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

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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.

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.

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 Sac1p(1-581)-GFP did not abolish ER retention (Fig. 1 B). In contrast, the deletion of
a COOH-terminal fragment including the second transmembrane domain in Sac1p(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 Sac1p(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 Sac1p(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 Sac1p(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 Sac1p(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
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 Dpm1/H9004C-Sec62p rescued the lethal phenotype in a dpm1/H9004 mutant. Proper ER localization of Dpm1/H9004C-Sec62p was confirmed by cofractionation with Sec61p in sucrose density gradients (Fig. 3 A).

Chemical cross-linking of microsomal membranes from the dpm1/H9004C-sec62 mutant produced several novel Sac1p-specific cross-linked bands, but no product between Sac1p and Dpm1/H9004C-Sec62p (Fig. 3 B). We also observed a significantly reduced ER localization of GFP-Sac1p in the dpm1Δ mutant (Fig. 3 C). The ER marker Sec63p-GFP showed typical ER staining in both wild-type and dpm1Δ cells (Fig. 3 D). 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).

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...
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 with Sec7p-RFP in a dpm1ΔC-sec62 strain. Bars (C and D), 2 μm.

The availability of Dpm1p-synthesized Dol-P-Man is critical for assembly of Glc3Man9GlcNAc2-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 Man5GlcNAc2-PP-Dol (M5N2), which lacks all luminal mannosyl modifications (Fig. 5, C and D; Orlean, 1990). Late log cells showed strongly reduced incorporation of [3H]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
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.
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 \(sac1\-GFP\), 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 (\(sac1\-GFP\)) and pFF6 (\(sac1\-GFP\)), were linearized with EcoRI before transformation of yeast strains.

To construct the \(dpm1\-sec62\) hybrid, a DNA fragment encoding residues \(149-283\) of Sec62p was amplified by PCR and cloned into pRS317 (Sikorski and Hieter, 1989), resulting in pFF1-1. 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 pFF1-2.

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 at 37°C before being labeled with \(^{3}H\)-mannose. Lipids were extracted and the mannosylated species were analyzed by HPLC.

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 Heps, 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, 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 Heps, 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 pDM-6 were provided by P. Orlean (University of Illinois at Urbana-Champaign, IL), YSC1021 was from C. Ungermann (University of Heidelberg, Heidelberg, Germany), and pMS329 was from M. See- dorf (University of Heidelberg, Heidelberg, Germany). Antibodies against glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich. [14C]myo-inositol and [3H]-mannose were purchased from PerkinElmer. BMH was obtained from Pierce Chemical Co. Antibodies against Dpm1p (SCS) and Vps10p (18CB) 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 phosphoinositide 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.

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