting regions within Sac1p, we constructed two GFP-tagged truncated versions of this protein (Fig. 1 B). The absence of the cytosolic COOH-terminal tail in Sac1(1-581)-GFP did not abolish ER retention (Fig. 1 B). In contrast, the deletion of

The Journal of Cell Biology, Vol. 168, No. 2, January 17, 2005 185–191
http://www.jcb.org/cgi/doi/10.1083/jcb.200407118

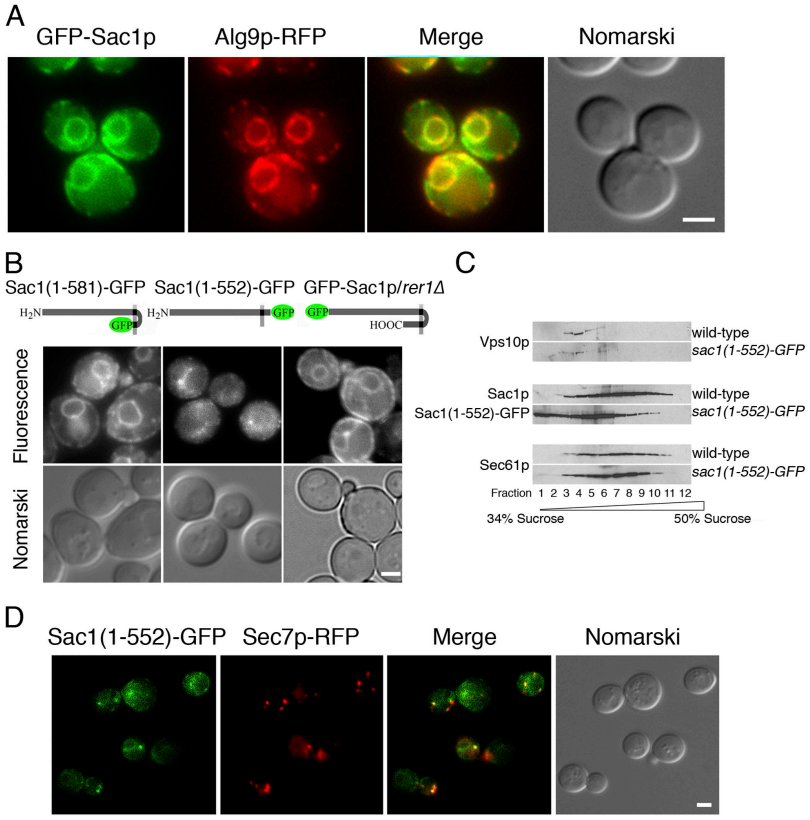

Figure 1. **Truncated versions of Sac1p localize differentially to ER and Golgi membranes.** (A) Colocalization of Alg9p-RFP with GFP-Sac1p expressed from a *CEN*-based plasmid in a *sac1Δ* background. (B) Intracellular localization of Sac1(1-552)-GFP and Sac1(1-581)-GFP in wild-type cells and of GFP-Sac1p in *rer1Δ* cells. (C) Membranes from wild-type and *sac1(1-552)-GFP* cells were fractionated using sucrose density centrifugation. Individual fractions were analyzed by immunoblotting using the indicated antibodies. (D) Colocalization of Sac1(1-552)-GFP and Sec7p-RFP. Bars (A, B, and D), 2 μm.

a COOH-terminal fragment including the second transmembrane domain in Sac1(1-552)-GFP caused reduced ER localization and increased punctate staining (Fig. 1 B and Fig. S1 A [available at http://www.jcb.org/cgi/content/full/jcb.200407118/DC1]). We have shown previously that a Sac1p variant lacking the second transmembrane domain is properly inserted into the membrane with the expected topology (Konrad et al., 2002). The portion of Sac1(1-552)-GFP present at punctate structures colocalized with the Golgi marker Sec7p-RFP (Fig. 1 D), suggesting that the second transmembrane domain contributes to ER localization of Sac1p and that loss of ER retention causes increased Golgi localization. The altered distribution of Sac1(1-552)-GFP was also observed when cell homogenates were analyzed by sucrose density centrifugation. The majority of Sac1p cosedimented with the ER marker Sec61p, whereas Sac1(1-552)-GFP was present at fractions with lower sucrose density that contained the Golgi marker Vps10p (Fig. 1 C). The reduced ER localization of Sac1(1-552)-GFP led to inositol auxotrophy (Fig. S1 B) and a moderate elevation in PtdIns(3)P and PtdIns(4)P levels (Fig. S1 D).

ER localization of Sac1p was independent of Rer1p, an adaptor targeting non-KKXX transmembrane proteins to COPI-mediated backward traffic (Sato et al., 1997; Fig. 1 B). To identify interacting factors required for ER retention of Sac1p, we performed chemical cross-linking experiments. Treatment of microsomal membranes with 1,6-bis-maleimidohexane (BMH) produced a single cross-linked product detected with anti-Sac1p antibodies (Fig. 2 A). Mass spectrometric analysis identified the Dol-P-Man synthase Dpm1p in the Sac1p-specific cross-linked complex (unpublished data). Anti-Dpm1p antibodies recognized a cross-linked product of the same size as the anti-Sac1p antiserum (Fig. 2 A). When Sac1p was overexpressed, the chemical cross-linking procedure yielded a significant increase in the Sac1p-Dpm1p product (Fig. 2 B). Deletion of the second transmembrane domain in Sac1p abolished the ability of this mutant protein Sac1pΔ553-573 to form a cross-link with Dpm1p (Fig. 2 B), suggesting that this region in Sac1p may be important for the contact with Dpm1p.

Dpm1p is an integral ER membrane protein that synthesizes Dol-P-Man, which serves as a mannosyl donor for glycosylation reactions in the ER lumen (Burda and Aebi, 1999). YFP-Sac1p colocalized extensively with a CFP-tagged version of Dpm1p (Fig. 2 C), which is consistent with an interaction of Dpm1p and Sac1p in the ER. To directly test whether Dpm1p recruits Sac1p to ER membranes, we used the temperature-sensitive *dpm1-6* mutant. A shift to 37°C caused rapid degradation of the Dpm1-6 protein (Fig. 2 D) and significantly reduced perinuclear localization of GFP-Sac1p (Fig. 2 F). In contrast, localization of the ER protein Sec63p-GFP remained unchanged at 37°C (Fig. 2 F). A similar mislocalization phenotype was observed in sucrose density gradient analysis of cell homogenates. When the *dpm1-6* strain was incubated at 25°C, the majority of Sac1p cosedimented with Sec61p (Fig. 2 E). A shift of *dpm1-6* cells to 37°C before homogenization caused a shift of Sac1p in the sucrose gradients toward lower density fractions (Fig. 2 E). The *dpm1-6* strain showed a moderate increase in PtdIns(4)P at 37°C (Fig. S1 E), corresponding to the mislocalization of Sac1p.

The second transmembrane region in Sac1p appeared to be involved in the interaction with Dpm1p. To characterize this

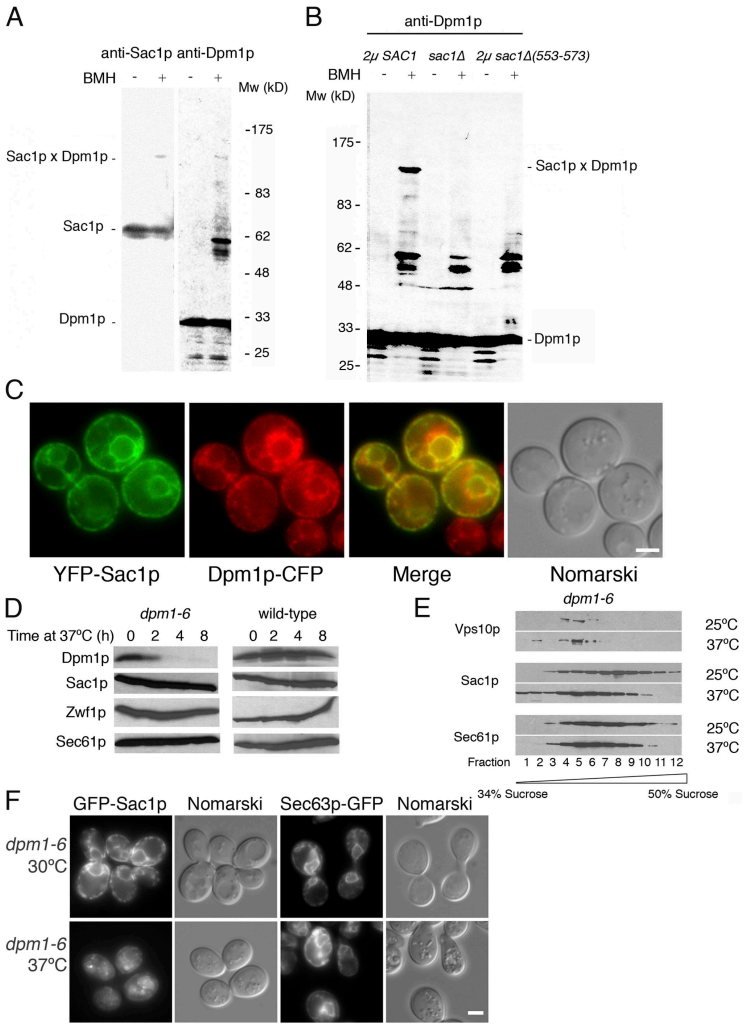

Figure 2. **Interaction with Dpm1p is required for ER localization of Sac1p.** (A and B) Microsomes from the strains shown were or were not treated with BMH. The cross-linked products were analyzed by immunoblotting using the indicated antibodies. (C) Colocalization of YFP-Sac1p and Dpm1p-CFP. (D) Wild-type and *dpm1-6* yeast cells were cultivated at 30°C. At the indicated times after shifting to 37°C, aliquots were removed and cell extracts were analyzed by immunoblotting using the indicated antibodies. (E) Membranes from *dpm1-6* cells before and after shift to 37°C were fractionated using sucrose density centrifugation. Individual fractions were analyzed by immunoblotting using the indicated antibodies. (F) Localization of GFP-Sac1p and Sec63p-GFP in a *dpm1-6* strain cultivated at 30°C or after 4 h incubation at 37°C. Bars (C and F), 2 μm.

interaction, we engineered a hybrid protein in which the transmembrane domain of Dpm1p was replaced by the transmembrane segments of Sec62p (Fig. 3 A). This COOH-terminal portion of Sec62p was shown previously to be sufficient for ER localization (Wittke et al., 1999). Expression of Dpm1ΔC-Sec62p rescued the lethal phenotype in a *dpm1Δ* mutant. Proper ER localization of Dpm1ΔC-Sec62p was confirmed by cofractionation with Sec61p in sucrose density gradients (Fig. 3 A). Chemical cross-linking of microsomal membranes from the *dpm1ΔC-sec62* mutant produced several novel Sac1p-specific cross-linked bands, but no product between Sac1p and Dpm1ΔC-Sec62p (Fig. 3 B). We also observed a significantly reduced ER localization of GFP-Sac1p in the *dpm1ΔC-sec62* strain (Fig. 3, C and D; and Fig. S1 A). The ER marker Sec63p-GFP showed typical ER staining in both wild-type and *dpm1ΔC-sec62* cells (Fig. 3 C), proving that the mislocalization of GFP-Sac1p was specific. Some GFP-Sac1p present at intensely stained puncta in the *dpm1ΔC-sec62* mutant colocalized with the Golgi marker Sec7p-RFP (Fig. 3 D), and we observed reduced punctate Golgi localization of the PtdIns(4)P-specific probe FAPP1-PH-GFP (Fig. S1 C). These results confirmed that Dpm1p recruits Sac1p to the ER, and that transmembrane regions of Dpm1p and Sac1p are important for this interaction.

To study the physiological role of Sac1p at ER and Golgi membranes, we examined yeast cells under different growth conditions. During exponential cell growth, secretory capacity is high and trafficking of secretory vesicles is largely polarized to the bud site (Finger and Novick, 1998). When nutrients become limited, protein synthesis is drastically reduced and secretory cargo delivery becomes more isotropic, mainly used to reorganize the cell wall (Werner-Washburne et al., 1993). GFP-Sac1p showed ER localization only during times of exponential growth. When cells were grown to late log phase, Sac1p was absent from the ER and instead colocalized with the Golgi marker Sec7p-RFP (Fig. 4, A and C). In contrast, a YFP-tagged version of Dpm1p showed ER localization during all growth conditions (Fig. 4 A). Consistent with this result, chemical cross-linking of microsomal membranes from late log cells yielded a significant decrease in the Sac1p-Dpm1p cross-linked product (Fig. 4 B). Depletion of glucose from culture media induces a rapid decrease in protein biosynthesis and cell growth of yeast (Werner-Washburne et al., 1993). A shift of exponentially growing cells to glucose-depleted media caused translocation of Sac1p from the ER to the Golgi (Fig. 4 D). The addition of glucose triggered a prompt redistribution of Sac1p to the ER (Fig. 4 E). Thus, the localization of Sac1p between the ER and

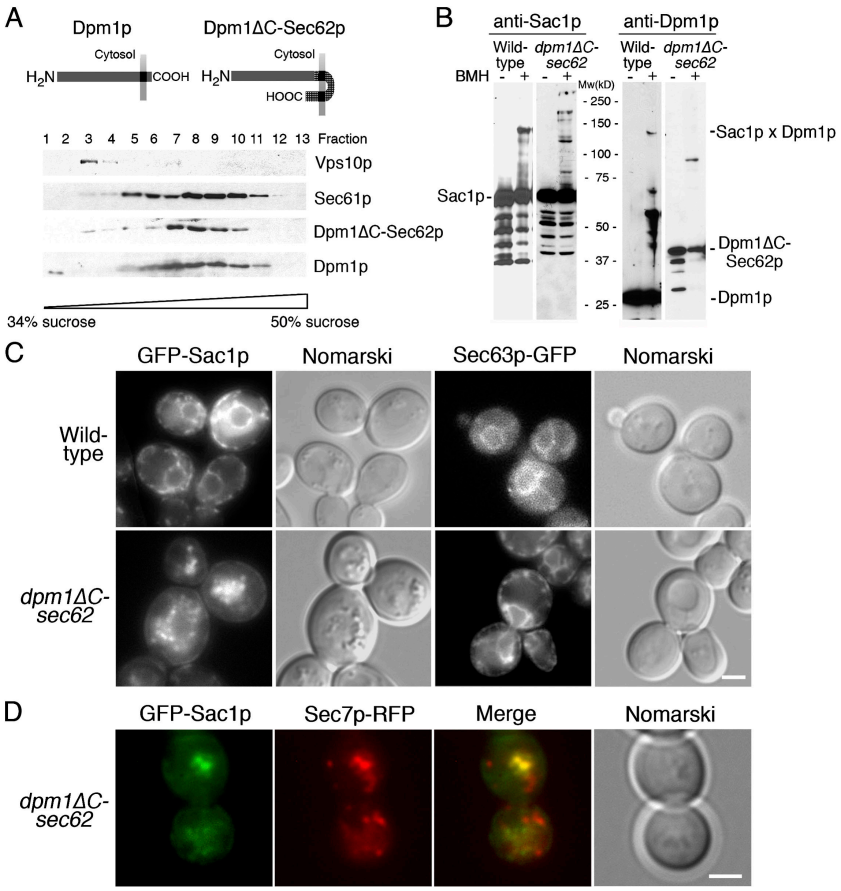

Figure 3. **The transmembrane domain of Dpm1p is required for interaction with Sac1p.** (A) Membranes from wild-type and *dpm1ΔC-sec62* cells were fractionated using sucrose density centrifugation. Individual fractions were analyzed by immunoblotting using the indicated antibodies. The predicted topologies of Dpm1p and Dpm1ΔC-Sec62p are shown (top). (B) Chemical cross-linking of microsomes from *dpm1ΔC-sec62* cells. Microsomes were or were not treated with BMH. Cross-linked products were analyzed by immunoblotting using the indicated antibodies. (C) Intracellular localization of GFP-Sac1p and Sec63p-GFP in wild-type and *dpm1ΔC-sec62* strains. (D) Colocalization of GFP-Sac1p with Sec7p-RFP in a *dpm1ΔC-sec62* strain. Bars (C and D), 2 μm.

the Golgi changes rapidly and reversibly in response to growth conditions. The introduction of an ER retention signal (KKRD) at the COOH terminus of Sac1p did not abolish the starvation-induced translocation out of the ER (Fig. S2 C, available at http://www.jcb.org/cgi/content/full/jcb.200407118/DC1). The cell growth–dependent mechanism that determines localization of Sac1p is apparently not affected by dilysine-mediated recycling.

Yeast cells cultivated in glucose-rich medium showed Golgi-specific localization of the PtdIns(4)P-specific probe FAPP1-PH-GFP (Fig. 4 F). In glucose-deprived cells, FAPP1-PH-GFP staining at Golgi structures was decreased, and this probe showed mainly diffuse cytosolic distribution with some accumulation at perinuclear ER regions (Fig. 4 F and Fig. S2 D). Similar localization of FAPP1-PH-GFP was observed in late log cells (Fig. S2 D). A *sac1Δ* strain showed accumulation of PtdIns(4)P at the ER, but also at other cellular membranes (Roy and Levine, 2004; Tahirovic et al., 2005). Golgi-localized PtdIns(4)P persisted in glucose-deprived *sac1Δ* cells (Fig. S2 D), which suggests that Sac1p contributes to the controlling of Golgi PtdIns(4)P in response to growth conditions. Because the total cellular levels of PtdIns(4)P decreased significantly in late log phase (Fig. 4 G), the increase in ER-localized PtdIns(4)P probably occurred at the expense of the Golgi pool of this lipid. This difference in distribution of PtdIns(4)P coincided with the translocation of the Sac1p phosphatase from the ER to the Golgi in late log cells. However, changes in lipid kinase activities may also contribute to this phenotype.

The availability of Dpm1p-synthesized Dol-P-Man is critical for assembly of $Glc_3Man_9GlcNAc_2$-PP-Dol (G3M9N2), which is the substrate for N-linked glycosylation. Because our data showed that Sac1p interacts with Dpm1p, we examined dolichol oligosaccharide biosynthesis under different growth conditions. A *dpm1-6* mutant deficient in Dol-P-Man biosynthesis showed accumulation of $Man_5GlcNAc_2$-PP-Dol (M5N2), which lacks all luminal mannosyl modifications (Fig. 5, C and D; Orlean, 1990). Late log cells showed strongly reduced incorporation of [^{3}H]mannose into oligosaccharides compared with that into exponentially growing cells (Fig. 5 A), and, thus, oligosaccharide biosynthesis is down-regulated when cell growth slows. Oligosaccharide biosynthesis in late log cells was not dependent on Sac1p because disruption of the *SAC1* gene did not influence oligosaccharide assembly under those conditions (Fig. 5 B). However, in exponentially growing cells, in which oligosaccharide biosynthesis was significantly more rapid, a *sac1Δ* mutant showed accumulation of M5N2 and decreased levels of G3M9N2 (Fig. 5, C and D). The phosphatase-deficient mutant *sac1-8* and a strain expressing Sac1(1-552)-GFP, which is largely absent from ER membranes (Fig. 1 B), also showed accumulation of M5N2 and decreased levels of G3M9N2 (Fig. 5 D). These defects were not caused by mislocalization or decreased levels of Dpm1p in these mutants (Fig. S2, A and B); thus, Sac1p phosphatase activity at the ER is required for efficient oligosaccharide biosynthesis during times of exponential growth. To study ER to Golgi trafficking in

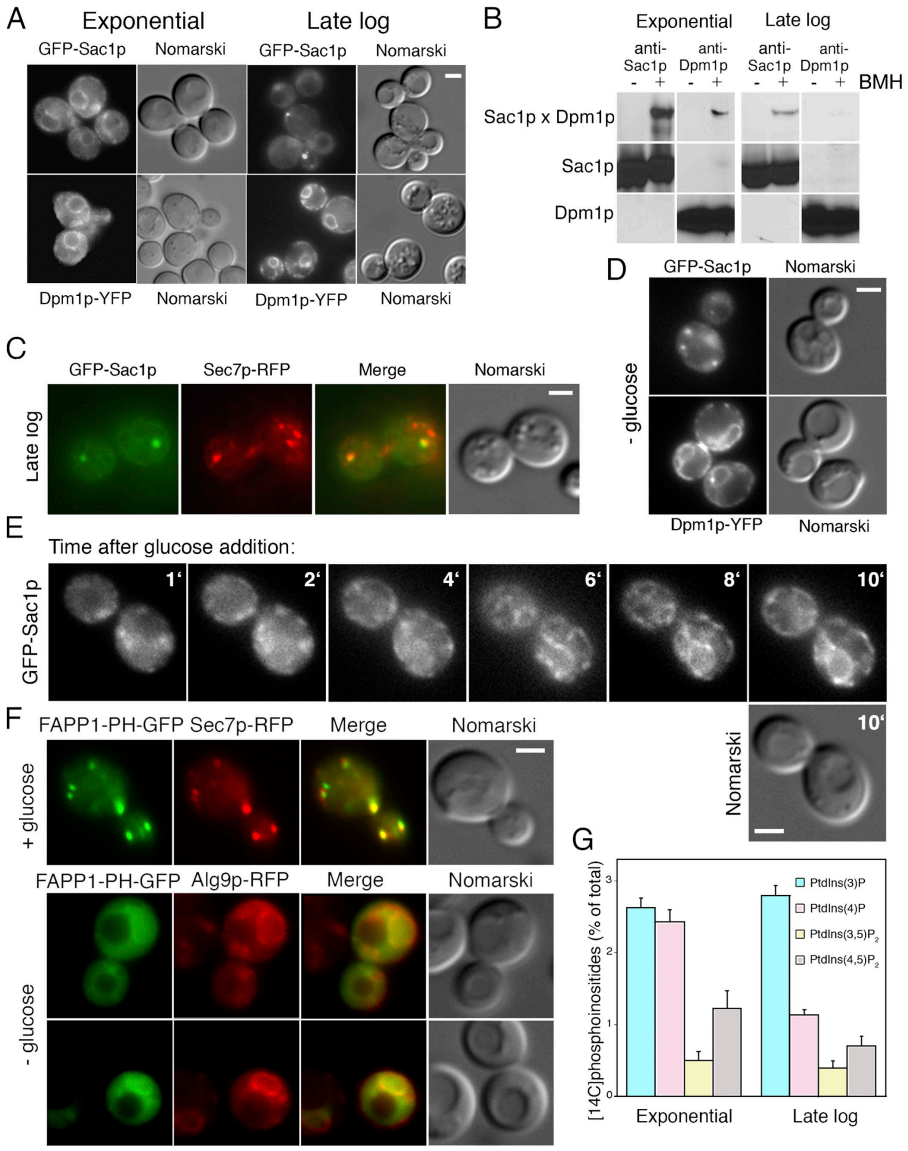

Figure 4. **Growth-dependent shuttling of Sac1p between the ER and the Golgi.** (A) Exponentially growing cells and cells in late log phase (after cultivation for 36 h) were analyzed by fluorescence microscopy. (B) Chemical cross-linking with or without BMH of microsomal fractions from cells grown as indicated. Cross-linked products were analyzed by immunoblotting using the indicated antibodies. (C) Colocalization of GFP-Sac1p with Sec7p-RFP in late log phase cells after cultivation for 36 h. (D and E) Localization of Sac1p and Dpm1p in response to glucose starvation. Yeast cells were grown exponentially for two doubling times and shifted to glucose-free medium for 30 min (D), which was followed by readdition of 2% glucose (E). (F) Colocalization of the PtdIns(4)P-specific probe FAPP1-PH-GFP with Sec7p-RFP and Alg9p-RFP in exponentially growing cells and in cells shifted to glucose-free medium for 30 min. FAPP1-PH-GFP was expressed from a 2μ plasmid. (G) Quantification of cellular phosphoinositides. Yeast strains were incubated with 4 μCi/ml [^{14}C]inositol for 6 h (exponential) or for 36 h (late log). Lipids were extracted and deacylated, and the ^{14}C-labeled glyceroinositol phosphate species were analyzed by HPLC. Data shown are means ± SD from three independent experiments. Bars (A, C, D, E, and F), 2 μm.

mutants displaying mislocalization of Sac1p, we analyzed the maturation of carboxypeptidase Y (CPY). This enzyme is N-glycosylated in the ER (Fig. 5 E, p1), further modified in the Golgi (Fig. 5 E, p2), and proteolytically cleaved in the vacuole to the mature form (Fig. 5 E, m). Both *sac1(1-552)-GFP* and *dpm1ΔC-sec62* mutant cells showed a delay of CPY passage to the Golgi, as indicated by a prolonged accumulation of the ER form (Fig. 5 E). Similarly delayed CPY transport kinetics was observed in *sac1Δ* cells (Fig. 5 E; Mayinger et al., 1995).

Our study demonstrates that ER localization of the lipid phosphatase Sac1p is mediated by interaction with Dpm1p. A direct contact between transmembrane domains of Dpm1p and Sac1p is likely to be required for this interaction. Therefore, recruitment by Dpm1p is a novel mechanism for localizing a lipid phosphatase to the ER. The Dpm1p-dependent localization of Sac1p to ER membranes was stimulated by rapid cell growth, which is characterized by active polarized secretion. Under these conditions, the presence of Sac1p was required for proper dolichol oligosaccharide biosynthesis and secretory protein trafficking at the ER. The characteristic accumulation of oligosaccharide intermediates in the phosphatase-deficient *sac1-8* mutant indicates that the catalytic activity of Sac1p is important for the regulation of oligosaccharide biosynthesis. Starvation-induced shutdown of cell proliferation was accompanied by rapid and reversible translocation of Sac1p to Golgi membranes. The rate of oligosaccharide biosynthesis declined significantly during starvation and became independent of Sac1p function. PtdIns(4)P is the major substrate for Sac1p in vivo (Foti et al., 2001; Konrad et al., 2002). Thus, cell growth–dependent shuttling of Sac1p between the ER and the Golgi may be important for reciprocal control of PtdIns(4)P at these organelles. Anterograde trafficking from the Golgi requires sufficient levels of PtdIns(4)P (Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000; Schorr et al., 2001), whereas secretory protein processing at the ER is reduced when PtdIns(4)P accumulates at ER membranes (Kochendorfer et al., 1999; Konrad et al., 2002). Therefore, Sac1p may function as a device for synchronizing the secretory capacities of the ER and the Golgi in response to different growth conditions.

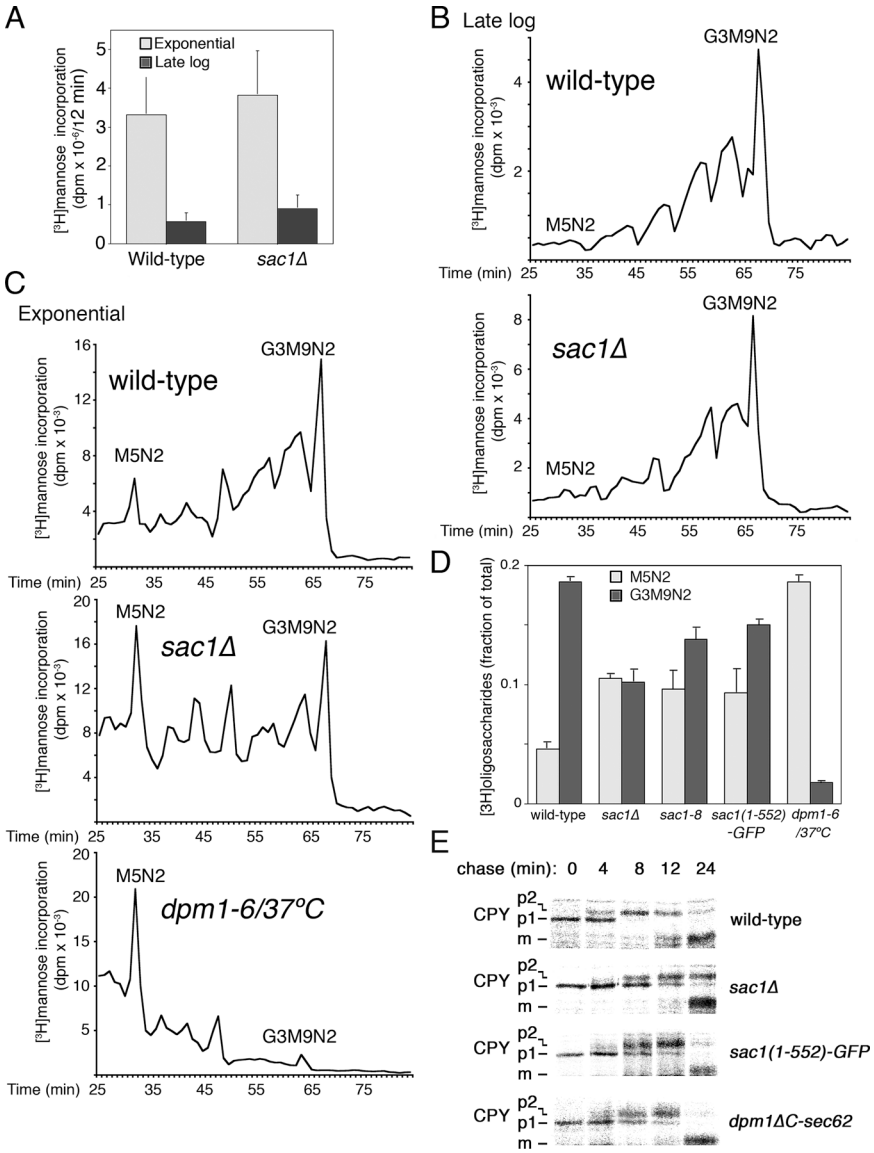

Figure 5. **ER localization of Sac1p is required for efficient oligosaccharide biosynthesis in exponentially growing cells.** (A) Incorporation of [^{3}H]mannose into oligosaccharides. Exponentially growing or late log yeast from wild-type and *sac1Δ* strains was incubated with 1.25 mCi/ml [^{3}H]D-mannose for 12 min at 30°C. Lipids were extracted and incorporated [^{3}H]mannose was quantified by scintillation counting. Data shown are means ± SD from two to four independent experiments. (B) Analysis of mannosylated lipids in wild-type and *sac1Δ* strains grown to late log phase. Yeast strains were incubated with 1.25 mCi/ml [^{3}H]D-mannose for 12 min at 30°C. Lipids were extracted and the mannosylated species were analyzed by HPLC. (C) Analysis of mannosylated lipids in exponentially growing yeast. Yeast strains were incubated with 1.25 mCi/ml [^{3}H]D-mannose for 12 min at 30°C. The *dpm1-6* strain was incubated for 2 h at 37°C before being labeled with [^{3}H]D-mannose. Lipids were extracted and the mannosylated species were analyzed by HPLC. (D) Quantification of M5N2 and G3M9N2 oligosaccharides in the indicated strains. Data shown are means ± SD from two to four independent experiments. (E) CPY trafficking. Yeast strains were pulsed with ^{35}S-labeled methionine and cysteine for 5 min at 25°C, and then subjected to a chase with nonlabeled amino acids for the times indicated. CPY species were immunoprecipitated from cell extracts and analyzed by SDS-PAGE and fluorography. p1, ER form; p2, Golgi form; m, mature CPY.

Materials and methods

GFP tagging of yeast proteins

To replace *DPM1* with a DNA fragment encoding Dpm1p-YFP or Dpm1p-CFP, a *DPM1* fragment from bp +233 to bp +801 was amplified by PCR and cloned into pPS1891 or pPS1890 (Damelin and Silver, 2000). The resulting plasmids, pFF1 (for tagging with YFP) and pFF3 (for tagging with CFP), were linearized with AvrII before transformation of yeast strains. Replacement of *SEC7* and *ALG9* with DNA fragments encoding Sec7p-CFP and Alg9-CFP was performed using PCR-amplified fragments of *SEC7* from bp +5401 to bp +6027 and of *ALG9* from bp +1001 to bp +1665. The fragments were cloned into pPS1890 (Damelin and Silver, 2000). The resulting plasmids, pGK63 and pFF18, were linearized with NcoI or Bsu36I before transformation of yeast strains. For expression of Sec7p-RFP and Alg9p-RFP, the CFP-coding regions in pGK63 and pFF18 were replaced by PCR-amplified DNA encoding RFP, resulting in pFF7 and pFF19. A plasmid containing RFP was provided by M. Knop (European Molecular Biology Laboratory, Heidelberg, Germany).

To create genomic versions of *sac1(1-552)-GFP* and *sac1(1-581)-GFP*, these *SAC1* regions were amplified by PCR and ligated next to the coding region for GFP in pAT9 (Tahirovic et al., 2003). The resulting plasmids, pFF5 (*sac1(1-552)-GFP*) and pFF6 (*sac1(1-581)-GFP*), were linearized with EcoRI before transformation of yeast strains.

To construct the *dpm1ΔC-sec62* hybrid, a DNA fragment encoding residues 149–283 of Sec62p was amplified by PCR and cloned into pRS317 (Sikorski and Hieter, 1989), resulting in pFF11-1. A DNA fragment containing the promoter region and the coding region for residues 1–234 of Dpm1p was amplified by PCR and cloned in-frame to the *SEC62* fragment into pFF11-1, resulting in vector pFF11-2.

Chemical cross-linking

Yeast microsomes were prepared as described previously (Mayinger and Meyer, 1993). For chemical cross-linking, 4 OD_{280}/ml of microsomes was suspended in 150 mM NaCl, 250 mM sucrose, 1 mM PMSF, and 20 mM Hepes, pH 7.4. BMH was added to a final concentration of 0.5 mM. The cross-linking reactions were incubated for 2 h on ice and quenched with 10 mM DTT for 5 min. Cross-linked products were analyzed by SDS-PAGE (with 6–16% polyacrylamide) and immunoblotting.

Density gradient fractionation

Yeast cells were homogenized in 250 mM sucrose, 50 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 0.5 mM PMSF, and 20 mM Hepes, pH 7.4. Extracts were centrifuged at 1,000 *g* for 2 min and the supernatant was loaded on a 1-M sucrose cushion. After centrifugation at 4,000 *g* for 15 min, the supernatant from this second centrifugation was loaded on top of a continuous sucrose gradient (34–50% sucrose, 50 mM potassium acetate, 1 mM DTT, 0.5 mM PMSF, and 20 mM Hepes, pH 7.4). The samples were centrifuged at 79,000 *g* for 20 h. 1-ml fractions were collected and analyzed by immunoblotting.

Fluorescence microscopy

Images were acquired using a microscope (model E800; Nikon) equipped with a Plan-Apo 100×/1.4 oil objective and a camera (Coolsnap HQ; Photometrics). Images were analyzed using Metamorph software (Universal Imaging Corp.).

Strains, reagents, and other procedures

Plasmids and strains are listed in Table SI (available at http://www.jcb.org/cgi/content/full/jcb.200407118/DC1). XD6-2B, dpm1-6, pDM6, and pDM8-6 were provided by P. Orlean (University of Illinois at Urbana-Champaign, Urbana, IL), YSC1021 was from C. Ungermann (University of Heidelberg, Heidelberg, Germany), and pMS329 was from M. Seedorf (University of Heidelberg, Heidelberg, Germany). Antibodies against glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich. [^{14}C]myo-inositol and [^{3}H]D-mannose were purchased from PerkinElmer. BMH was obtained from Pierce Chemical Co. Antibodies against Dpm1p (5C5) and Vps10p (18C8) were obtained from Molecular Probes. Western blotting and phosphoinositide analysis were performed as described by Schorr et al. (2001). Analysis of oligosaccharides was conducted as described previously (Zufferey et al., 1995). Maturation of CPY was analyzed as in Mayinger et al. (1995). A polyclonal antiserum against CPY was provided by D.I. Meyer (University of California, Los Angeles, Los Angeles, CA).

Online supplemental material

Supplemental figures show that mislocalization of Sac1p causes changes in cellular phophoinositide levels and depict the controls for localization of Dpm1p, Sac1p, and PtdIns(4)P. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200407118/DC1.

We thank Armin Bosserhoff and Thomas Ruppert for mass spectrometry. We are indebted to Peter Orlean, Christian Ungermann, Michael Knop, Matthias Seedorf, and David I. Meyer for yeast strains and plasmids. We thank Peter Gillespie and Teresa Nicolson for comments on the manuscript.

This work was funded by a grant (Ma1363/5-2) from the Deutsche Forschungsgemeinschaft to P. Mayinger. F. Faulhammer was supported by a PhD. scholarship from the Boehringer Ingelheim Fonds.

Submitted: 19 July 2004
Accepted: 3 December 2004

References

Audhya, A., M. Foti, and S.D. Emr. 2000. Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. *Mol. Biol. Cell.* 11:2673–2689.

Burda, P., and M. Aebi. 1999. The dolichol pathway of N-linked glycosylation. *Biochim. Biophys. Acta.* 1426:239–257.

Damelin, M., and P.A. Silver. 2000. Mapping interactions between nuclear transport factors in living cells reveals pathways through the nuclear pore complex. *Mol. Cell.* 5:133–140.

De Matteis, M., A. Godi, and D. Corda. 2002. Phosphoinositides and the Golgi complex. *Curr. Opin. Cell Biol.* 14:434–447.

Finger, F.P., and P. Novick. 1998. Spatial regulation of exocytosis: lessons from yeast. *J. Cell Biol.* 142:609–612.

Foti, M., A. Audhya, and S.D. Emr. 2001. Sac1 lipid phosphatase and stt4 phosphatidylinositol 4-kinase regulate a pool of phosphatidylinositol 4-phosphate that functions in the control of the actin cytoskeleton and vacuole morphology. *Mol. Biol. Cell.* 12:2396–2411.

Hama, H., E.A. Schnieders, J. Thorner, J.Y. Takemoto, and D.B. DeWald. 1999. Direct involvement of phosphatidylinositol 4-phosphate in secretion in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* 274:34294–34300.

Kochendorfer, K.U., A.R. Then, B.G. Kearns, V.A. Bankaitis, and P. Mayinger. 1999. Sac1p plays a crucial role in microsomal ATP transport, which is distinct from its function in Golgi phospholipid metabolism. *EMBO J.* 18:1506–1515.

Konrad, G., T. Schlecker, F. Faulhammer, and P. Mayinger. 2002. Retention of the yeast Sac1p phosphatase in the endoplasmic reticulum causes distinct changes in cellular phosphoinositide levels and stimulates microsomal ATP transport. *J. Biol. Chem.* 277:10547–10554.

Mayinger, P., and D.I. Meyer. 1993. An ATP transporter is required for protein translocation into the yeast endoplasmic reticulum. *EMBO J.* 12:659–666.

Mayinger, P., V.A. Bankaitis, and D.I. Meyer. 1995. Sac1p mediates the adenosine triphosphate transport into yeast endoplasmic reticulum that is required for protein translocation. *J. Cell Biol.* 131:1377–1386.

Orlean, P. 1990. Dolichol phosphate mannose synthase is required in vivo for glycosyl phosphatidylinositol membrane anchoring, O mannosylation, and N glycosylation of protein in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 10:5796–5805.

Rohde, H.M., F.Y. Cheong, G. Konrad, K. Paiha, P. Mayinger, and G. Boehmelt. 2003. The human phosphatidylinositol phosphatase SAC1 interacts with the coatomer I complex. *J. Biol. Chem.* 278:52689–52699.

Roy, A., and T.P. Levine. 2004. Multiple pools of PtdIns 4-phosphate detected using the pleckstrin homology domain of Osh2p. *J. Biol. Chem.* 279:44683–44689.

Sato, K., M. Sato, and A. Nakano. 1997. Rer1p as common machinery for the endoplasmic-reticulum localization of membrane-proteins. *Proc. Natl. Acad. Sci. USA.* 94:9693–9698.

Schorr, M., A. Then, S. Tahirovic, N. Hug, and P. Mayinger. 2001. The phosphoinositide phosphatase Sac1p controls trafficking of the yeast Chs3p chitin synthase. *Curr. Biol.* 11:1421–1426.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* 122:19–27.

Simonsen, A., A.E. Wurmser, S.D. Emr, and H. Stenmark. 2001. The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* 13:485–492.

Tahirovic, S., M. Schorr, A. Then, J. Berger, H. Schwarz, and P. Mayinger. 2003. Role for lipid signaling and the cell integrity MAP kinase cascade in yeast septum biogenesis. *Curr. Genet.* 43:71–78.

Tahirovic, S., M. Schorr, and P. Mayinger. 2005. Regulation of intracellular phosphatidylinositol-4-phosphate by the Sac1 lipid phosphatase. *Traffic.* 6:116–130.

Walch-Solimena, C., and P. Novick. 1999. The yeast phosphatidylinositol-4-OH kinase pik1 regulates secretion at the Golgi. *Nat. Cell Biol.* 1:523–525.

Werner-Washburne, M., E. Braun, G.C. Johnston, and R.A. Singer. 1993. Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.* 57:383–401.

Wittke, S., N. Lewke, S. Muller, and N. Johnsson. 1999. Probing the molecular environment of membrane proteins in vivo. *Mol. Biol. Cell.* 10:2519–2530.

Zufferey, R., R. Knauer, P. Burda, I. Stagljar, S. te Heesen, L. Lehle, and M. Aebi. 1995. STT3, a highly conserved protein required for yeast oligosaccharyl transferase activity in vivo. *EMBO J.* 14:4949–4960.