Syt1p promotes activation of Arl1p at the late Golgi to recruit Imh1p

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Summary

In yeast, Arl3p recruits Arl1p GTPase to regulate Golgi function and structure. However, the molecular mechanism involved in regulating activation of Arl1p at the Golgi is unknown. Here, we show that Syt1p promoted activation of Arl1p and recruitment of a golgin protein, Imh1p, to the Golgi. Deletion of *SYT1* resulted in the majority of Arl1p being distributed diffusely throughout the cytosol. Overexpression of Syt1p increased Arl1p-GTP production in vivo and the Syt1-Sec7 domain promoted nucleotide exchange on Arl1p in vitro. Syt1p function required the N-terminal region, Sec7 and PH domains. Arl1p, but not Arl3p, interacted with Syt1p. Localization of Syt1p to the Golgi did not require Arl3p. Unlike *arl1*Δ or *arl3*Δ mutants, *syt1*Δ did not show defects in Gas1p transport, cell wall integrity or vacuolar structure. These findings reveal that activation of Arl1p is regulated in part by Syt1p, and imply that Arl1p activation, by using more than one GEF, exerts distinct biological activities at the Golgi compartment.

Key words: Golgi complex, ADP-ribosylation factor, GTPase, Vesicular trafficking, GEF, Sec7

Introduction

ADP-ribosylation factor (ARF) and ARF-like (ARL) proteins belong to the ARF family, which regulate several vesicular trafficking pathways as well as cytoskeletal organization (D'Souza-Schorey and Chavrier, 2006; Kahn et al., 2006; Gillingham and Munro, 2007b). ARF function requires cycling between the cytosolic GDP-bound and the membrane-associated GTP-bound forms. Nucleotide switching results in conformational changes to two regions of the protein (Schurmann et al., 1994). In the GTP-bound form, an N-terminal amphipathic helix is exposed from a surface pocket, enhancing ARF interaction with the lipid bilayer, stabilizing membrane association of the GTPase and recruiting effector proteins. Inactivation of ARFs by hydrolysis of bound GTP is facilitated by GTPase-activating proteins (GAPs) (Kahn et al., 2008), releasing ARFs from membranes; some GDP-bound forms of ARF and ARL proteins can, however, weakly associate with membranes (Casanova, 2007). Although the spontaneous exchange of GDP for GTP on ARFs occurs very slowly under physiological conditions, guanine-nucleotide exchange factors (GEFs) are required to activate ARFs (Donaldson and Jackson, 2000; Shin and Nakayama, 2004; Casanova, 2007).

There are fifteen recognizable human ARF GEFs, eight in *Arabidopsis*, five in *Drosophila*, five in *Caenorhabditis elegans* and five in *Saccharomyces cerevisiae* (Casanova, 2007). All ARF GEFs contain a region of approximately 200 amino acids, known as the Sec7 domain (Jackson and Casanova, 2000; Shin and Nakayama, 2004), that is responsible for GEF activity. The regions of the protein outside of the Sec7 domain, which interact with partner proteins, have been reported to play important biological roles, including membrane localization, substrate specificity and upstream regulation (Cox et al., 2004). In yeast, there are five members of the Sec7-domain family – Sec7p, Gea1p, Gea2p, Syt1p and Yel1p – and one homolog of the Sec7 GEFs – Mon2p. Sec7p, Gea1p, and Gea2p are known to have roles in secretion (Peyroche et al., 1996; Wolf et al., 1998). Sec7p localizes to the trans-Golgi apparatus and is involved in COPI-mediated intra-Golgi transport (Deitz et al., 2000). Gea1p and Gea2p overlap functionally and neither is required for viability; however, the double mutant is lethal (Peyroche et al., 1996). Studies using a temperature-sensitive *GEA1* or *GEA2* mutant suggest that they are involved in ER-Golgi and intra-Golgi transport (Peyroche et al., 2001). Yel1p acts as a GEF for Arf3p and is involved in Arf3p-dependent actin-patch polarization (Gillingham and Munro, 2007a; Tsai et al., 2008). Syt1p acts as a GEF for Arf2p in vitro and could be involved in secretion (Jones et al., 1999). Mon2p (also named Ysl2p) has been shown to interact biochemically and genetically with Arl1p and has been postulated to be an Arl1p GEF (Jochum et al., 2002; Efe et al., 2005; Gillingham et al., 2006). However, it has been reported that Mon2p is not required for membrane recruitment and activation of Arl1p (Gillingham et al., 2006). Of the five GEFs listed above, only the Gea1p, Gea2p and Sec7p proteins are conserved in eukaryotes (Cox et al., 2004; Gillingham and Munro, 2007b). Syt1p and Yel1p contain conserved pleckstrin homology (PH) domains, whereas Gea1p, Gea2p and Sec7p do not. As almost every ARF has been assigned to one or more GEFs based on either predictions or experimental data in yeast and mammals (Cox et al., 2004), the identities of ARL-GEFs are currently unknown.

Arl1p and Arl3p (homologous to ARFRP1 in mammals) are localized to the trans-Golgi in both mammalian cells and yeast (Lowe et al., 1996; Lu et al., 2001; Lu and Hong, 2003; Behnia et al., 2004; Setty et al., 2004; Liu et al., 2005), and might be involved in the regulation of Golgi structure and function (Lu et al., 2001; Liu et al., 2005; Shin et al., 2005; Zahn et al., 2008). Previous

studies have shown that Golgi-associated, GTP-bound Arl3p acts upstream of Arl1p and regulates association of Arl1p with the Golgi. Arl1p via its own GTPase cycle regulates Golgi localization of the golgin protein Imh1p (Jackson, 2003; Setty et al., 2003), a GRIP domain protein thought to play a role in vesicular transport between an endosomal compartment and distal Golgi compartment (Tsukada et al., 1999). The release of Arl1p from the Golgi is accelerated by Gcs1p (Liu et al., 2005). Previous studies from our laboratory have demonstrated that Arl1p and Arl3p are involved in maintenance of cell wall integrity and the transport of Gas1p, a GPI-anchored protein, from the late Golgi to the plasma membrane (Liu et al., 2006). To obtain additional clues to the regulation of Arl1p activation, we investigated a potential GEF regulator for Arl1p.

In this study, we provide both in vivo and in vitro evidence that the yeast Arf-GEF, Syt1p, is also an Arl1p GEF. Syt1p regulates Golgi localization and activation of Arl1p. Syt1p-dependent Arl1p activation results in the recruitment of Imh1p to the Golgi but does not affect Arl1p-mediated cell wall integrity, vacuolar biogenesis or Gas1p transport. Our findings indicate that activation of Arl1p is regulated in part by Syt1p, and imply that Arl1p activation at the Golgi exerts distinct biological activities.

Results

Syt1p is involved in localization of Arl1p and Imh1p to the Golgi

Based on the hypothesis that the absence of the Arl1p-GEF would affect GTP and GDP binding equilibrium of Arl1p and its Golgi membrane association, we examined the localization of Arl1-mRFP in three ARF GEFs mutants (*SYT1*, *GEA1*, *GEA2*) and a GEF-like (*MON2*) deletion mutant. As previously reported (Liu et al., 2005), Arl1-mRFP colocalized with the late-Golgi marker Sft2p in *arl1*Δ cells and had a diffuse cytosolic localization in *arl3*Δ cells (Fig. 1A). The localization of Arl1-mRFP to the Golgi was not affected in yeast cells lacking Gea1p, Gea2p or Mon2p (data not shown); however, the fraction of Arl1p localized to the late Golgi in *syt1*Δ cells was reduced in comparison to *SYT1* cells (Fig. 1A). By contrast, the localization of GFP-Sft2 appeared normal in the *syt1*Δ mutant, suggesting that the Golgi structure was intact. The fluorescence intensity profiles from line scan are shown in the right panels. Quantification of Arl1-mRFP and GFP-Sft2 fluorescence punctuate signals confirmed that Arl1p localized to the Golgi to a higher extent in *syt1*Δ cells than in *arl3*Δ cells (Fig. 1B). Diminished localization of endogenous Arl1p to Golgi in *syt1*Δ cells was also observed (supplementary material Fig. S1A), although the endogenous Arl1p detected by our purified anti-Arl1p antibody has some background signal. These results suggested that a minor fraction of Arl1p remained activated in *syt1*Δ cells. Ectopically expressed Syt1p restored the Golgi localization of Arl1-mRFP or endogenous Arl1p in *syt1*Δ mutants (Fig. 1C,D; supplementary material Fig. S1B).

We next examined whether *MON2*, *GEA1* or *GEA2* might be involved in the activation of Arl1p in *syt1*Δ mutants. Like in *syt1* mutants, *mon2syt1*Δ, P_{GAL1}-*GEA1/syt1*Δ and P_{GAL1}-*GEA2/syt1*Δ cells displayed a weak Arl1-mRFP signal localized at the late-Golgi (supplementary material Fig. S2A), suggesting that Mon2p, Gea1p and Gea2p are not involved in the activation of Arl1p in *syt1*Δ cells.

Imh1p, a downstream effector of activated Arl1p-GTP, was previously shown to lose its Golgi localization in *arl1*Δ cells (Panic et al., 2003; Setty et al., 2003). Because less Arl1p protein is recruited to the Golgi in *syt1*Δ cells, we examined whether GFP-Imh1 might lose its Golgi localization in *syt1*Δ cells. GFP-Imh1 displays a Golgi-like distribution in wild-type cells, but a diffuse pattern in *syt1*Δ or *arl1*Δ cells (Fig. 1E). Moreover, overexpression of Syt1p restored Golgi localization of GFP-Imh1 in *syt1*Δ yeast (Fig. 1F). Together, our findings demonstrate that Syt1p regulates the localization of Arl1p and its downstream effector Imh1p to the Golgi.

Previous studies have shown that activated Arl3p-GTP is required for the intracellular localization and activation of Arl1p (Setty et al., 2003). Therefore, we examined whether the loss of Golgi localization of Arl1p in *syt1*Δ yeast is due to lack of Arl3p activation. In both *arl3*Δ and *arl3*Δ*syt1*Δ cells, Arl3-mRFP was present in a few punctate structures partially colocalized with GFP-Sft2 (supplementary material Fig. S2B), suggesting that Syt1p has no effect on Arl3p activation.

Syt1p has been reported to act as a GEF for Arf2p (Jones et al., 1999). We next tested whether lack of Syt1p could affect the localization of Arf1p or Arf2p. In wild-type and *syt1*Δ cells, Arf1-mRFP and Arf2-mRFP localized to punctate organelles and partially colocalized with the early-Golgi marker, Sed5p (supplementary material Fig. S2C,D), suggesting that Syt1p was not involved in regulating the localization of ARF proteins. Therefore, the effects of Syt1p on the localization of Arf and Arl proteins appear to be specific for Arl1p.

Syt1p localizes at Golgi compartments

To identify Syt1p in vivo, we performed indirect immunofluorescence on cells overexpressing HA-tagged Syt1p; Arl1p (late Golgi), Sft2p (late Golgi), Arf1p (early Golgi) and Pep12p (prevacuolar endosome) were used as the localization markers. Like Arl1p, Sft2p and Arf1p, Syt1p was detected in typical Golgi-like punctate structures (Fig. 2A) and was partially colocalized with Arl1p (~65%), Sft2p (~70%) and Arf1p (~30%), but not with Pep12p (Fig. 2A,B). These results indicate that Syt1p predominately localizes to the late Golgi. We generated an anti-Syt1p specific antibody, which recognizes endogenous Syt1p by western blot (supplementary material Fig. S4A), but failed to detect endogenous Syt1p by immunostaining using this anti-Syt1p antibody. Thus, we further analyzed the subcellular localization of Syt1p by sedimentation centrifugation. We fractionated homogenized spheroplast lysates into a heavy membrane-rich fraction (P13) and a microsome-Golgi-rich and soluble fraction (S13). Surprisingly, the majority of Syt1p was detected in the P13 fraction, but was largely absent from the S13 fraction (Fig. 2C). However, these fractionation results were inconsistent with the results from the indirect immunofluorescence experiments (Fig. 2A). Therefore, to evaluate further the association of Syt1p with membranes, we treated the P13 fraction with 1 M NaCl, 0.1 M Na_2CO_3 (pH 11.5), or 1% Triton X-100 (Fujiki et al., 1982). Unlike the peripheral membrane Golgi protein Gcs1p, Syt1p was not solubilized by Na_2CO_3 (Fig. 2D); however, like an integral mitochondria membrane protein porin, Syt1p was solubilized by Triton-X-100. Syt1p is not an integral protein based on analysis of its amino acid composition, therefore we suggest that Syt1p mainly localizes to Golgi compartments and might aggregate with integral membrane proteins only when cells are lysed.

Syt1p increases the GTP-bound form of Arl1p

To determine whether Syt1p could increase the GTP-bound form of Arl1p in vivo, we performed a pull-down assay taking

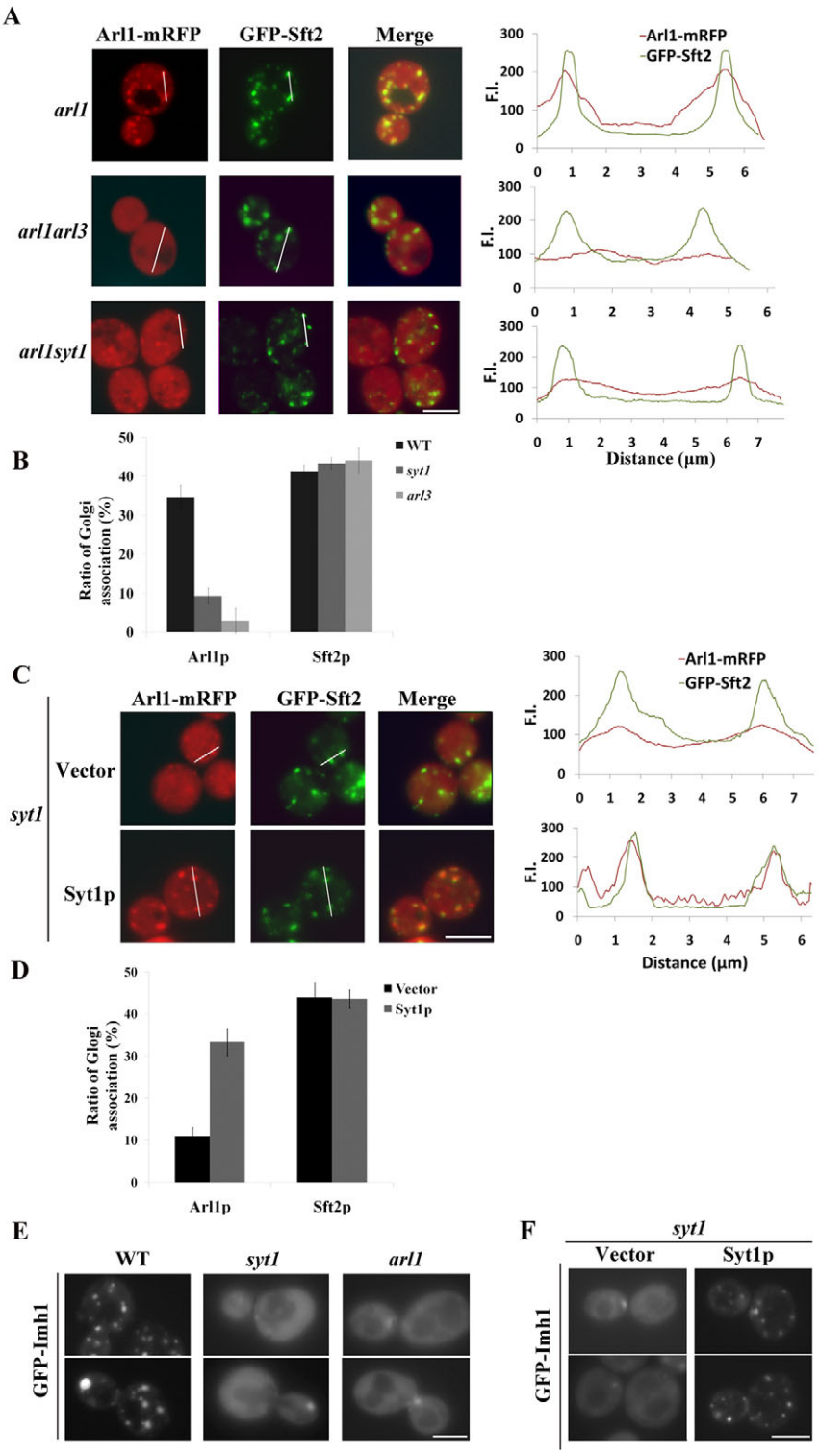

Fig. 1. Syt1p is involved in the localization of Arl1p and Imh1p to the Golgi. (**A**) Syt1p alters the Golgi localization of Arl1p. Arl1-mRFP and GFP-Sft2 were coexpressed in *arl1*Δ, *arl1arl3*Δ or *arl1syt1*Δ yeast. The fluorescence intensity (F.I.) profiles from line scan are shown in the right panels. (**B**) Quantification of the Golgi association ratio of Arl1p. Signals in A were quantified using Axio Vision Rel. 4.2 software. The fluorescence intensities of the Golgi punctate signals were summed and divided by the whole cell signal fluorescence intensity (n=50, P<0.05) as described in the Materials and Methods. The bar graph presents the mean ± s.e.m. of three independent experiments. (**C**) Expression of Syt1p restores the localization of Arl1p to the Golgi. Syt1p was coexpressed with Arl1-mRFP and GFP-Sft2 in *arl1syt1*Δ yeast. Live cells during the mid-log phase were observed using fluorescence microscopy. (**D**) Quantification of the Golgi association ratio of Arl1p. Signals in C were quantified as described in B. (**E**) Syt1p affects the localization of Imh1p. GFP-Imh1 expressed under the control of the *GAL1* promoter was transformed into wild-type or *syt1*Δ yeast. After induction with 2% galactose medium, live cells during the mid-log phase were observed by fluorescence microscopy. (**F**) The expression of Syt1p restores the Golgi-like localization of Imh1p. The localization of GFP-Imh1 in *syt1*Δ yeast expressing Syt1p or vector control is shown. Scale bars: 5 μm.

advantage of the GTP-dependent interaction of Arl1p with Imh1p. The specificity of this assay was tested using *arl1*Δ cells expressing either wild-type Arl1p (Arl1WT), the GTP binding-defective Arl1T32N (Arl1TN) or the GTP hydrolysis-deficient Arl1Q72L (Arl1QL) proteins (Gietz and Sugino, 1988). As shown in Fig. 3A, Arl1QL, but not Arl1WT or Arl1TN, bound GST-Imh1. Thus, this assay can be used to detect the amount of Arl1p-GTP in vivo.

To measure whether Syt1p can increase the active form of Arl1p in cells, we replaced the endogenous promoter of *SYT1* with the *GAL1* promoter. As shown in Fig. 3B, more Arl1p bound to GST-Imh1 was observed in extracts from Syt1p overexpressing yeast than in extracts from Syt1p-suppressed yeast, indicating that the overexpression of Syt1p increased Arl1p-GTP in vivo. To confirm that Syt1p regulates Arl1p activation, we ectopically expressed a constitutively active form of Arl1p (Arl1QL) in *syt1*Δ cells. As

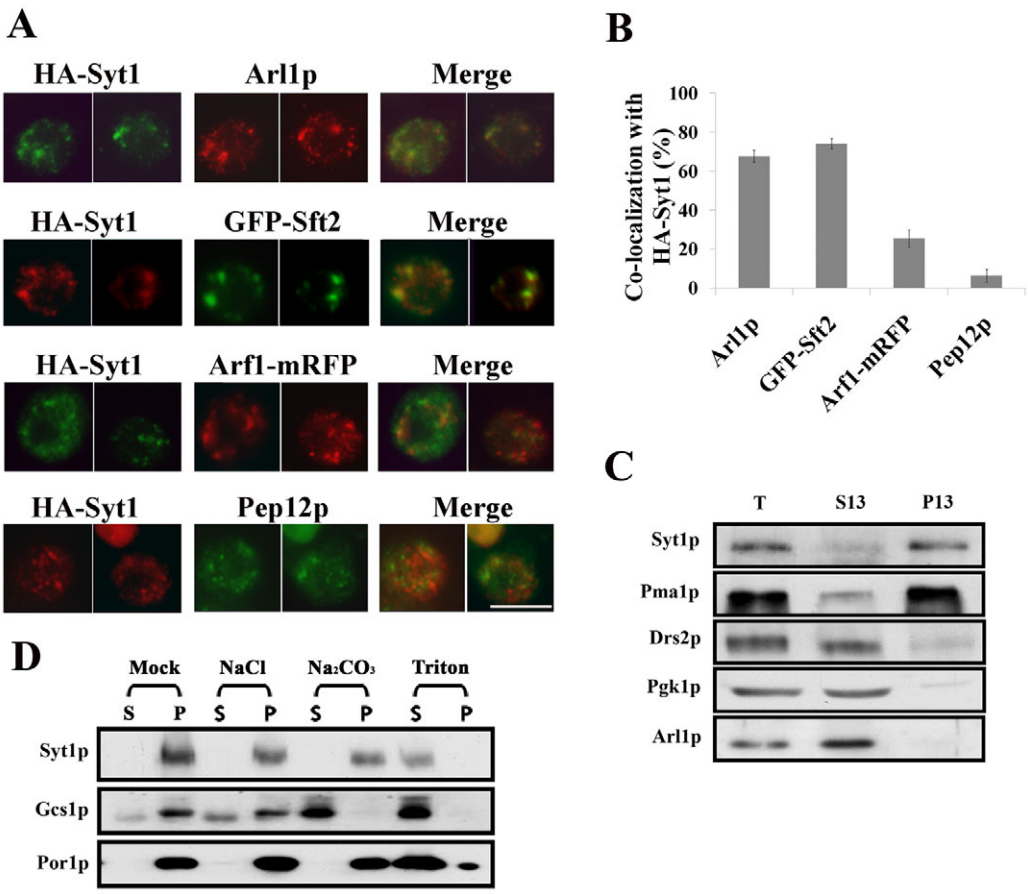

Fig. 2. Syt1p localizes to Golgi compartments. (**A**) Syt1p partially localizes to early and late-Golgi. Arl1p (late-Golgi), GFP-Sft2 (late-Golgi), Arf1-mRFP (early Golgi) and Pep12p (endosome) were used to identify the localization of HA-Syt1 using immunofluorescence microscopy. The observations were replicated in three experiments. Scale bar: 5 μm. (**B**) The percentage of colocalization between HA-Syt1 and each Golgi or endosome marker is given. In each experiment, 40 cells were examined. The bar graph presents the mean ± s.e.m. of three independent experiments. (**C**) Subcellular localization of endogenous Syt1p. Spheroplasts from cells were subjected to velocity sedimentation and were separated into P13 and S13 fractions as described in the Materials and Methods. Equal amounts of protein were analyzed using western blot with the indicated marker proteins: Pma1p (plasma membrane marker), Drs2p (Golgi marker), Pgk1p (cytosol marker) and Arl1p. (**D**) Syt1p was solubilized by Triton X-100 treatment. The P13 fraction from wild-type yeast was treated with NaCl, Na_2CO_3, Triton X-100 or buffer (Mock), followed by centrifugation at 150,000 ***g***.

shown in Fig. 3C, expression of Arl1QL could restore the localization of Imh1p to the Golgi in *syt1*Δ cells. These results suggest that Syt1p promotes the activation of Arl1p, which determines the localization of Imh1p in vivo.

We next determined whether the isolated Sec7 domain of Syt1p could display nucleotide exchange activity towards Arl1p. Because the Syt1-Sec7 domain expressed in *E. coli* was insoluble (data not shown), GST, the GST-Syt1-Sec7 domain and the GST-Yel1-Sec7 domain were expressed in yeast and isolated to perform in vitro GEF activity assays (Fig. 3D; supplementary material Fig. S3). The catalytic activity of the Syt1-Sec7 domain can be expressed as the fold-stimulation over the spontaneous exchange activity of the G protein. Therefore, we monitored nucleotide exchange on Arf1p, Arf2p, Arl1p and Arl3p after incubation with the isolated Sec7 domain protein for 2 minutes. GST-Syt1-Sec7 (10 nM) stimulated nucleotide exchange on Arf1p and Arf2p nearly 12-fold, and 7-fold on Arl1p. GST or GST-Yel1-Sec7 did not stimulate nucleotide exchange on Arf1p, Arf2p or Arl1p (Fig. 3D). Both GST-Syt1-Sec7 and GST-Yel1-Sec7 did not stimulate nucleotide exchange on Arl3p. Together, our results suggest Syt1p can increase the GTP-bound form of Arl1p in vivo and in vitro.

Syt1p function requires the N-terminal region, PH domain, and Sec7 domain

SYT1 encodes a ~135 kDa protein that contains a Sec7 domain and PH domain. PH domains are functional motifs found in many signal-transduction and cytoskeletal proteins and have been shown to mediate phosphoinositide and protein interactions (Gibson et al., 1994). To identify the specific domains of Syt1p responsible for its function and localization, a series of Syt1p-truncated mutants were generated (Fig. 4A) and expressed at similar levels (supplementary material Fig. S4B). The expression of Syt1p and Syt1dC, but not Syt1dSec7, Syt1dPH or Syt1dN, restored the localization of Arl1-mRFP and GFP-Imh1 to the Golgi in *syt1*Δ cells (Fig. 4B,C). These data indicate that the N-terminal region, Sec7 domain and PH domain are necessary for Syt1p function. No conserved domains or motifs were found within the N-terminal region of Syt1p.

To determine whether the Sec7 domain of Syt1p is required for the activation of Arl1p in vivo, we expressed Syt1p or Syt1dSec7 in *syt1*Δ strains and quantified the amount of active Arl1p using a pull-down assay. As shown in Fig. 4D, significantly more Arl1p bound to GST-Imh1 in yeast overexpressing Syt1p compared with yeast overexpressing Syt1dSec7, indicating that the Sec7 domain of Syt1p is required to promote activation of Arl1p in vivo.

We next examined the localization of the Syt1p mutants by immunofluorescence. The majority of HA-Syt1, HA-Syt1dSec7, HA-Syt1dC and HA-Syt1dN, but not HA-Syt1dPH, colocalized with GFP-Sft2 (Fig. 5A), indicating that the PH domain is required for the proper localization of Syt1p. We also performed sedimentation centrifugation to verify the localization of these mutants. Interestingly, Syt1dPH was predominantly detected in the P13 fraction, whereas Syt1dN was more prevalent in the S13 fraction (Fig. 5B). Moreover, Syt1dN cofractionated with the Golgi marker Drs2p in the P100 fraction (Fig. 5C), indicating that the N-terminal region of Syt1p might be important for interaction with the heavy membrane fractions. Activated Arl3p has been proposed to recruit a GEF to the late Golgi to activate Arl1p (Behnia et al., 2004). We next examined whether Arl3p could affect the

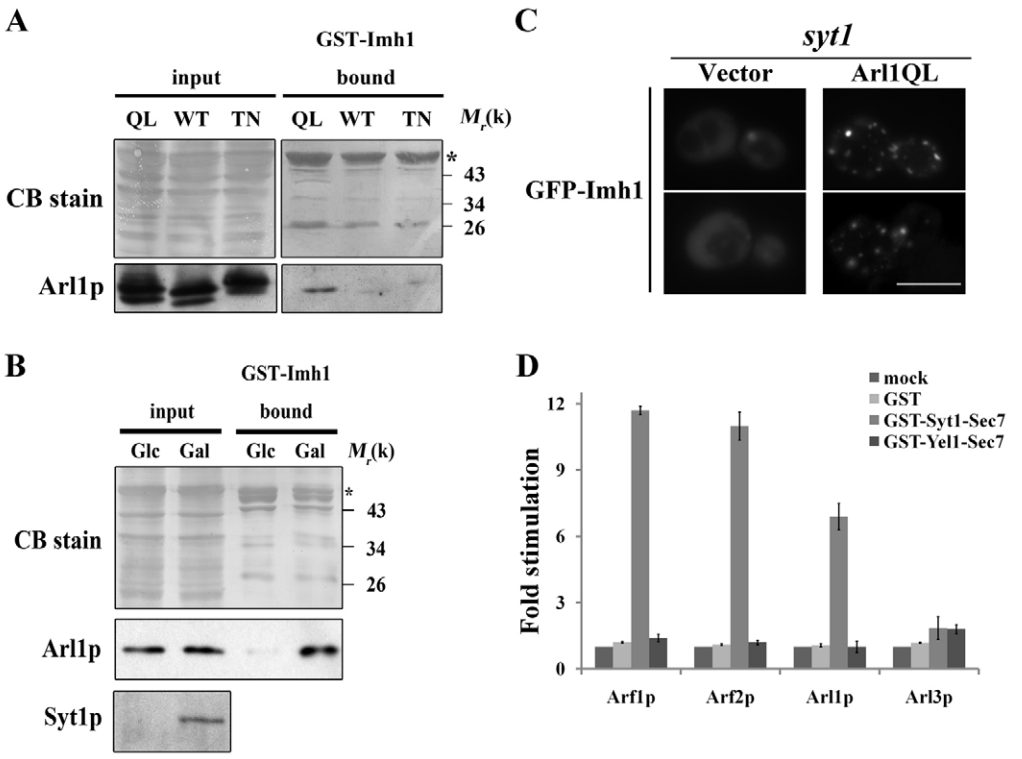

Fig. 3. Syt1p is required for the activation of Arl1p. (**A**) GST-Imh1 pulls down Arl1p-Q72L specifically. A lysate from *arl1* mutants expressing Arl1WT, Arl1QL or Arl1TN was incubated with purified recombinant GST-Imh1 and then washed before being analyzed using western blot with Arl1p-specific antibody (bottom). (**B**) Overexpression of Syt1p resulted in increased pull-down of active Arl1p by GST-Imh1. Yeast expressing Arl1p were integrated with GST-Syt1p under the *GAL1* promoter. Galactose was added to induce GST-Syt1p, yeast lysate was prepared and analysis of bound proteins was performed. (**C**) Exogenous expression of constitutively active Arl1p (Arl1QL) suppressed the localization of Imh1p in *syt1*Δ yeast. Arl1QL was coexpressed with GFP-Imh1 in *syt1*Δ yeast. Live cells in the mid-log phase were observed following induction with galactose. Scale bar: 5 μm. (**D**) Fold-stimulation of nucleotide exchange on Arf1p, Arf2p, Arl1p and Arl3p by the Syt1-Sec7 domain. GST, GST-Syt1-Sec7 and GST-Yel1-Sec7 were overexpressed and isolated from yeast. His-tagged-Arf1p, -Arf2p, -Arl1p and -Arl3p were expressed and purified from *E. coli*. Nucleotide exchange assays were performed as described in the Materials and Methods. Fold-stimulation is shown as the difference in GDP to GTP exchange at 2 minutes. The bar graph presents the mean ± s.e.m. (n=3).

localization of Syt1p. We found that the localization Syt1p to the Golgi was similar (~70%) in wild-type and *arl3*Δ mutants (supplementary material Fig. S5A,B), indicating that the Golgi localization of Syt1p does not require Arl3p. Together, these results indicate that the PH domain is required for the localization of Syt1p to the Golgi and suggest that the N-terminal region might associate with the heavy membrane-enriched fraction.

Syt1p interacts with Arl1p and Arf1p but not with Arl3p

To determine whether Arl1p interacts with Syt1p directly, we used a two-hybrid analysis to examine the interaction between N-terminal deletion of Arl1p or Arl3p with the Sec7 domain of ARF GEF proteins (Sec7p, Gea1p, Gea2p and Syt1p). As shown in Fig. 6A, Arl1d17N interacted with the Sec7 domain of Syt1p and weakly interacted with the Sec7 domain of Gea2p, but not Sec7p or Gea1p. We also found that Arl1d17N did not interact with the Sec7 domain of Yel1p (data not shown). Moreover, Arl3d17N failed to interact with the Sec7 domain of these ARF GEF proteins. We next examined the specific interaction between Arl1p and Syt1p. The N-terminal region of Syt1p (1–458), the Sec7 domain (459–633) and the C-terminal region (634–1226) were tested for their interactions with different forms of Arl1p and Arl3p in a two-hybrid assay (Table 1). Only the N-terminal deletion mutant of Arl1p interacted with Syt1p, but full-length Arl1p did not. The interaction of Arl1d17N with the N-terminal region of Syt1p was much stronger than interaction with other regions or full-length of Syt1p. Arl3p failed to interact with any of the different regions or full-length of Syt1p. These results suggest that Arl1p interacts with Syt1p and that Arl3p does not interact with Syt1p.

We further examined whether Arl1p interacts with Syt1p in vivo. We replaced the endogenous promoter of *SYT1* with the *GAL1* promoter in an N-terminal 3HA-tagged construct and expressed GST-tagged Arl1d17N (WT, Q72L or T32N) in yeast. GST-Arl1 proteins were pulled down by glutathione Sepharose, and the presence of bound HA-Syt1 was assessed by immunoblotting (Fig. 6B). Our data showed that HA-Syt1 interacted with Arl1T32Nd17N and Arl1WTd17N, and to a much lesser extent with Arl1Q72Ld17N. Consistent with a previous report, Imh1p could be pulled down by Arl1Q72Ld17N, but not Arl1T32Nd17N. Moreover, HA-Syt1 had a much stronger interaction with Arf1T32Nd17N than Arf1Q71Ld17N (Fig. 6C). Together, these results suggest that Syt1p prefers to interact with GDP-bound forms of Arl1p and Arf1p in vivo.

Syt1p-dependent Arl1p activation does not alter cell wall integrity or vacuole structure

A previous report demonstrated that Arl1p is involved in the maintenance of cell wall integrity and participates in the transport of

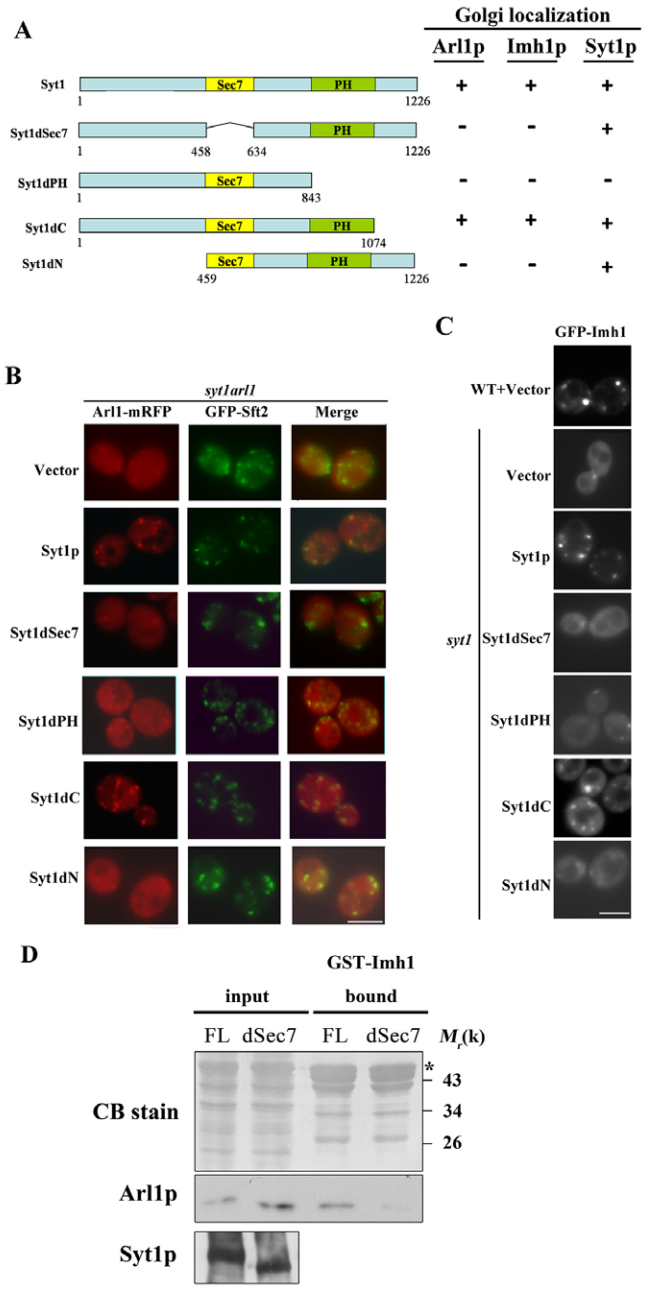

Fig. 4. The PH domain, Sec7 domain and N-terminal region of Syt1p are required for the function of Syt1p. (**A**) Diagram of Syt1p and the generated deletion constructs. The constructs used in B and C are shown with their amino acid designations. (**B**) Overexpression of Syt1dSec7, Syt1dPH or Syt1dN did not restore localization of Arl1p to the Golgi. Different forms of Syt1p were coexpressed with Arl1-mRFP and GFP-Sft2 in *arl1syt1*Δ yeast. Live cells in mid-log phase were observed using fluorescence microscopy. (**C**) Overexpression of Syt1dSec7, Syt1dPH or Syt1dN did not restore localization of GFP-Imh1 to the Golgi. (**D**) Overexpression of Syt1dSec7 resulted in decreased pull-down of active Arl1p by GST-Imh1. Yeast expressing Arl1p coexpressed with Syt1 (FL) or Syt1dSec7. Yeast lysates were prepared and analysis of bound proteins was performed as described in the Materials and Methods. Scale bars: 5 μm.

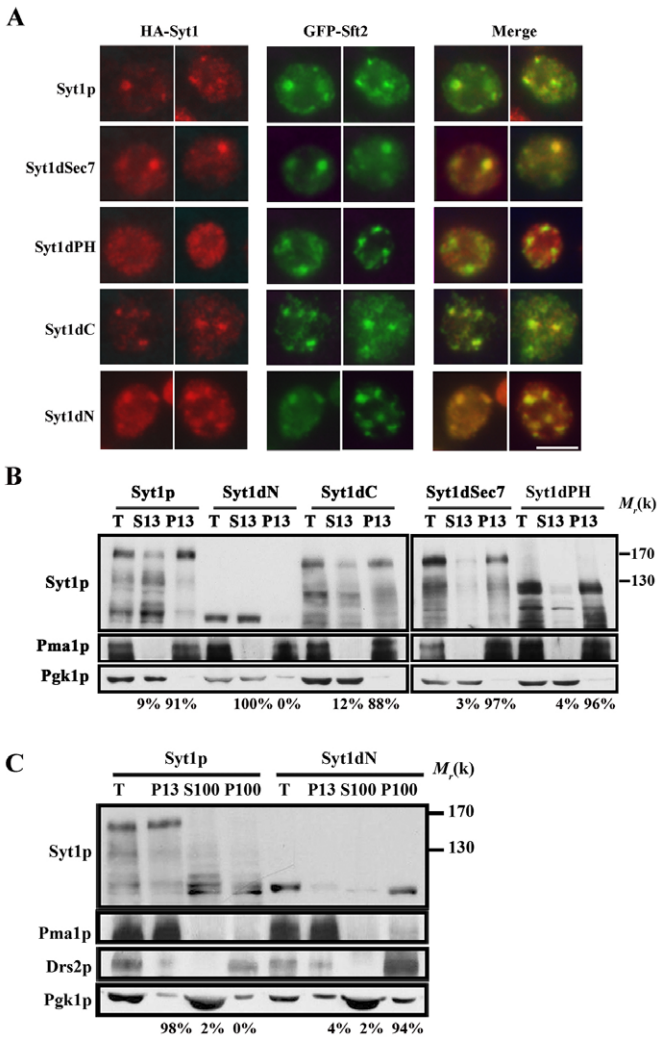

Fig. 5. The PH domain of Syt1p is required for the Golgi localization of Syt1p. (**A**) Localization of different Syt1p mutants. Different Syt1p mutants were transformed into *syt1* mutants expressing GFP-Sft2. The indirect immunofluorescence staining was performed as described in the Materials and Methods. The spheroplasts were prepared for reaction with monoclonal anti-HA antibody and polyclonal anti-GFP antibodies. Scale bar: 5 μm. (**B**) Differential centrifugation analysis of different Syt1p mutants. Different constructs of Syt1p were expressed in *syt1*Δ yeast. The cells were grown in selection medium, subjected to velocity sedimentation and separated into P13 and S13 fractions as described in the Materials and Methods. (**C**) Most of Syt1dN proteins distributed into the Golgi-rich membrane fraction. Lysates from cells overexpressing Syt1 or Syt1dN grown at 30°C were prepared as described in the Materials and Methods and centrifuged sequentially at 13,000 ***g*** for 10 minutes (P13 pellet) and 100,000 ***g*** for 1 hour (P100 pellet and S100 supernatant). Each fraction was analyzed by immunoblot to detect Syt1p, Drs2p, Pma1p or Pgk1p with specific antibodies.

a GPI-anchored protein, Gas1p (Liu et al., 2006). Therefore, we determined whether Syt1p is also involved in Gas1p transport. Gas1p is a β-1, 3-glucanosyltransferase, which is localized to lipid rafts and important for cell wall integrity (Kopecka and Gabriel, 1992; Mouyna et al., 2000). GFP-GPI (Gas1p) expressed in *arl1*Δ cells accumulated in Golgi-like structures but not in wild-type or *syt1*Δ cells (Fig. 7A). We next determined whether Syt1p is required for the transport of a non-GPI-anchored plasma membrane protein, Gap1. The $Gap1^{K9K16}$ mutant was used because the mutation abolishes its ubiquitylation and sorting to the vacuole; thus, the protein remains fully stable at the plasma membrane (Soetens et al., 2001). Similar to *arl1*Δ cells,

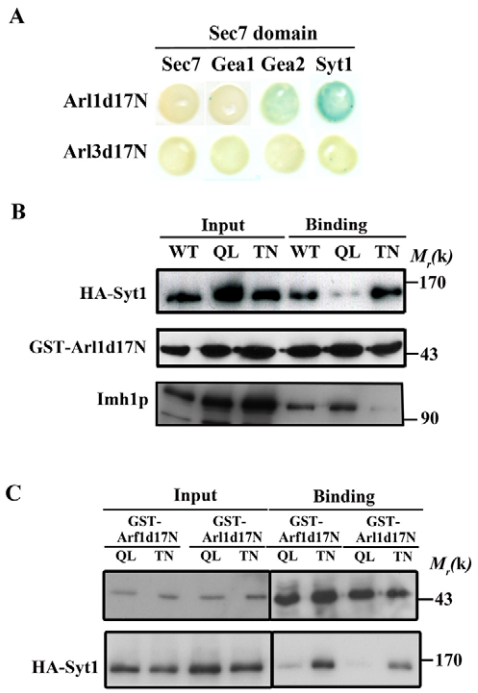

Fig. 6. Syt1p interacts with Arl1p and Arf1p. (**A**) Interactions between Arl3d17N and Arl1d17N with the Sec7 domain of GEFs. Bait plasmids pEG202 that encode Arl3d17N and Arl1d17N and pJG4-5 plasmids that encode the Sec7 domain of various ARF GEFs were cotransformed into YEM1α and the interactions were determined using a β-galactosidase assay at 30°C for 6 hours. (**B**) Interaction of Arl1d17N with Syt1p in a GDP-dependent manner. The cells were lysed with glass beads. After centrifugation, the cleared lysates were incubated with glutathione-Sepharose beads at 4°C for 4 hours and then washed three times with binding buffer before analysis of bound proteins. (**C**) Arf1d17N and Arl1d17N interact with Syt1p. Yeast lysates were prepared and analyzed as described in the Materials and Methods.

the loss of Syt1p did not affect the plasma membrane localization of Gap1p (Fig. 7B). These results indicated that Syt1p was not involved in Arl1p-mediated transport of Gas1p.

The deletion of *ARL1* is known to affect cell wall integrity, resulting in hypersensitivity to Calcofluor White or Congo Red (Liu et al., 2006). We next examined whether the *syt1*Δ mutant is hypersensitive to Congo Red. The *arl1*Δ and *arl3*Δ mutants, but not the *syt1*Δ mutant, were hypersensitive to Congo Red (Fig. 7C), suggesting that the deletion of *SYT1* did not affect cell wall integrity, consistent with the finding that Syt1p is not involved in Gas1p transport. Furthermore, exogenously expressed Syt1p could not suppress the hypersensitivity to Congo Red in *arl3*Δ cells (Fig. 7D). Previous studies have shown that Arl1p and Arl3p are required for normal vacuole formation (Bonangelino et al., 2002). Therefore, we examined whether Syt1p or Imh1p was involved in vacuole biogenesis. Consistent with previous reports, we detected vacuole fragmentation in *arl1*Δ, *arl3*Δ and *ypt6*Δ cells; however, the vacuoles were normal and rounded in WT, *imh1*Δ and *syt1*Δ cells (Fig. 7E; data not shown). These results indicate that Syt1p does not regulate cell wall integrity or vacuole structure.

Deletion of both *IMH1* and *YPT6* has been shown to lead to a severely fragmented vacuole (Tsukada et al., 1999). To determine whether *syt1*Δ, like *imh1*Δ, causes synthetic vacuolar fragmentation in *ypt6*Δ cells, we replaced the endogenous promoter of *SYT1, IMH1* and *ARL1* with the *GAL1* promoter in *ypt6*Δ cells and examined vacuole morphology in yeast grown in glucose- or galactose-containing medium. Repression of Syt1p, Imh1p, or Arl1p protein in P_{GAL1}-HA-*SYT1/ypt6*Δ, P_{GAL1}-*IMH1/ypt6*Δ or P_{GAL1}-*ARL1/ypt6*Δ cells in glucose-containing medium showed severely fragmented vacuoles compared with cells grown in galactose (50%, 49%, 64% versus 30%, 36%, 32%, respectively; Fig. 7E,F). These results suggest that Syt1p and Imh1p on the Golgi can affect Ypt6-dependent vacuolar fragmentation.

Discussion

We report here that Syt1p at the Golgi promotes the activation of Arl1p. We found that the fraction of Arl1p at the Golgi relative to cytosol was reduced in *syt1*Δ cells compared with wild-type cells; however, no difference was detected in the localization of Arl3p, Arf1p or Arf2p. We also found that the N-terminal region, PH domain and Sec7 domain of Syt1p were required for localization of Arl1p and Imh1p to the Golgi. Overexpression of Syt1p, but not Syt1p that lacked the Sec7 domain, increased the level of Arl1p-GTP in vivo. The Sec7 domain of Syt1p is sufficient to stimulate nucleotide exchange on Arl1p in vitro. In addition, Arl1p, but not Arl3p, interacts with Syt1p and the localization of Syt1p to the Golgi does not require Arl3p. Lastly, Syt1p, like Imh1p, did not participate in Arl1p-mediated cell wall integrity or vacuole biogenesis. Thus, our study demonstrates that Syt1p promotes activation of Arl1p at the late-Golgi to recruit Imh1p, rather than to maintain cell wall integrity or vacuolar biogenesis.

Syt1p regulates Golgi localization and activation of Arl1p

Yeast cells contain five structurally related Sec7 domain-containing proteins with the potential to provide GEF activity for ARF family proteins involved in vesicular transport and actin remodeling (Casanova, 2007; Anders and Jurgens, 2008; Tsai et al., 2008). Sec7p, Gea1p, Gea2p and Syt1p have been shown to be Arf1/2 GEFs and Yel1p acts as an Arf3p-GEF (Jones et al., 1999; Gillingham and Munro, 2007a; Gillingham and Munro, 2007b). Previous studies have also shown that Sec7p and Yel1p are not

Table 1. Syt1p interacts with Arl1p, but not Arl3p, in a yeast two-hybrid assay[a]

	Pray plasmids			
Bait plasmids	Syt1 (1-1226)	Syt1-N (1-458)	Syt1-Sec7 (459-633)	Syt1-C (634-1226)
Arl1	–	–	–	–
Arl1d17N	+	++++	++	+
Arl3	–	–	–	–
Arl3d17N	–	–	–	–

[a]Bait plasmids pEG202 encoding different forms of Arl1p or Arl3p and pJG4-5 plasmids encoding the full-length, N-terminal, Sec7 domain or C-terminal of Syt1p were cotransformed into YEM1α and the interactions were determined using a β-galactosidase assay. The scores of the interactions between the various Syt1p constructs with different forms of Arls are given. Interaction, +; no interaction, –. Strong interactions are indicated by additional '+' signs.

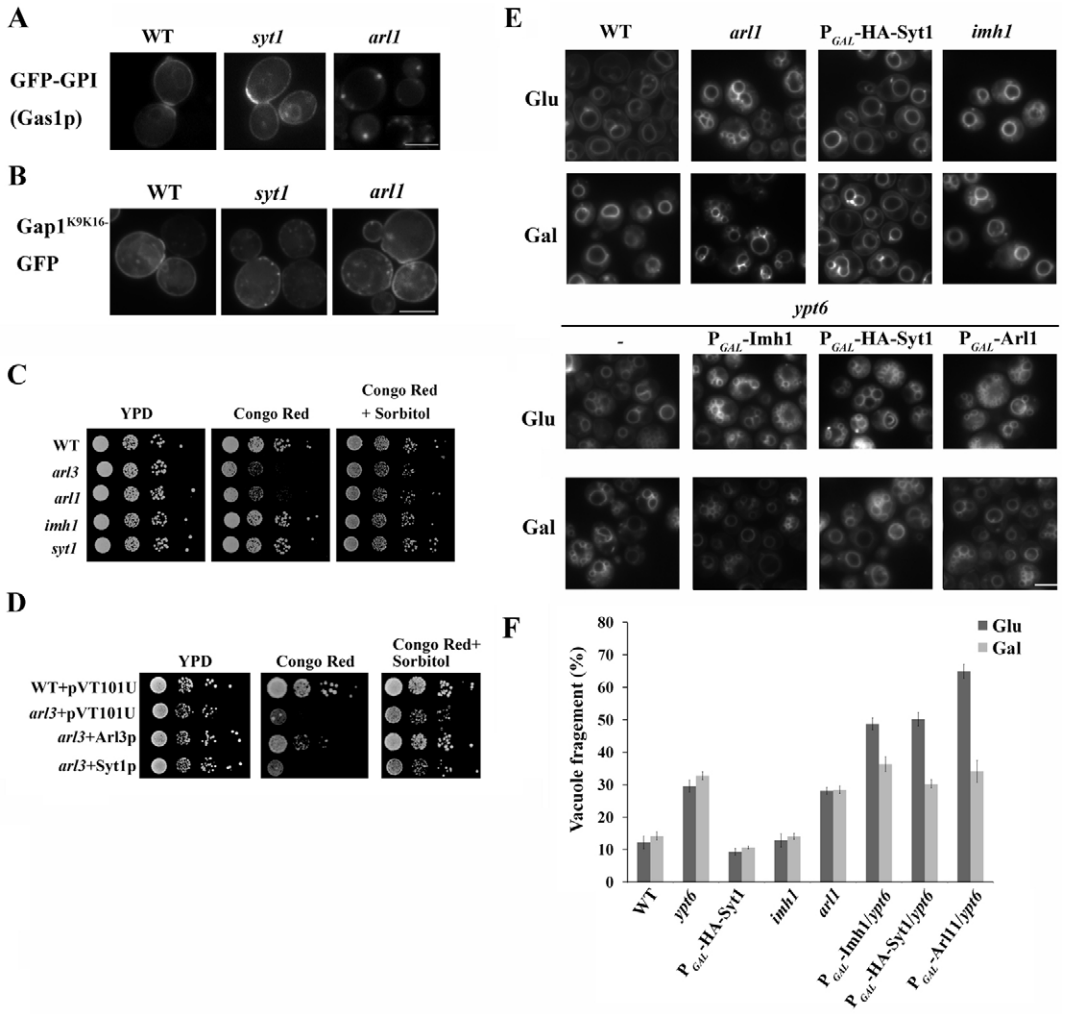

Fig. 7. Syt1p does not regulate cell wall integrity or vacuole biogenesis. (**A**) Localization of GFP-GPI (Gas1p) in *syt1*Δ or *arl1*Δ mutant cells. GFP-GPI was expressed in wild-type, *syt1*Δ or *arl1*Δ mutants, and live cells in mid-log phase were observed using fluorescence microscopy. (**B**) Localization of Gap1^{K9K16}-GFP in *arl1*Δ or *syt1*Δ mutant cells. (**C**) Congo Red hypersensitivity assay. Different mutant yeast were serially diluted ten-fold as indicated and spotted on plates containing Congo Red or Congo Red with 1.2 M sorbitol to analyze their sensitivity to cell-wall interference. (**D**) Congo Red hypersensitivity of *arl3* mutants with overexpressed Syt1p. Arl3p and Syt1p were expressed in *arl3*Δ cells and transformants were serially diluted and spotted on plates containing Congo Red or Congo Red with 1.2 M sorbitol to examine their hypersensitivity. (**E**) In vivo vacuole morphology. *GAL1* promoter was integrated upstream of *SYT1*, *IMH1* or *ARL1* genes in *ypt6*Δ cells and upstream of *SYT1* gene in wild-type yeast. Wild-type and mutant yeast grown in glucose or galactose medium were labeled with the endocytotic tracer dye FM4-64. Note that only vacuoles are visible under these conditions. (**F**) Quantification of fragment vacuole ratio in different mutant cells. In each experiment, 100 cells were examined. The bar graph presents the mean ± s.e.m. of three independent experiments.

colocalized with Arl1p (Liu et al., 2005; Gillingham and Munro, 2007a) and are therefore unlikely to be Arl1p-GEFs. Consistent with these findings, our study showed that the Sec7 domain of Yel1p and Sec7p did not interact with Arl1p. Mon2p is distantly related to the Arf-GEFs and has been suggested to be an Arl1p-GEF (Jochum et al., 2002; Wicky et al., 2004); however, consistent with a previous report demonstrating that Mon2p is not required for activation of Arl1p (Gillingham et al., 2006), we found that Mon2p is not involved in recruitment of Arl1p and Imh1p.

SYT1 was initially identified through its genetic interaction with Ypt31 and Ypt32p, which are involved in the exocytic pathway (Jones et al., 1999). Ypt31 and Ypt32p are required for the exit of secretory vesicles from the late Golgi and for recycling of proteins between the late Golgi and early endosomes (Jedd et al., 1997). Overexpression of *SYT1* suppressed the loss of Ypt31 and Ypt32p function and Syt1-Sec7 from yeast lysate-stimulated nucleotide exchange on Arf1p approximately 3-fold (Jones et al., 1999). Our data show that Syt1p affected the localization of Arl1p and Imh1p, but not Arf1/2p or Arl3p, to the Golgi and that Syt1p promoted the activation of Arl1p in vivo to recruit Imh1p. Moreover, overexpression of Arl1QL restored the localization of Imh1p to the Golgi in *syt1*Δ cells (Fig. 3C), suggesting that Syt1p might not act as an Arl1p effector to recruit Imh1p to the Golgi. These results suggest that Syt1p might function as a GEF for Arl1p.

We further provided biochemical evidence that isolated Syt1-Sec7 expressed in yeast could stimulate nucleotide exchange on Arl1p, Arf1p and Arf2p, but not Arl3p in vitro. We have tried to purify recombinant Sec7 domain of Syt1p from an *E. coli* expression system; however, the His-tagged Syt1-Sec7 was insoluble. Although *E. coli*-expressed ARNO-Sec7 could increase GTPγS binding on Arf1p nearly 15-fold, it has no activity on Arl1p (our unpublished results). We infer that the properties of Syt1-Sec7

prepared from a yeast expression system are different from ARNO-Sec7 prepared from *E. coli*.

Although it is important to use full-length Syt1p to directly demonstrate that Syt1p possesses GEF activity for Arl1p in vitro, the association of Syt1p with the heavy membrane fraction makes it difficult to purify the soluble form. Our preliminary data also show that Syt1p might form dimer or oligomers in vivo, which might be important for assaying its GEF activity. The structure outside of the Sec7 domain has been suggested to be important for ARF specificity and to regulate GEF activity (Donaldson and Jackson, 2000; Casanova, 2007). Consistent with this notion, our preliminary data also show that the N-terminal region of Syt1p, which can interact with Arl1p directly, is important for Arl1p activation and Imh1p localization. Although we do not have evidence that Syt1p can activate Arf1p or Arf2p in vivo, partial colocalization and strong interaction of Arf1p and Syt1p implies that Syt1p might also act as a GEF for Arf1p or Arf2p where they have roles in unidentified membrane or vesicular trafficking under specific physiological conditions.

Dual localizations suggests multiple roles for Syt1p

ARF GEFs and ARFs are not highly colocalized (Gillingham and Munro, 2007a; Chun et al., 2008). We found that the majority of Syt1p was colocalized with the late-Golgi and that some Syt1p colocalized with the early Golgi. Syt1p disruption did not affect Golgi localization of Arf1p and Arf2p. Therefore, Syt1p might only activate a minor fraction of Arf1p or Arf2p, whereas the majority of Arf1p or Arf2p are activated by other GEFs (Sec7p, Gea1p or Gea2p). The ability of Syt1p both to act as an ARF GEF and to suppress the *ypt31/32* mutation suggests that Syt1p does have a role in Golgi protein transport (Jones et al., 1999), which is consistent with our finding that Syt1p promoted Arl1p activation. Because Arl1p is more closely related to Arf1p and Arf2p than to Arl3p or any other GTPase, it is not surprising that the Syt1p ARF GEF promoted Arl1p activation. Consistent with this finding, Gcs1p acts as a GAP for Arl1p and Arf1p, but not Arl3p (Liu et al., 2005). These data led us to infer that Syt1p is a multifunctional molecule, which might act as multiple targets at different intracellular membranes to regulate intracellular membrane dynamics.

Most GEFs interact with either the nucleotide-free or GDP-bound forms of their target GTPases (Macia et al., 2001; Niu et al., 2005). Consistent with this, our data also showed that, in yeast, Syt1p prefers to interact with Arl1T32Nd17N (Fig. 6B,C). Furthermore, this interaction in vivo might involve other proteins or phospholipids on the Golgi membrane. The N-terminally truncated ARF proteins are soluble and retain full nucleotide exchange activity (Paris et al., 1997); moreover, their structure mimics the membrane-bound structure of ARFs. Syt1p interacts with the N-terminal deletion mutant, but not with full-length Arl1p, suggesting that Arl1p interacts with Syt1p at the Golgi membrane. The interaction between Arl1d17N and N-terminal region of Syt1p is much stronger than that of full-length Syt1p. We infer that conformation of Syt1p needs to be altered before the N-terminal region can interact with Arl1p.

Multiple domains are required for the function of Syt1p

Regions of GEF proteins outside of the Sec7 domain have been reported to play important biological roles (Cox et al., 2004). Deletion of the Sec7 domain of Syt1p prevents the activation of Arl1p, suggesting that the Sec7 domain plays a catalytic role in the protein. PH domains are involved in binding phosphoinositides. The lack of sequence similarity between the PH domains probably reflects binding specificity to various phosphoinositides on different cellular membranes. Several Sec7 domain-containing GEFs have been shown to be recruited to the plasma membrane by PH domains (Klarlund et al., 2000). Despite the fact that the PH domain of yeast Syt1p has poorer homology to the consensus PH domain (Cox et al., 2004), we found that it was necessary for the localization of Syt1p to the Golgi. Unlike Syt1p, Yel1p required both its Sec7 domain and PH domain for targeting to membrane regions of polarized growth in yeast, where it colocalized with Arf3p (Gillingham and Munro, 2007a).

The N-terminal region of human BIG1 has been shown to be sufficient for Golgi targeting (Mansour et al., 1999). In addition, mutations near the Sec7 domain in the N-terminal region of Gea2p result in defects in membrane trafficking, but not Gea2p targeting or activation of Arf1p (Park et al., 2005). Although the N-terminal region of Syt1p has not been previously found to contain any conserved domains or motifs, it was reported to contain eight phosphorylated residues (Gruhler et al., 2005; Chi et al., 2007; Li et al., 2007; Smolka et al., 2007; Albuquerque et al., 2008). The N-terminal region, but not the PH domain, is responsible for the association of Syt1p with the heavy membrane fraction (Fig. 5B). The association of Syt1p with the heavy membrane is disrupted after treatment with Triton X-100, but not with Na_2CO_3. There are, albeit few, examples of peripheral membrane proteins that resist extraction with high pH (Feldheim and Schekman, 1994). The N-terminal region of Syt1p has neither a membrane-associated motif or transmembrane motif or domain, nor is it considerably hydrophobic. This abnormal association of Syt1p with the heavy membrane might be due to the strong interaction of its N-terminal region with membrane proteins that interact with Syt1p. Consistent with this hypothesis, deletion of the N-terminal region of Syt1p resulted in fractionation with the Golgi membrane-rich fraction. Previous studies have suggested that the DCB (dimerization and cyclophilin binding) domain in the N-terminal region of GEF is required for the dimerization of GEF (Claude et al., 1999; Grebe et al., 2000; Ramaen et al., 2007). Therefore, it is possible that the N-terminal region of Syt1p might be involved in dimerization, intra-molecular protein-protein interaction or clustering with integral membrane proteins on the Golgi. It is also possible that the interaction of Arl1p with Syt1p might stabilize the GTP-bound state, resulting in more Arl1p-GTP at steady state.

Syt1p-dependent Arl1p activation reveals a dual role for Arl1p

Arf1p is thought to function at multiple locations to generate different classes of carrier vesicles in the secretory pathway and accumulating evidence suggests that different Arf1p-GEFs control its activation at each site (Kawamoto et al., 2002; Zhao et al., 2002; Garcia-Mata et al., 2003). Mammalian Arl1 has been shown to recruit different golgins to different subdomains of the trans-Golgi network to act on distinct cargo transport and suggested that the subcellular localization of GEFs is a crucial determinant for the spatiotemporal activation of ARFs (Lock et al., 2005). Consistent with previous findings, our data also suggest a model in which Arl1p can be activated by more than one GEF to play distinct roles in regulating the structure and function of the Golgi (Fig. 8). Syt1p-GEF activates Arl1p to recruit Imh1p, whereas Syt1p-independent activation of Arl1p,

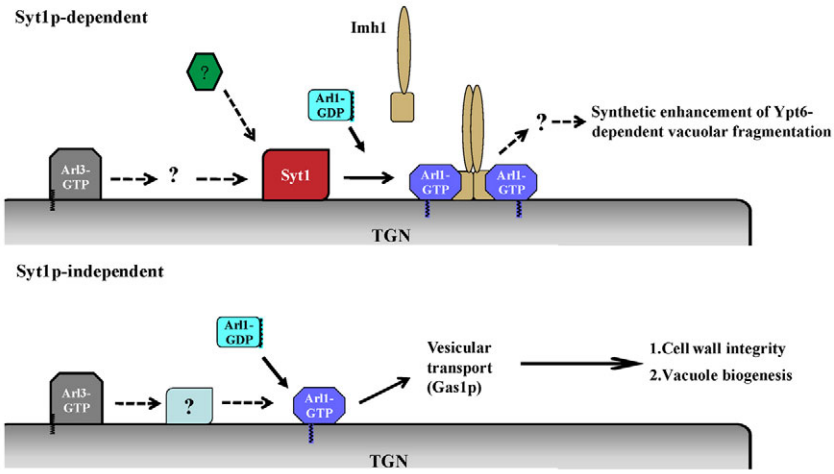

Fig. 8. A model for the Arl3p-Syt1p-Arl1p-Imh1p pathway. Arl1p can be activated by more than one GEF to play distinct roles in regulating the structure and function of the Golgi. Syt1p might activate Arl1p to recruit Imh1p, which is subsequently involved in synthetic enhancement of Ypt6p-dependent vacuole fragmentation. Arl1p can be activated by another GEF to promote vesicular transport to maintain cell wall integrity and vacuole biogenesis. Arl3p or other factors might, through an unknown mechanism, trigger Syt1p for promoting the activation of Arl1p at the late Golgi, which in turn leads to the recruitment of Imh1p.

presumably activated by another GEF, promotes vesicular transport for cell wall integrity and vacuole biogenesis (Fig. 8). A previous study showed that deletion of both *IMH1* and *YPT6* lead to a severely fragmented vacuole (Tsukada et al., 1999). Consistent with this, *ypt6*Δ cells lacking Syt1p also showed synthetic enhancement of Ypt6-dependent vacuolar fragmentation. A role for both Imh1p and Ypt6p in vesicle tethering has been suggested; however, no physical interaction between these proteins has been found. Further study on the function of Imh1p will undoubtedly reveal the role of Syt1p-dependent activation of Arl1p on Golgi function.

In conclusion, we have uncovered a new regulator, Syt1p, involved in the activation of Arl1p to recruit Imh1p to the late-Golgi. It seems likely that Arl3p regulates Arl1p activation at more than one late-Golgi site via Syt1p and other unidentified GEFs, each in specific molecular complexes that localize, respond to and integrate multiple signals (Fig. 8). However, we cannot rule out that Syt1p could be acting in a parallel pathway to the Arl3-Arl1 cascade for recruitment of Imh1p to the Golgi. Defining the molecular mechanism of how Syt1p stimulates guanine-nucleotide exchange on Arl1p in vivo and identifying other GEFs for Arl1p will require further considerable work. Further analysis of Syt1p and its regulators will improve our understanding of the novel mechanism of the activation of Arl1p in regulating Golgi function.

Materials and Methods

Strains, media and plasmids

Supplementary material Table S1 lists the yeast strains used in this study. Yeast culture media were prepared as described (Sherman et al., 1986). Yeast extract peptone dextrose (YPD) medium contained 1% Bactoyeast extract, 2% Bactopeptone and 2% glucose. Synthetic dextrose (SD) medium contained 0.17% Difco yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate and 2% glucose. Nutrients essential for auxotrophic strains were supplied at specified concentrations (Sherman et al., 1986). Yeast strains were transformed using the lithium acetate method (Ito et al., 1983). The plasmids used in this study are listed in supplementary material Table S2. The GFP-GPI (Gas1p; MBQ31) and Gap1K9K16-GFP (TPQ88) expression plasmids were kindly provided by K. Simons (MPICBG, Dresden, Germany). The GFP-Sft2p expression plasmid was a gift from Hugh R. B. Pelham (MRC-LMB, Cambridge, UK).

Yeast two-hybrid analysis

Yeast two-hybrid assays were performed using the 'interaction-trap' system (Golemis and Khazak, 1997). In this system, the bait (Arf1p, Arl1p or Arl3p) was fused with the DNA-binding domain of LexA in pEG202 (bait plasmid). The Sec7 domain from different GEFs or different regions of Syt1p [N-terminal region (1–458), Sec7 domain (459–633), and C-terminal region (634–1226)] were constructed using the vector pJG4-5 (pray plasmid), which uses the inducible yeast *GAL1* promoter to express the protein fused to an acidic domain that functions as a transcriptional activation motif. The reporter yeast, YEM1α, expressing interacting proteins, can transactivate two reporter genes, *LacZ* and *LEU2*, allowing for the expression of β-galactosidase and growth on minimal medium lacking leucine.

Expression and purification of recombinant proteins and polyclonal antibody production

Extended Sec7 domains from yeast Syt1p (424–665) were N-terminally tagged with a histidine tag using the vector pET32a. His-tagged proteins were then purified using the Ni^{+} NTA resin (Qiagen, Valencia, CA) as previously described (Huang et al., 1999). The denatured purified recombinant Syt1 Sec7 domain was isolated from an SDS-PAGE gel and used as an antigen for polyclonal antibody production in rabbits. The antibody against Syt1p is shown in supplementary material Fig. S4.

Microscopy

Images of live cells containing GFP-tagged or mRFP-tagged proteins were obtained after growth in synthetic medium to the mid-log phase. All fluorescent protein-tagged chimeras were cultured in selection medium with 2% glucose, except for cells expressing GFP-GPI (Gas1p) and Gap1K9K16-GFP, which were induced with 2% galactose. After overnight culture or induction, mid-log-phase cells were examined and images were taken using a Zeiss Axioskop microscope equipped with a Cool Snap FX camera. Quantification of the ratio of Golgi association (RGA) for Arl1p and Sft2p was calculated by measuring the fluorescence intensity values (arbitrary units) using Axio Vision Rel. 4.2 software: (1) punctate structure (PS) signal greater than 0.2 μm diameter; and (2) fluorescence signal of the whole cell (C). RGA was then calculated as a percentage of the total fluorescence of both areas: RGS=(PS/C)×100. Measurements were performed on images acquired with the same microscope intensity settings (Matheson et al., 2007).

Indirect immunofluorescence

Cells were grown and prepared for indirect immunofluorescence, as previously described (Lee et al., 1997). The antibodies used included affinity-purified anti-Syt1p and monoclonal anti-GFP antibody (Sigma). The secondary antibodies included Alexa-Fluor-488-conjugated goat anti-mouse IgG and Alexa-Fluor-594-conjugated goat anti-rabbit IgG (Molecular Probes), used at a dilution of 1:1000 and 1:2000, respectively. The preparations were visualized with a Zeiss Axioskop microscope and images were processed using Image Pro Plus software.

Protein interaction analysis

To analyze the in vivo interaction of Arl1p and Syt1p, yeast cells (~150 A_{600} units) coexpressing P_{GAL}-3HA-Syt1p and various GST-Arl1d17N or GST-Arf1d17N mutants were harvested and lysed with glass beads in 1 ml of binding buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% 50 mM EDTA, 5 mM NaF and a protease inhibitor cocktail) at 4°C for 4 hours and centrifuged at 700 ***g*** for 10 minutes to obtain the cleared lysate (1 ml). Beads were washed three times with binding buffer and boiled in sample buffer (50 μl), and samples of proteins were separated by SDS-PAGE in 10% gel before blotting.

G-protein pull-down assay

Activation of Arl1p was assayed using a novel pull-down assay. Arl1 (Arl1WT) and point mutations Arl1Q72L (Arl1QL) or Arl1T32N (Arl1TN) were ectopically expressed in an *arl1*Δ mutant strain from pYIplac128 derivatives and the transformed yeast were lysed with glass beads at 4°C in 0.65 ml of 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$ and protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin,

1 μg/ml pepstatin, 1 μM benzamidine and 1 mM phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation at 10,000 ***g*** for 5 minutes. The clarified lysate (0.5 ml) was incubated for 2 hours at 4°C with 20 μg of either GST or GST-Imh1 containing the C-terminal 177 amino acids of Imh1p, each bound to glutathione-Sepharose beads (GE Healthcare). The beads were washed three times with lysis buffer and the bound proteins were eluted with 40 μl SDS-PAGE sample buffer. This sample was assayed for the presence of Arl1p by western blotting.

To pull down activated Arl1p, yeast expressing Arl1p under the control of the *ADH* promoter were integrated into a strain carrying GST-Syt1p under the control of the *GAL1* promoter. The expression of GST-Syt11p was induced for 6–12 hours by the addition of 2% (final concentration) galactose to rich media. Yeast lysates were prepared and the analysis of bound proteins was performed as described above.

Nucleotide-binding assays

Purified His-tagged small G-proteins from *E. coli* were incubated with 100 μM GDP for 45 minutes at 37°C to insure that all the small G-protein bound to GDP and then were stabilized by the addition of 10 mM $MgCl_2$. GST, GST-Syt1-Sec7 and GST-Yel1-Sec7 were overexpressed and isolated from yeast (~150 A_{600} units) by glutathione-Sepharose in 1 ml binding buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 15 mM EDTA and protease inhibitors). Small G-proteins (1 μM) were incubated at 30°C with [^{35}S]GTPγS (8 μM, <800 CPM/pmol) in GEF assay buffer (20 mM Tris-HCl, pH 7.5, 40 mM KCl, 2 mM $MgCl_2$, 250 mM sucrose, 2 mM DTT and 1 mM EDTA). GST-tagged proteins were added at a final concentration of 10 nM. Samples (20 μl) were removed at various timepoints, diluted with 2 ml ice-cold stop buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT) and filtered on MF-membrane nitrocellulose filters (Millipore). Filters were washed three times in the same buffer, dried and counted in a liquid scintillation counter (Beckman, LS6000IC).

Velocity subcellular fractionation

The cell lysate was prepared as previously described (Liu et al., 2006) and centrifuged (600 ***g***) for 7 minutes to remove debris and unbroken cells. The cleared lysate was centrifuged (13,000 ***g*** for 10 minutes) to collect pellet fraction P13 and supernatant fraction S13. Samples were precipitated with 10% trichloroacetic acid, resuspended in SDS sample buffer, separated by SDS-PAGE and analyzed using western blot.

Extraction of Syt1p from membrane fraction

The cell lysate was prepared as previously described (Liu et al., 2006) and centrifuged (600 ***g***) for 7 minutes to remove debris and unbroken cells. The cleared lysate was centrifuged (13,000 ***g*** for 10 minutes at 4°C) to pellet fraction P13, which was suspended in the same buffer (800 μl). To perform protein extractions, 200 μl of the P13 fraction was mixed with an equal volume of either lysis buffer or one of the three extraction solutions (2% Triton X-100 in lysis buffer, 2 M NaCl in lysis buffer or 0.2 M Na_2CO_3 in H_2O) and then incubated on ice for 30 minutes. The extracts were layered on top of 0.3 M sucrose in 10 mM Tris-HCl (pH 7.4) and centrifuged at 150,000 ***g*** for 1 hour in a Beckman SW55 rotor at 4°C. The samples were then analyzed using western blot as described above.

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Supplementary material available online at
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