The Yeast Synaptojanin-like Proteins Control the Cellular Distribution of Phosphatidylinositol (4,5)-Bisphosphate

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Phosphoinositides (PI) are synthesized and turned over by specific kinases, phosphatases, and lipases that ensure the proper localization of discrete PI isoforms at distinct membranes. We analyzed the role of the yeast synaptojanin-like proteins using a strain that expressed only a temperature-conditional allele of *SJL2*. Our analysis demonstrated that inactivation of the yeast synaptojanins leads to increased cellular levels of phosphatidylinositol (3,5)-bisphosphate and phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P₂), accompanied by defects in actin organization, endocytosis, and clathrin-mediated sorting between the Golgi and endosomes. The phenotypes observed in synaptojanin-deficient cells correlated with accumulation of PtdIns(4,5)P₂, because these effects were rescued by mutations in *MSS4* or a mutant form of *Sjl2p* that harbors only PI 5-phosphatase activity. We utilized green fluorescent protein-pleckstrin homology domain chimeras (termed FLAREs for fluorescent lipid-associated reporters) with distinct PI-binding specificities to visualize pools of PtdIns(4,5)P₂ and phosphatidylinositol 4-phosphate in yeast. PtdIns(4,5)P₂ localized to the plasma membrane in a manner dependent on *Mss4p* activity. On inactivation of the yeast synaptojanins, PtdIns(4,5)P₂ accumulated in intracellular compartments, as well as the cell surface. In contrast, phosphatidylinositol 4-phosphate generated by *Pik1p* localized in intracellular compartments. Taken together, our results demonstrate that the yeast synaptojanins control the localization of PtdIns(4,5)P₂ in vivo and provide further evidence for the compartmentalization of different PI species.

INTRODUCTION

Phosphoinositides (PIs) control several cellular processes including cell signaling, cell growth, vesicular trafficking, transcription, and actin cytoskeletal arrangement (Fruman *et al.*, 1998; Martin, 2001; Simonsen *et al.*, 2001). Phosphatidylinositol (PtdIns) can be differentially phosphorylated on its inositol head group to form seven different PI isoforms that

act as second messengers in various cellular pathways. Accordingly, PI synthesis is regulated by specific kinases localized to distinct membrane sites. In this manner, individual PI isoforms can recruit isoform-specific PI-binding proteins to distinct membranes. Moreover, the reversible phosphorylation of these lipids make them ideally suited to act as temporal and spatial regulators of numerous cellular processes. Correspondingly, a set of phosphatases and lipases regulate PI turnover, thereby controlling the duration and distribution of signaling events mediated by PI second messengers.

The spatial and temporal regulation of PI signaling is illustrated by several previous studies utilizing protein domains with distinct PI-binding specificities (Balla *et al.*, 2000; Czech, 2000; Hurley and Meyer, 2001). For example, pleckstrin homology (PH) domains present in numerous cell-signaling and cytoskeletal proteins display a wide range of PI-binding specificities. The PH domain of phospholipase C $\delta 1$ (PLC δ) binds phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)-P₂) with high specificity and is efficiently recruited to the plasma membrane (Kavran *et al.*, 1998; Stauffer *et al.*, 1998; Honda *et al.*, 1999; Botelho *et al.*, 2000). Other PH domains present in proteins such as Akt/PKB and Grp1 have specificity for phosphatidylinositol (3,4,5)-triphosphate

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Abbreviations used: ALP, alkaline phosphatase; *CEN*, centromeric; CPY, carboxypeptidase Y; FLARE, fluorescent lipid-associated reporter; FM4-64, *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl)hexatrienyl pyridinium dibromide; 5-FOA, 5-fluoroorotic acid; FYVE domain, for Fab1, YGL023, Vps27, and EEA1; GFP, green fluorescent protein; 5-Pase, phosphoinositide 5-phosphatase; PH, pleckstrin homology; PI, phosphoinositide; PLC δ , phospholipase C $\delta 1$; PPIPase, polyphosphoinositide phosphatase; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3,5)P₂, phosphatidylinositol (3,5)-bisphosphate; PtdIns(4,5)P₂, phosphatidylinositol (4,5)-bisphosphate; *Sjl*, synaptojanin-like; TGN, trans-Golgi network.

Figure 1. Growth of yeast synaptojanin mutant cells lacking either *SacI*-like PIPase or 5-Pase activities. (A) Schematic representations of the domain structures of yeast PI 5-Pases. The *SacI* domains in Sjl1p, Sjl2p, and Sjl3p are shown by open ovals. The asterisks above the *SacI*-like domain of Sjl1p are to indicate that Sjl1p does not possess intrinsic PIPase activity. The 5-Pase domains in Sjl1p, Sjl2p, Sjl3p, and Inp54p are indicated by gray bars. (B) The residues present in the conserved catalytic motif (CX₅RTN) of the Sjl2p *SacI* domain are shown. The arrow indicates the substitution of the conserved cysteine residue that inactivates the PIPase activity of Sjl2p in vivo. Conserved residues present in separate regions of the Sjl2p 5-Pase domain are shown. The arrow indicates the substitution of the conserved aspartate residue that inactivates the 5-Pase activity of Sjl2p in vivo. *sjl1Δ sjl2Δ sjl3Δ* cells harboring a *URA3 CEN SJL2* plasmid (YCS156) were transformed with *LEU2*-marked *CEN* plasmids carrying the *SJL2*, *sjl2C446S*, or *sjl2D850S* allele. Ten-fold serial dilutions of cells were spotted onto either YPD (left) or media containing 5-FOA (right) and incubated for 2 d at 30°C.

and play a role in recruiting these proteins to the cell surface in response to external stimuli (Kavran *et al.*, 1998; Venkateswarlu *et al.*, 1998; Meili *et al.*, 1999; Servant *et al.*, 2000). In contrast, the FYVE (for Fab1, YGL023, Vps27, and EEA1) domain present in a number of membrane-trafficking proteins binds phosphatidylinositol 3-phosphate (PtdIns(3)P) with high specificity and is sufficient for endosomal targeting (Burd and Emr, 1998; Gaullier *et al.*, 1998; Gillooly *et al.*, 2000).

In particular, PtdIns(4,5)P₂ controls several cellular processes, including actin cytoskeletal organization and membrane trafficking, through interactions with PtdIns(4,5)P₂-binding proteins (Janmey, 1994; Martin, 2001). PtdIns(4,5)P₂-mediated signaling events are initiated by specific PI kinases and are terminated in part by a family of PI 5-phosphatases (5-Pases). PI 5-Pases regulate cellular levels of PtdIns(4,5)P₂ by removing the phosphate at the D5 position of the inositol head group (Majerus *et al.*, 1999). Several mammalian 5-Pases have been identified thus far with various substrate specificities for PtdIns(4,5)P₂, PtdIns(3,4,5)-triphosphate, and soluble inositol phosphates (Majerus *et al.*, 1999). Recently, the crystal structure of a PI 5-Pase domain was determined, suggesting a novel conserved catalytic mechanism for this family of phosphatases (Tsujishita *et al.*, 2001).

Four PI 5-Pases are present in the yeast *Saccharomyces cerevisiae* (Figure 1A). Three enzymes, Sjl1p, Sjl2p, and Sjl3p (also named Inp51p, Inp52p, and Inp53p), contain a *SacI*-like domain and a 5-Pase domain, similar to mammalian synaptojanin. The *SacI*-like domains of mammalian synaptojanin, Sjl2p, and Sjl3p, but not Sjl1p, possess polyphosphoinositide phos-

phatase (PIIPase) activity that dephosphorylates PtdIns(3)P, phosphatidylinositol 4-phosphate (PtdIns(4)P), and phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P₂) to PtdIns in vitro (Guo *et al.*, 1999). The fourth yeast PI 5-Pase, Inp54p, possesses only 5-Pase activity and localizes to the endoplasmic reticulum (Wiradjaja *et al.*, 2001). Although the yeast 5-Pase domains hydrolyze PtdIns(4,5)P₂ to form PtdIns(4)P, they do not act on soluble inositol phosphates in vitro (Stolz *et al.*, 1998b; Guo *et al.*, 1999; Ooms *et al.*, 2000; Raucher *et al.*, 2000; Wiradjaja *et al.*, 2001). Consistent with these in vitro activities, deletion of *SJL1* leads to an increase in cellular PtdIns(4,5)P₂ levels (Stolz *et al.*, 1998b), whereas double deletion of *SJL2* and *SJL3* causes an increase in PtdIns(3,5)P₂ levels in vivo (Guo *et al.*, 1999).

Deletion of all three yeast synaptojanin-like (*SJL*) genes is lethal (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998a), suggesting that the yeast synaptojanins have essential but overlapping functions. Accordingly, single *sjl1*, *sjl2*, *sjl3*, and *inp54* null mutants are viable and display no obvious phenotypes. However, *sjl1Δ sjl2Δ* and *sjl2Δ sjl3Δ* double mutants demonstrate several phenotypes, including impaired cell growth, defects in actin cytoskeletal organization, and aberrant cell surface and vacuole morphologies (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998a). In contrast, *sjl1Δ sjl3Δ* double mutants display relatively few phenotypes (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998a), suggesting that Sjl2p may provide overlapping functions in these cellular processes. Consistent with this, *sjl1Δ sjl2Δ* and *sjl2Δ sjl3Δ* cells display endocytic defects but not *sjl1Δ sjl3Δ* cells (Singer-Kruger *et al.*, 1998). Moreover, although *S. cerevisiae* encodes multiple PI 5-Pases, genetic evidence suggests that Sjl1p and Sjl3p may have

Table 1. Strains used in this study

Strains	Genotypes	Reference or source
SEY6210	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson et al., 1988
SEY6210.1	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson et al., 1988
SEY6210 α /a	<i>MATα/MATα homozygous diploid strain from a SEY6210/SEY6210.1 cross</i>	Robinson et al., 1988
6210 <i>arf1Δ</i>	Same as SEY6210 except <i>arf1Δ::HIS3</i>	Gaynor et al., 1998
JGY131	Same as SEY6210 except <i>sjl3Δ::TRP1</i>	Foti et al., 2001
JGY132	Same as SEY6210 except <i>sjl2Δ::HIS3 sjl3Δ::TRP1</i>	Foti et al., 2001
YCS62	Same as SEY6210.1 except <i>sjl1Δ::HIS3</i>	This study
YCS66	Same as SEY6210.1 except <i>sjl1Δ::HIS3 sjl2Δ::HIS3</i>	This study
YCS156	Same as JGY132 except <i>sjl1Δ::hisG</i> and harboring pRS415S/JL2 (<i>LEU2 CEN6 S/JL2</i>)	This study
YCS157	Same as YCS156 instead harboring pRS416S/JL2 (<i>URA3 CEN6 S/JL2</i>)	This study
YCS176	Same as YCS156 except harboring pRS415S/jl2 ^{ts} -8 (<i>LEU2 CEN6 sjl2^{ts}-8</i>)	This study
AA1102	Same as SEY6210 except <i>stt4Δ::HIS3</i> and harboring pRS415stt4-4 (<i>LEU2 CEN6 stt4-4</i>)	Audhya et al., 2000
AA1104	Same as SEY6210 except <i>pik1Δ::HIS3</i> and harboring pRS314pik1-83 (<i>TRP1 CEN6 pik1-83</i>)	Audhya et al., 2000
AA1106	Same as SEY6210 α /a except heterozygotic for <i>mss4Δ::HIS3MX6/MSS4</i>	Audhya et al., 2000
AA1143	Same as JGY132 except <i>sac1Δ::TRP1</i> and harboring pRS416sac1-23 (<i>URA3 CEN6 sac1-23</i>)	Foti et al., 2001
AA1201	Same as SEY6210 except <i>mss4Δ::HIS3MX6</i> and harboring pRS416MSS4 (<i>URA3 CEN6 MSS4</i>)	This study
AA1202	Same as AA1201 except harboring YCplac111mss4 ^{ts} -102 (<i>LEU2 CEN6 mss4^{ts}-102</i>)	This study
AA1219	Same as AA1202 except <i>sjl1Δ::HIS3 sjl2Δ::HIS3</i>	This study
AA1219.2	Same as AA1219 except harboring pRS416MSS4 (<i>URA3 CEN6 MSS4</i>)	This study

primary functions. Specific genetic interactions exist between *sjl1* mutations and mutations in genes that encode actin-regulatory proteins, such as *PAN1* and *SAC6* (Singer-Kruger *et al.*, 1998; Wendland and Emr, 1998). In contrast, *Sjl3p* is specifically implicated in clathrin-mediated protein sorting at the trans-Golgi network (TGN; Bensen *et al.*, 2000).

Because previous work has suggested that *Sjl2p* provides overlapping functions, we generated a strain that expressed only a temperature-sensitive allele of *S/JL2*. We found that inactivation of the yeast synaptojanins resulted in pleiotropic phenotypes, including effects on actin cytoskeletal organization, endocytic transport, cell surface morphology, and clathrin-dependent transport between the TGN and endosomes. Consequently, we examined whether regulation of PtdIns(4,5)P₂ levels by the 5-Pase domain or control of other PI isoforms by the *SacI*-like domain of *Sjl2p* contributed to these phenotypes. Furthermore, we created two FLAREs to visualize pools of PtdIns(4,5)P₂ and PtdIns(4)P in yeast by fusing GFP to PH domains with distinct PI-binding specificities. Accordingly, we examined the role of the yeast synaptojanins in regulating the steady-state distribution of these PIs in vivo. We found that PtdIns(4,5)P₂ inappropriately accumulated in intracellular compartments on inactivation of the yeast synaptojanins, providing the first demonstration that these PI phosphatases were necessary to restrict the steady-state distribution of PtdIns(4,5)P₂ to the plasma membrane. We propose that this spatial control of PtdIns(4,5)P₂ within cells is critical for normal cell morphology and membrane trafficking.

MATERIALS AND METHODS

Reagents and Media

Enzymes used for recombinant DNA techniques were purchased from commercial sources and used as recommended by the suppliers. Standard recombinant DNA techniques were performed as

previously described (Sambrook *et al.*, 1989). Sources of growth media for yeast and bacterial strains have been described elsewhere (Gaynor *et al.*, 1998), and standard yeast genetic methods were used throughout (Sherman *et al.*, 1979). *S. cerevisiae* strains used in this study are listed in Table 1 and their constructions are described below. Primers used in this study are available upon request.

Plasmids, Strains, and Mutagenesis

A 5.6-kb *SalI-SpeI* fragment containing *S/JL2* was subcloned from a YEp24INP52 plasmid (generously supplied by J. York) into either pRS415 or pRS416 (Sikorski and Hieter, 1989), which had been cleaved with *SpeI* and *SalI* to create pRS415S/JL2 and pRS416S/JL2, respectively. A 3.8-kb *SacI-PstI* fragment containing *S/JL1* was subcloned from pRS316INP51 (Stolz *et al.*, 1998b) into pRS415, which had been cleaved with *SacI* and *PstI* to create pRS415S/JL1. To create an *S. cerevisiae* strain that expressed synaptojanin activity from *S/JL2* alone, a plasmid was first created to generate a chromosomal deletion of the *S/JL1* gene. This plasmid, pRS415S/jl1 Δ , was constructed by inserting a *BamHI/BglII* fragment containing a *hisG-URA3-hisG* cassette (Alani *et al.*, 1987) into pRS415S/JL1, which had been cleaved with *BglII*. The resulting construct was then digested with *EcoRI* and transformed into an *sjl2::HIS3 sjl3::TRP1* strain (JGY132) carrying pRS415S/JL2 to eliminate *S/JL1*-coding sequences. PCR was used to confirm the *S/JL1* deletion. This strain was grown on media containing 5-fluoroorotic acid (5-FOA) to create an *sjl1::hisG sjl2::HIS3 sjl3::TRP1* strain carrying pRS415S/JL2 (YCS156). YCS156 was transformed with pRS416S/JL2, and plasmid shuffle experiments were performed to isolate an *sjl1 Δ sjl2 Δ sjl3 Δ* strain carrying pRS416S/JL2 alone (YCS157).

To inactivate the *SacI*-like PIPase and 5-Pase activities of *Sjl2p* individually, we created two substitutions in *Sjl2p*, C446S and D850S, respectively. Mutations encoding the C446S and D850S substitutions were separately created in pRS415S/JL2 by site-directed mutagenesis. The *HpaI* fragment containing the mutation encoding the C446S substitution was sequenced and subcloned into pRS416S/JL2, which had been cleaved with *HpaI* to ensure that no other mutations were present in the pRS416S/jl2C446S plasmid used in these studies. Accordingly, the *NarI/SpeI* fragment containing the mutation encoding the D850S substitution was sequenced and subcloned into pRS416S/JL2, which had been cleaved with *NarI* and *SpeI* to create pRS416S/jl2D850S. Finally, the 5.6-kb *SalI/SpeI* fragments

containing only mutations encoding either the C446S or D850S substitutions were subcloned into pRS415, which had been cleaved with *Sall* and *SpeI* to create pRS415*sjl2C446S* and pRS415*sjl2D850S*, respectively.

A strain expressing only a temperature-conditional allele of *Sjl2* was generated in the following manner. *Sjl2*-coding sequences were amplified by error-prone PCR (Muhlrads *et al.*, 1992) and co-transformed with *Bgl*II-gapped pRS415*Sjl2* into YCS157. Ura⁺ Leu⁺ prototrophic transformants were selected and screened for growth on 5-FOA at 26 and 38°C. Cells now lacking pRS416*Sjl2* that grew at 26°C but not at 38°C on 5-FOA-containing media were selected for further study. From >12,000 transformants, four putative pRS415*sjl2^{ts}* plasmids were isolated in *Escherichia coli*, retransformed into YCS157, retested for growth at 26°C but not 38°C on media containing 5-FOA, and tested for PI phosphatase activities at the nonpermissive temperature. A strain harboring the *sjl2^{ts}-8* allele (YCS176) displayed strong temperature-sensitive phenotypes and was selected for further characterization.

A strain expressing a temperature-conditional allele of *Mss4* was generated in the following manner. *Mss4*-coding sequences were amplified by error-prone PCR and cotransformed with *NdeI*/*StuI*-gapped YCplac111*Mss4* into AAY201. Transformants were selected and screened for growth on 5-FOA at 26 and 37°C. Transformants, now lacking pRS416*Mss4*, that grew at 26°C but not at 37°C on 5-FOA-containing media were selected for further study. From >13,000 transformants, six putative YCplac111*mss4^{ts}* plasmids were isolated in *E. coli*, retransformed into AAY201, and retested for growth at 26°C but not 37°C on media containing 5-FOA and PtdIns(4)P 5-kinase activities at the nonpermissive temperature. One particular strain, AAY202, harboring the *mss4-102* allele displayed the strongest temperature-sensitive phenotypes and was selected for further characterization.

To create YCS62, pMS1 (Wendland and Emr, 1998) was digested with *FspI* and *AseI* and transformed into SEY6210.1 to eliminate *Sjl1*-coding sequences. PCR was used to confirm the *Sjl1* deletion. To construct an *sjl1Δ sjl2Δ* double mutant strain (YCS66), *sjl1Δ* cells (YCS62) and *sjl2Δ sjl3Δ* cells (JGY132) were crossed and sporulated, and tetrads were dissected. PCR was used to confirm disruptions in spores harboring the appropriate markers. To construct an *sjl1Δ sjl2Δ mss4^{ts}* mutant strain (AAY219), *mss4^{ts}* cells (AAY202) and *sjl1Δ sjl2Δ* cells (YCS66) were crossed and sporulated, and tetrads were dissected. With two different sets of primers for each gene, PCR was used to confirm disruptions in spores harboring the appropriate markers.

To visualize PtdIns(4)P and PtdIns(4,5)P₂ in vivo, we constructed fusions between GFP and PH domains with distinct PI-binding specificities. To create a PtdIns(4,5)P₂-specific FLARE, we fused two tandem copies of the PLCδ1 PH domain (Kavran *et al.*, 1998) to GFP. PCR was used to generate sequences encoding two PLCδ1 PH domains flanked by either *Bam*HI/*Eco*RI or *Eco*RI/*Sall* restriction sites. These PCR products were digested with *Bam*HI/*Eco*RI or *Eco*RI/*Sall* and cloned in-frame into pGO35 (Burd and Emr, 1998), which had been cut with *Bgl*II and *Sall* to create a plasmid harboring a GFP-2×PH(PLCδ) fusion, pRS426GFP-2×PH(PLCδ). The PtdIns(4)P-binding PH domain of FAPP1 (Dowler *et al.*, 2000) was also fused to the C terminus of GFP to create a PtdIns(4)P-specific FLARE. A *Bam*HI fragment containing the PH domain of FAPP1 was fused in-frame to GFP-coding sequences in the yeast GFP expression plasmid pGO35, which had been cleaved with *Bgl*II to create pRS426GFP-PH(FAPP1).

In Vivo PI Analysis

Analysis of PI levels was done essentially as described previously (Audhya *et al.*, 2000; Foti *et al.*, 2001). Briefly, before labeling, cells were grown in synthetic medium with the appropriate amino acids. Five OD₆₀₀ units of cells from a log-phase culture were harvested, washed, and resuspended in inositol-free synthetic medium. Cells were next shifted to the appropriate temperature for 10 min, fol-

lowed by the addition of 50 μCi of *myo*-[2-³H]inositol (Nycomed Amersham, Buckinghamshire, UK), and incubated for an additional 50 min at the appropriate temperature. Cells were lysed by mechanical agitation with glass beads in 4.5% perchloric acid to generate extracts. Further processing of extracts was as described previously (Stack *et al.*, 1993). Analysis of ³H-labeled glycerophosphoinositols was performed by separation on a Beckman (Fullerton, CA) System Gold HPLC and quantitated by liquid scintillation counting after either collecting fractions eluting from the HPLC column (Whatman, Clifton, NJ) every 0.66 min or by an on-line radiomatic detector (Packard Instrument, Meriden, CT).

Fluorescence and Electron Microscopy

For localization of actin, cells were grown to early log phase, shifted to the appropriate temperature for 2 h, fixed in 3.7% formaldehyde, and stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR) as described previously (Audhya *et al.*, 2000).

Labeling with FM4-64 (*N*-[3-(triethylammoniumpropyl)-4-[*p*-diethylaminophenyl]hexatrienyl] pyridinium dibromide; Molecular Probes) was done essentially as described by Vida and Emr (1995). Briefly, cells were grown to early log phase in YPD and shifted to the appropriate temperature for 90 min. Cells (2 OD₆₀₀ units) were harvested by centrifugation and labeled with 16 nM FM4-64 and 100 nM CMAC (Molecular Probes, Eugene, OR) in YPD prewarmed to the appropriate temperature, followed by a chase in YPD without the vital dyes at the appropriate temperature for either 0 or 30 min. Cells were concentrated and visualized by fluorescence microscopy.

To visualize PtdIns(4)P and PtdIns(4,5)P₂ in vivo, cells expressing GFP-2×PH(PLCδ) or GFP-PH(FAPP1) were grown to early log at the permissive temperature. After shift to the appropriate temperature, cells were concentrated and visualized by fluorescence microscopy. For colabeling studies, cells expressing GFP-2×PH(PLCδ), which had been incubated at the appropriate temperature for 30 min, were killed with NaN₃ plus NaF, stained with FM4-64 on ice, and observed by fluorescence microscopy.

All fluorescent images were observed using an Axiovert S1002TV inverted fluorescent microscope (Carl Zeiss, Thornwood, NY) and acquired and subsequently processed using a Delta Vision deconvolution system (Applied Precision, Seattle, WA). Observations were based on the examination of at least 100 cells.

For ultrastructural analysis, 50 OD₆₀₀ units of log-phase cells incubated at the appropriate temperature were harvested from YPD medium and fixed in 3% glutaraldehyde, 0.1 M Na cacodylate (pH 7.4), 5 mM CaCl₂, 5 mM MgCl₂, and 2.5% sucrose for 1 h. Cells were further processed for electron microscopy as described previously (Rieder *et al.*, 1996). Observations were based on the examination of at least 50 cells.

Metabolic Labeling and Immunoprecipitation

Cell labeling and immunoprecipitations were performed as described previously (Gaynor *et al.*, 1998) with noted variations. Log-phase cultures were concentrated to 1–2 OD₆₀₀/ml and labeled with 2 μl/OD₆₀₀ Tran³⁵S label (DuPont New England Nuclear, Boston, MA) for 10 min in YNB containing 100 μg/ml BSA and 20 μg/ml 2-macroglobulin. Cells were then chased with 5 mM methionine, 2 mM cysteine, and 0.2% yeast extract for the indicated times, and proteins were precipitated with 9% trichloroacetic acid. Temperature preshifts were conducted for 60 min at 38°C when appropriate. Extracts were immunoprecipitated with antisera against the mating pheromone α-factor, carboxypeptidase Y (CPY), alkaline phosphatase (ALP), or Hsp150p, which have been characterized previously (Gaynor *et al.*, 1998; Audhya *et al.*, 2000). Immunoprecipitated proteins were resuspended in sample buffer for resolution by SDS-PAGE and subjected to autofluorography.

Table 2. Phosphoinositide levels in wild-type and various PI phosphatase and kinase mutant cells

Strain	PtdIns levels (% of total ³ H-labeled PI)			
	PtdIns(3)P	PtdIns(4)P	PtdIns(3,5)P ₂	PtdIns(4,5)P ₂
<i>sac1^{tsf} sjl2Δ sjl3Δ</i> 38°C	0.75 ± 0.09	27.4 ± 3.9	0.80 ± 0.13	0.97 ± 0.17
<i>sac1^{tsf} sjl2Δ sjl3Δ (CEN sjl2C446S)^a</i> 38°C	0.88 ± 0.12	28.1 ± 4.4	0.75 ± 0.16	1.04 ± 0.11
<i>sac1^{tsf} sjl2Δ sjl3Δ (CEN SJL2)^a</i> 38°C	0.68 ± 0.15	12.0 ± 1.7	0.27 ± 0.09	1.18 ± 0.24
<i>sjl1Δ sjl2^{ts} sjl3Δ</i> 38°C	0.90 ± 0.10	1.21 ± 0.12	0.20 ± 0.04	2.93 ± 0.30
<i>sjl1Δ sjl2^{ts} sjl3Δ (CEN sjl2D850S)^b</i> 38°C	0.87 ± 0.13	1.10 ± 0.11	0.10 ± 0.03	2.78 ± 0.16
<i>sjl1Δ sjl2^{ts} sjl3Δ (CEN SJL2)^b</i> 38°C	0.94 ± 0.11	0.80 ± 0.10	0.10 ± 0.02	1.30 ± 0.10
Wild type 26°C	0.88 ± 0.03	0.99 ± 0.08	0.05 ± 0.01	0.89 ± 0.05
Wild type 38°C	0.85 ± 0.04	1.14 ± 0.10	0.08 ± 0.02	1.28 ± 0.14
<i>mss4^{ts}</i> 26°C	0.86 ± 0.08	0.88 ± 0.06	0.02 ± 0.01	0.51 ± 0.06
<i>mss4^{ts}</i> 38°C	0.79 ± 0.05	1.05 ± 0.08	0.09 ± 0.01	0.17 ± 0.05
<i>sjl1Δ sjl2Δ</i> 26°C	0.69 ± 0.12	0.91 ± 0.11	0.04 ± 0.02	1.90 ± 0.26
<i>sjl1Δ sjl2Δ mss4^{ts}</i> 26°C	0.74 ± 0.07	0.86 ± 0.09	0.06 ± 0.02	0.94 ± 0.13

Yeast strains incubated at the indicated temperatures were labeled with *myo*-[³H]inositol. Lipids were extracted and deacylated for analysis by HPLC as described in Materials and Methods. The mean peak area (cpm) of each PtdIns species is reported as a percentage of total ³H-labeled PIs. The values reported are the means (± SD) of at least two or three independent experiments. The appropriate PI species in each subset of experiments have been indicated in bold text to highlight relevant comparisons.

^a Derived from AAY143 (Foti *et al.*, 2001) by transformation with pRS415SJL2 or pRS415sjl2C446S.

^b Derived from YCS176 by transformation with pRS415SJL2 or pRS415sjl2D850S.

RESULTS

The PI 5-Pase Activity of the Yeast Synaptojanins Is Essential for Cell Growth

Yeast encode four proteins that possess a PI 5-Pase domain (Figure 1A). Simultaneous deletion of *SJL1*, *SJL2*, and *SJL3* is lethal, but double deletion of *SJL2* and *SJL3* is not (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998a), suggesting that the 5-Pase activity is essential because Sjl1p lacks Sac-like PPIPase activity (Guo *et al.*, 1999). To test this, we constructed a yeast strain that lacked the chromosomal copies of *SJL1*, *SJL2*, and *SJL3* but carried *SJL2* on a *URA3*-marked centromeric (*CEN*) plasmid. We chose to add back *SJL2* on a *CEN* plasmid rather than *SJL1* or *SJL3* because *sjl1Δ sjl3Δ* cells display fewer effects on cell viability, morphology, and vesicular transport compared with *sjl1Δ sjl2Δ* and *sjl2Δ sjl3Δ* cells. Next, mutant forms of Sjl2p that bore substitutions in highly conserved residues of either the Sac1-like domain (C446S) or 5-Pase domain (D850S) were generated (Figure 1B). Each substitution was sufficient to impair either the Sac1-like PPIPase activity or the 5-Pase activity of Sjl2p in vivo, respectively, as assessed below. However, neither of these substitutions affected the steady-state expression level of Sjl2p (Stefan, Audhya, and Emr, unpublished results).

We performed two tests to demonstrate that the C446S substitution inactivated the Sac1-like PPIPase activity of Sjl2p in vivo. Deletion of *SAC1* and *SJL3* in combination has been shown to be lethal, suggesting that the Sac1 domains of these proteins were indispensable for viability (Foti *et al.*, 2001). Likewise, *sac1^{tsf} sjl2Δ sjl3Δ* cells have been shown to be viable at 26°C but were unable to grow at 38°C (Foti *et al.*, 2001). Similarly, *sac1^{tsf} sjl2Δ sjl3Δ* cells expressing *sjl2C446S* from a *LEU2 CEN* plasmid were viable at 26°C but were unable to grow at 38°C (Stefan,

Audhya, and Emr, unpublished results). In contrast, the growth defect of *sac1^{tsf} sjl2Δ sjl3Δ* cells was complemented by coexpression of the *SJL2* or *sjl2D850S* alleles. Moreover, *sac1^{tsf} sjl2Δ sjl3Δ* cells have been shown to accumulate very high levels of PtsIns(4)P and PtdIns(3,5)P₂ at the nonpermissive temperature (Foti *et al.*, 2001). At the nonpermissive temperature, *sac1^{tsf} sjl2C446S sjl3Δ* cells accumulated PtsIns(4)P and PtdIns(3,5)P₂ at levels similar to those in *sac1^{tsf} sjl2Δ sjl3Δ* cells (Table 2). In contrast, the increases in PtdIns(4)P and PtdIns(3,5)P₂ observed in *sac1^{tsf} sjl2Δ sjl3Δ* cells were partly complemented by coexpression of *SJL2* (Table 2), similar to levels previously described in *sac1^{tsf} sjl3Δ* cells (Foti *et al.*, 2001). Taken together, these results indicated that the *sjl2C446S* allele behaved as a null allele with regard to the Sac1-like activity of Sjl2p. Confirmation that the D850S substitution impaired the 5-Pase activity of Sjl2p in vivo is described below.

Next, we performed plasmid shuffle experiments to determine which mutant form of Sjl2p was sufficient for viability. *LEU2*-marked *CEN* plasmids carrying the *SJL2*, *sjl2D850S*, or *sjl2C446S* alleles were transformed into *sjl1Δ sjl2Δ sjl3Δ* triple mutant cells also harboring a *URA3 CEN SJL2* plasmid. As shown in Figure 1B, *sjl1Δ sjl2Δ sjl3Δ* cells expressing wild-type Sjl2p from the *LEU2*-marked *CEN* plasmid were able to grow on 5-FOA plates after loss of the *URA3 CEN* plasmid carrying *SJL2*. Likewise, *sjl1Δ sjl2Δ sjl3Δ* cells expressing *sjl2C446S* from a *LEU2*-marked *CEN* plasmid were able to form colonies on 5-FOA plates after the loss of the *URA3 CEN SJL2* plasmid. However, *sjl1Δ sjl2Δ sjl3Δ* cells carrying *sjl2D850S* on a *LEU2*-marked *CEN* plasmid were unable to grow on media containing 5-FOA and thus were unable to lose the *URA3 CEN SJL2* plasmid (Figure 1B). These results indicated that the 5-Pase activity was in fact the essential function conferred by *SJL1*, *SJL2*, and *SJL3*.

Figure 2. *sjl1Δ sjl2^{ts} sjl3Δ* cells display growth defects and generate increased levels of PtdIns(4,5)P₂ at the restrictive temperature. (A) *sjl1Δ sjl2Δ sjl3Δ* cells expressing either *SJL2* (YCS157) or *sjl2^{ts}* (YCS176) from *LEU2* *CEN* plasmids were grown in YPD at the permissive temperature. Tenfold serial dilutions of cells were spotted onto YPD and grown for 2 d at 26°C or 38°C. (B and C) Cells were preincubated at either 26°C or 38°C for 10 min and labeled with *myo*-[2-³H]inositol for 50 min at the permissive or nonpermissive temperatures. Lipids were then deacylated from cellular membranes, and glycerophosphoinositols were extracted and analyzed by HPLC. (B) Quantitative comparisons of glycerophosphoinositols generated by *sjl1Δ SJL2 sjl3Δ* cells (YCS157; open columns) and *sjl1Δ sjl2^{ts} sjl3Δ* cells (YCS176; black columns) at 26°C (left) and 38°C (right) are shown. These data represent the means $\pm$ SD of three independent experiments. (C) Typical HPLC elution profiles are shown for *sjl1Δ SJL2 sjl3Δ* cells (YCS157; left) and *sjl1Δ sjl2^{ts} sjl3Δ* cells (YCS176; right) at 38°C. Peaks corresponding to glycerol-Ins(3)P, glycerol-Ins(4)P, glycerol-Ins(3,5)P₂, and glycerol-Ins(4,5)P₂ are indicated.

The Yeast Synaptojanins Regulate Cellular PtdIns(3,5)P₂ and PtdIns(4,5)P₂ Levels

Our initial results suggested that proper homeostasis of cellular PtdIns(4,5)P₂ levels was necessary for viability. To examine the immediate effects of inactivating the yeast synaptojanins, we generated temperature-conditional alleles of *SJL2*. The *sjl1Δ sjl2Δ sjl3Δ* strain harboring *SJL2* on a *URA3*-marked *CEN* plasmid (YCS157) was transformed with pools of PCR-mutagenized *SJL2* carried on *LEU2*-marked *CEN* plasmids. We then performed plasmid shuffle experiments to isolate mutants that were able to form colonies on 5-FOA plates at 26°C but not at 38°C. Approximately 12,000 transformants were screened for temperature-sensitive growth on media containing 5-FOA. Plasmids from transformants that were positive in this assay were recovered in *E. coli* and rescreened in YCS157 for the ability to confer temperature-sensitive growth on 5-FOA. One strain harboring an *sjl2^{ts}* allele that conferred a strong temperature-sensitive growth phenotype (Figure 2A) was chosen for further characterization.

Next, we analyzed changes in PI synthesis/turnover rates in *sjl1Δ sjl2Δ sjl3Δ* cells expressing either the *SJL2* or *sjl2^{ts}* alleles (hereafter referred to as *SJL2* and *sjl2^{ts}* cells, respectively). *SJL2* and *sjl2^{ts}* cells were pulse-labeled with *myo*-[2-³H]inositol at 26 and 38°C. Subsequent analysis of labeled lipids by HPLC enabled us to monitor the levels of glycerophosphoinositols derived from PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P₂, and PtdIns(4,5)P₂. At the permissive temperature, PI levels in *sjl2^{ts}* cells were nearly identical to those in *SJL2* cells, except for an increase (twofold) in PtdIns(3,5)P₂ levels (Figure 2B). Moreover, a shift to the restrictive temperature resulted in a specific increase in PtdIns(4,5)P₂ levels (2.2-fold) in *sjl2^{ts}* cells compared with *SJL2* cells (Figure 2, B and C), consistent with our initial results demonstrating a requirement for the 5-Pase activity of Sjl2p for viability.

On sequence analysis of the *sjl2^{ts}* allele used in these studies, we found several mutations throughout the *SJL2*-coding region. Subsequent mapping experiments indicated that substitutions in the Sac1 domain of Sjl2p were responsible for both the temperature-independent and temperature-sensitive phenotypes conferred by this *sjl2^{ts}* allele

(Stefan, Audhya, and Emr, unpublished results). The temperature-independent increases in PtdIns(3,5)P₂ levels observed in *sjl2^{ts}* cells suggested that these substitutions impaired the Sac1-like activity of Sjl2p in vivo, consistent with the increase in PtdIns(3,5)P₂ levels previously observed in *sjl2Δ sjl3Δ* cells (Guo *et al.*, 1999). More importantly, these substitutions caused an increase in PtdIns(4,5)P₂ levels only at the restrictive temperature, consistent with the temperature-sensitive growth phenotype observed for these cells.

We used the *sjl2^{ts}* cells to examine the effect of the Sjl2D850S substitution on the Sac1-like and 5-Pase activities of Sjl2p. At the nonpermissive temperature, *sjl1Δ sjl2Δ sjl3Δ* cells expressing both the *sjl2^{ts}* and *sjl2D850S* alleles did not exhibit an increase in PtdIns(3,5)P₂ levels as compared with *SJL2* cells, but still displayed a twofold increase in PtdIns(4,5)P₂ levels (Table 2). In contrast, coexpression of *SJL2* complemented the effects on PtdIns(3,5)P₂ and PtdIns(4,5)P₂ levels observed in *sjl2^{ts}* cells at the nonpermissive temperature (Table 2). These results indicated that the Sjl2D850S substitution impaired the 5-Pase activity of Sjl2p without affecting the Sac1-like PIPase activity in vivo.

By measuring total cellular PI levels, we found that the yeast synaptojanin-like proteins regulated both PtdIns(3,5)P₂ and PtdIns(4,5)P₂ levels in vivo. Next, we assessed the physiological consequences of inactivating the yeast synaptojanins. Moreover, we addressed whether the Sac1-like PIPase or 5-Pase activities were specifically involved in the control of these various cellular processes.

The Yeast Synaptojanins Control Organization of the Actin Cytoskeleton by Regulating Cellular PtdIns(4,5)P₂ Levels

PIs, particularly PtdIns(4,5)P₂, have been implicated in the organization of the actin cytoskeleton (Janmey, 1994). Because temperature shift of *sjl2^{ts}* cells resulted in a significant increase in PtdIns(4,5)P₂ levels, we investigated whether this resulted in effects on the actin cytoskeleton. At both the permissive and restrictive temperatures, *SJL2* and *sjl2^{ts}* cells were fixed, stained with rhodamine-phalloidin, and observed by fluorescence microscopy. At the permissive temperature, *SJL2* and *sjl2^{ts}* cells displayed actin cables in the mother cell aligned toward cortical actin patches concentrated in the bud, similar to previously published patterns of actin filaments in wild-type cells (Figure 3A; Karpova *et al.*, 1998). However, after a shift to restrictive temperature, >90% of *sjl2^{ts}* cells displayed actin patches randomly distributed throughout both mother and daughter cells, indicating that cells lacking synaptojanin activity fail to properly repolarize their actin cytoskeletons after exposure to high temperature (Figure 3A). After an identical temperature shift, >80% of *SJL2* cells exhibited normal patterns of actin cables and cortical patches (Figure 3A). These results demonstrated that the yeast synaptojanins control actin organization and that the defects in actin cytoskeletal organization observed in *sjl2^{ts}* cells correlated with increases in PtdIns(4,5)P₂ levels.

We further examined whether the actin defects observed in yeast synaptojanin mutants were directly due to increased levels of PtdIns(4,5)P₂ or due to indirect effects of *sjl* null mutations. Previous work (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998a) and our own studies have demonstrated that *sjl1Δ*

Figure 3. Cells deficient in synaptojanin-encoded PI 5-Pase activity fail to properly organize their actin cytoskeletons. (A) Actin cytoskeletal organization in *sjl1Δ SJL2 sjl3Δ* cells (YCS157; top) and *sjl1Δ sjl2^{ts} sjl3Δ* cells (YCS176; bottom) at 26°C (left column) and 38°C (right column). Cells were grown to early log phase, shifted to the appropriate temperature for 2 h, fixed with 3.7% formaldehyde, and labeled with rhodamine-phalloidin to visualize actin filaments. (B) Quantitative comparisons of PtdIns(4,5)P₂ levels synthesized by wild-type (SEY6210), *sjl1Δ sjl2Δ MSS4* (AAY219.2), *mss4^{ts}* (AAY202), and *sjl1Δ sjl2Δ mss4^{ts}* (AAY219) cells at 26°C are shown. These data represent the means $\pm$ SD of at least two independent experiments. (C) Actin cytoskeletal organization in *sjl1Δ sjl2Δ MSS4* cells (AAY219.2; left) and *sjl1Δ sjl2Δ mss4^{ts}* cells (AAY219; right) at 26°C.

sjl2Δ mutant cells possess increased PtdIns(4,5)P₂ levels (Figure 3B) and defects in organization of the actin cytoskeleton (Figure 3C). Thus, we created an *sjl1Δ sjl2Δ mss4^{ts}* triple mutant strain. *MSS4* encodes the major yeast PtdIns(4)P 5-kinase (Desrivieres *et al.*, 1998). The *mss4^{ts}* allele used in this study conferred a weak defect in PtdIns(4,5)P₂ synthesis even at the permissive temperature of 26°C compared with cells expressing wild-type *MSS4* (Figure 3B; Table 2). At the nonpermissive temperature, *mss4^{ts}* cells displayed an approximately sevenfold decrease in PtdIns(4,5)P₂ synthesis compared with *MSS4* cells identically treated (Table 2). Importantly, at the permissive temperature, *sjl1Δ sjl2Δ mss4^{ts}* triple mutant cells synthesized PtdIns(4,5)P₂ at levels similar to wild-type cells (Figure 3B; Table 2).

To visualize actin, *sjl1Δ sjl2Δ* and *sjl1Δ sjl2Δ mss4^{ts}* cells grown at the permissive temperature were fixed, stained with rhodamine-phalloidin, and observed by fluorescence microscopy. Whereas *sjl1Δ sjl2Δ* cells displayed random distribution of actin patches in both mother and daughter cells, proper organization of the actin cytoskeleton was restored in *sjl1Δ sjl2Δ mss4^{ts}* cells, with actin cables in mother cells properly aligned toward cortical actin patches in the bud and septum (Figure 3C). These results indicated that increased cellular PtdIns(4,5)P₂ levels directly affected organization of the actin cytoskeleton in *sjl1Δ sjl2Δ* cells, because *sjl1Δ sjl2Δ mss4^{ts}* cells possessed normal PtdIns(4,5)P₂ levels at the permissive temperature.

sjl2^{ts} Cells Exhibit Impaired Rates of Endocytosis and an Aberrant Cell Surface Morphology

PIs have previously been implicated in intracellular trafficking and membrane dynamics (Simonsen *et al.*, 2001). We investigated whether endocytic trafficking was affected in *sjl2^{ts}* cells when incubated at the nonpermissive temperature. To do this, we examined rates of transport of the lipophilic dye FM4-64 to the vacuole. This vital dye is internalized from the plasma membrane into punctate intracellular compartments and ultimately is delivered to the vacuole membrane. Accordingly, *SJL2* and *sjl2^{ts}* cells were grown at the permissive temperature and then further incubated at the permissive or nonpermissive temperatures. These cells were pulse-labeled with FM4-64 and CMAC (CMAC stains the vacuole lumen) for 15 min, washed, and chased in media not containing FM4-64 or CMAC for either 0 or 30 min at the appropriate temperature. At the permissive temperature, *SJL2* and *sjl2^{ts}* cells displayed similar rates of transport of FM4-64 to the vacuole membrane (Stefan, Audhya, and Emr, unpublished results).

After a chase at restrictive temperature for 30 min, most *SJL2* cells (>75%) displayed FM4-64 fluorescence only in the vacuole membrane (Figure 4A, top). In contrast, *sjl2^{ts}* cells clearly displayed defects in endocytic delivery of FM4-64 to the vacuole (Figure 4A, bottom). In >80% of these cells, FM4-64 fluorescence accumulated in intracellular, punctate structures clearly distinct from the vacuole, as defined by CMAC staining. These results indicated that yeast synaptotagmin activity is required for efficient transport of FM4-64 to the vacuole membrane and suggested that the endocytic defect correlated with increases in PtdIns(4,5)P₂ levels because the effect was observed only after a shift to the nonpermissive temperature.

At the nonpermissive temperature, *sjl2^{ts}* cells displayed large, aberrant cell surface structures stained with FM4-64 at the 0-min chase time (Stefan, Audhya, and Emr, unpublished results), likely corresponding to large invaginations at the cell surface previously observed in *sjl1Δ sjl2Δ* mutants (Srinivasan *et al.*, 1997; Singer-Kruger *et al.*, 1998; Stolz *et al.*, 1998a). To determine whether the yeast synaptotagmins play a primary role in regulating membrane dynamics at the cell surface, *SJL2* and *sjl2^{ts}* cells were examined at the ultrastructural level. Electron microscopy revealed that *sjl2^{ts}* cells were similar to *SJL2* cells at the permissive temperature, except for the presence of slightly fragmented vacuoles in some cells (Stefan, Audhya, and Emr, unpublished results). However, *sjl2^{ts}* cells formed large, abnormal invaginations at the plasma membrane (indicated by arrowheads in Figure 4B) in contrast to cells expressing *SJL2* at the restrictive temperature.

To determine whether the abnormal cell surface morphology observed in yeast synaptotagmin mutants was directly due to increased levels of PtdIns(4,5)P₂ or caused by indirect effects of *sjl* null mutations, we examined *sjl1Δ sjl2Δ* and *sjl1Δ sjl2Δ mss4^{ts}* cells at the ultrastructural level. Electron microscopy revealed that, whereas >70% of *sjl1Δ sjl2Δ* cells displayed large, abnormal invaginations at the cell surface (indicated by arrowheads in Figure 4C), normal morphology of the plasma membrane was restored in >90% *sjl1Δ sjl2Δ mss4^{ts}* cells grown at the permissive temperature (Figure 4C). These results indicated that increased cellular PtdIns(4,5)P₂ levels directly affected membrane dynamics at the cell surface, because *sjl1Δ sjl2Δ mss4^{ts}* cells possessed normal PtdIns(4,5)P₂ levels at the permissive temperature (Figure 3B).

The 5-Pase Activity of the Yeast Synaptotagmins Regulates TGN/Endosomal Sorting via Clathrin-coated Vesicles

Sjl3p has been implicated in clathrin-dependent transport between the TGN and endosomes (Bensen *et al.*, 2000). To address whether *Sac1*-like PIPase or 5-Pase activities were involved in the control of this pathway, we took advantage of the *sjl2C446S* and *sjl2D850S* alleles that inactivate the *Sac1*-like or 5-Pase activities of *Sjl2p*, respectively. These *sjl2* alleles were expressed in *sjl2Δ sjl3Δ* cells and examined for effects on processing of secreted α -factor. Maturation of precursor α -factor is mediated by the protease Kex2p. Kex2p cycles between the TGN and endosomes in a clathrin-dependent manner. If clathrin is impaired, Kex2p becomes mislocalized and inefficient maturation of α -factor precursor occurs (Bensen *et al.*, 2000). Thus, the extent of α -factor maturation serves as a measure of clathrin function at the TGN. To monitor α -factor maturation, cells expressing various *SJL2* alleles were labeled with [³⁵S]methionine, and secreted α -factor was immunoprecipitated from the medium.

Whereas wild-type cells secreted fully mature pheromone, *sjl3Δ* cells displayed a slight impairment in α -factor processing (19% precursor form; Figure 5, lanes 1 and 2), consistent with previously published observations (Bensen *et al.*, 2000). Moreover, although deletion of *SJL2* had no effect on α -factor processing (Bensen *et al.*, 2000), deletion of *SJL2* and *SJL3* conferred additive effects upon

Figure 4. Synaptojanin PI 5-Pase activity is required for normal endocytic transport and plasma membrane morphology. (A) *sjl1Δ SJL2 sjl3Δ* cells (YCS157; top) or *sjl1Δ sjl2^{ts} sjl3Δ* cells (YCS176; bottom) were incubated at 38°C for 90 min and then labeled with the vital dyes FM4-64 and CMAC for 15 min. After labeling, cells were chased for 30 min at the nonpermissive temperature. The left column shows cells under fluorescent illumination in the rhodamine channel (FM4-64). The middle column shows cells as observed by CMAC fluorescence (CMAC) to visualize the lumen of vacuoles. The right column shows cells as observed by Nomarski optics (DIC). (B) Ultrastructure of *sjl1Δ SJL2 sjl3Δ* cells (YCS157; left) and *sjl1Δ sjl2^{ts} sjl3Δ* cells (YCS176; right) at the restrictive temperature. YCS157 and YCS176 were grown to early log phase, shifted to 38°C for 90 min, fixed with 3% glutaraldehyde, and processed for electron microscopy. (C) Ultrastructure of *sjl1Δ sjl2Δ MSS4* cells (AAY219.2; left) and *sjl1Δ sjl2Δ mss4^{ts}* cells (AAY219; right) at 26°C. (B and C) Arrowheads indicate abnormal invaginations at the cell surface; v, vacuole; n, nucleus; bar, 0.5 μ m.

α -factor maturation, because *sjl2Δ sjl3Δ* cells secreted ~40% of α -factor in the precursor form (Figure 5, lane 3). In *sjl2Δ sjl3Δ* cells expressing wild-type *SJL2* from a *LEU2*-marked *CEN* plasmid, α -factor processing was similar to that in *sjl3Δ* cells (16% precursor form), as expected. Importantly, *sjl2Δ sjl3Δ* cells expressing *sjl2C446S* from a *CEN* plasmid did not display impaired α -factor process-

ing, compared with *sjl3Δ* cells (13% precursor form; Figure 5, lane 5). In contrast, *sjl2Δ sjl3Δ* cells expressing *sjl2D850S* secreted the precursor form of α -factor at levels nearly identical to that of *sjl2Δ sjl3Δ* cells (Figure 5, lane 6). Taken together, these results indicated that the 5-Pase activity of Sjl2p regulated clathrin-dependent protein sorting between the TGN and endosomes.

Figure 5. Inactivation of *Sjl2*-encoded PI 5-Pase activity affects α -factor maturation. Wild-type (WT; SEY6210; lane 1), *sjl3* Δ (JGY131; lane 2), *sjl2* Δ *sjl3* Δ (JGY132; lane 3), *Sjl2* *sjl3* Δ (JGY132 carrying pRS416*Sjl2*; lane 4), *sjl2C446S* *sjl3* Δ (JGY132 carrying pRS416*sjl2C446S*; lane 5), and *sjl2D850S* *sjl3* Δ (JGY132 carrying pRS416*sjl2D850S*; lane 6) cells were metabolically labeled at 26°C for 45 min, and secreted α -factor was immunoprecipitated from the culture supernatants. Samples were analyzed by SDS-PAGE and fluorography. Precursor levels of α -factor were quantified by phosphorimager analysis. The levels of secreted precursor α -factor indicated are the means of two independent experiments; SD < 15%.

We investigated whether the effects of inactivating the yeast synaptojanins were specific for clathrin-mediated protein transport pathways from the TGN. Accordingly, we examined roles for the yeast synaptojanins in additional protein-trafficking pathways, including two TGN to vacuole pathways and the secretory pathway. First, we determined whether the yeast synaptojanins were required for vacuole protein sorting and transport. Using the *sjl1* Δ *sjl2* Δ *sjl3* Δ strains expressing either the *Sjl2* or *sjl2*^{ts} alleles, we examined the transport and processing of two vacuolar proteins, CPY and ALP. However, even after an extended preshift to the nonpermissive temperature, neither CPY nor ALP transport was affected in *sjl2*^{ts} cells compared with *Sjl2* control cells (Stefan, Audhya, and Emr, unpublished results). Finally, we investigated a role for the yeast synaptojanins in secretion. By performing pulse-chase experiments, we tested whether Hsp150p, a high-molecular-weight glycoprotein that is rapidly secreted, was affected upon inactivation of the yeast synaptojanins. However, no significant impairment of Hsp150p glycosylation or secretion was observed in *sjl2*^{ts} cells after shift to the nonpermissive temperature compared with cells expressing *Sjl2* (Stefan, Audhya, and Emr, unpublished results). Taken together, these results indicated that inactivation of the yeast synaptojanins does not confer

rapid, nonspecific pleiotropic effects on protein transport from the Golgi complex.

The Yeast Synaptojanins Control the Intracellular Distribution of PtdIns(4,5)P₂

Because our results indicated that the yeast synaptojanins control several cellular processes by regulating PtdIns(4,5)P₂, we wished to localize this PI in vivo. For this purpose, we took advantage of the PH domain from PLC δ , which has been shown to bind PtdIns(4,5)P₂ in vitro (Kavran *et al.*, 1998). We fused two tandem copies of the PLC δ PH domain to GFP to create a PtdIns(4,5)P₂-specific FLARE, GFP-2 $\times$ PH(PLC δ). In wild-type cells, GFP-2 $\times$ PH(PLC δ) fluorescence was observed at the plasma membrane and weakly in the cytosol but not on intracellular compartments (Figure 6A). To demonstrate the specificity of this FLARE for PtdIns(4,5)P₂ in vivo, we expressed GFP-2 $\times$ PH(PLC δ) in *mss4*^{ts} mutant cells. At the permissive temperature, GFP-2 $\times$ PH(PLC δ) localized to the plasma membrane similar to wild-type cells (Stefan, Audhya, and Emr, unpublished results). On shift to the nonpermissive temperature, GFP fluorescence became diffuse in *mss4*^{ts} cells, mainly throughout the cytosol, indicating that recruitment of GFP-2 $\times$ PH(PLC δ) to the plasma membrane was dependent on Mss4p activity (Figure 6A).

Next, we examined the role of the yeast synaptojanins in regulating the cellular location of PtdIns(4,5)P₂. In *Sjl2* cells expressing GFP-2 $\times$ PH(PLC δ), GFP fluorescence was observed at the plasma membrane at both the permissive and nonpermissive temperatures (Figure 6B, left). Likewise, localization of GFP-2 $\times$ PH(PLC δ) was restricted to the plasma membrane in *sjl2*^{ts} cells at the permissive temperature (Figure 6B, top right). Interestingly, GFP fluorescence at the plasma membrane became punctate in *sjl2*^{ts} cells expressing GFP-2 $\times$ PH(PLC δ) when shifted to the nonpermissive temperature (Figure 6B, bottom right). Moreover, at the nonpermissive temperature, GFP-2 $\times$ PH(PLC δ) was observed on intracellular compartments as well as the plasma membrane in *sjl2*^{ts} cells (Figure 6B, bottom right). To confirm that these structures were indeed intracellular compartments, *sjl2*^{ts} cells expressing GFP-2 $\times$ PH(PLC δ), which had been incubated at the nonpermissive temperature for 30 min, were killed by the addition of NaN₃ and NaF to inhibit further internalization from the plasma membrane and stained with FM4-64 on ice. Under these conditions, punctate intracellular compartments containing GFP fluorescence were observed that clearly did not colocalize with FM4-64 fluorescence at the plasma membrane (Figure 6C). However, colocalization was observed between GFP and FM4-64 fluorescence on punctate, intracellular compartments in metabolically active *sjl2*^{ts} cells expressing GFP-2 $\times$ PH(PLC δ) after a brief labeling and chase with FM4-64 at the nonpermissive temperature, suggesting that these structures may be endocytic compartments (Stefan, Audhya, and Emr, unpublished results). Taken together, these results indicated that the yeast synaptojanins are essential to restrict the steady-state accumulation of PtdIns(4,5)P₂ to the plasma membrane.

Our results using the PtdIns(4,5)P₂-specific FLARE indicated that the yeast synaptojanins control the distribution of PtdIns(4,5)P₂ within cells. As a control, we utilized another PH domain from the mammalian protein FAPP1 that specifically bound PtdIns(4)P in vitro (Dowler *et al.*, 2000). This

Figure 6. The steady-state localization of a PtdIns(4,5)P₂-specific FLARE at the plasma membrane in yeast is dependent on Mss4p and synaptojanin-like activities. (A) Wild-type (SEY6210; left) or *mss4^{ts}* cells (AAY202; right) expressing a GFP-2×PH(PLCδ) fusion were incubated at 38°C for 45 min and observed by fluorescent microscopy using a DeltaVision deconvolution system. (B) *sjl1Δ Sjl2 sjl3Δ* cells (YCS157; left) or *sjl1Δ sjl2^{ts} sjl3Δ* cells (YCS176; right) expressing GFP-2×PH(PLCδ) were incubated for 0 or 60 min at the nonpermissive temperature. (C) *sjl1Δ sjl2^{ts} sjl3Δ* cells (YCS176) expressing GFP-2×PH(PLCδ) incubated at the nonpermissive temperature for 30 min were killed by the addition of NaN₃ and NaF to inhibit further internalization from the plasma membrane, stained with FM4-64 on ice, and observed by fluorescent microscopy. DIC, Nomarski optics.

PH domain was fused to GFP to create a PtdIns(4)P-specific FLARE, GFP-PH(FAPP1), and expressed in yeast. In wild-type cells expressing GFP-PH(FAPP1), GFP fluorescence

was observed on punctate, intracellular compartments but not at the plasma membrane (Figure 7). Next, we examined the localization of GFP-PH(FAPP1) in various yeast PI ki-

Figure 7. The intracellular localization of a PtdIns(4)P-specific FLARE to the Golgi is dependent on Pik1p activity. Wild-type cells (SEY6210), *pik1^{ts}* cells (AAY104), *mss4^{ts}* cells (AAY202), or *arf1Δ* cells (6210*arf1Δ*) expressing GFP-PH(FAPP1) were incubated at 26°C (left column) or 38°C (right column) for 20 min as indicated and observed by fluorescent microscopy using a DeltaVision deconvolution system. DIC, Nomarski optics.

nase mutants. Interestingly, GFP-PH(FAPP1) was diffuse throughout the cytosol in *pik1^{ts}* cells (Audhya *et al.*, 2000) when incubated at the nonpermissive temperature. Thus, PIK1-encoded PtdIns 4-kinase activity was necessary for the punctate localization of GFP-PH(FAPP1) (Figure 7). In contrast, Mss4p activity was not required for the localization of GFP-PH(FAPP1) to puncta, because GFP-PH(FAPP1) displayed wild-type localization in *mss4^{ts}* cells at the nonpermissive temperature (Figure 7). Moreover, we found that the localization of GFP-PH(FAPP1) did not change in *sjl2^{ts}* cells at the nonpermissive temperature (Stefan, Audhya, and Emr, unpublished results), providing additional evidence for the PI-binding specificities of the FLAREs used in these studies.

Because Pik1p has been implicated in transport from the Golgi (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000), we examined whether the structures that contained GFP-PH(FAPP1) corresponded to Golgi com-

partments. To do this, we expressed GFP-PH(FAPP1) in *arf1Δ* mutant cells. In *arf1Δ* cells, atypical membrane ring structures accumulate that contain known Golgi proteins (Gaynor *et al.*, 1998). Similarly, GFP-PH(FAPP1) localized to ring-like structures in *arf1Δ* cells (Figure 7, arrows), suggesting that GFP-PH(FAPP1) recognized PtdIns(4)P generated by Pik1p on intracellular Golgi compartments.

DISCUSSION

In this study, we examined the consequences of inactivating the yeast synaptojanins by generating a strain that expresses only a temperature-sensitive *SJL2* allele. Impairment of the yeast synaptojanins resulted in effects on the actin cytoskeleton, endocytic transport, cell surface morphology, and clathrin-dependent transport between the TGN and endosomes, consistent with previously published reports. Al-

though *sjl2^{ts}* cells accumulated increased levels of both PtdIns(3,5)P₂ and PtdIns(4,5)P₂, our studies indicated that these phenotypes correlated with accumulation of PtdIns(4,5)P₂. Moreover, we found that PtdIns(4,5)P₂ accumulated on intracellular compartments, as well as the plasma membrane when the yeast synaptojanins were inactivated, providing the first demonstration that the yeast synaptojanins control the subcellular localization of PtdIns(4,5)P₂. This spatial control of PtdIns(4,5)P₂ was essential for normal cell morphology and membrane transport.

The Yeast Synaptojanins Regulate Cellular PtdIns(3,5)P₂ and PtdIns(4,5)P₂ Levels

By measuring total cellular PI levels, our data confirmed that the yeast synaptojanins regulated both PtdIns(3,5)P₂ and PtdIns(4,5)P₂ levels in vivo. In *sjl2^{ts}* cells, PtdIns(3,5)P₂ levels were increased (twofold) at both the permissive and restrictive temperatures. More importantly, a specific increase in PtdIns(4,5)P₂ levels was observed in *sjl2^{ts}* cells at the restrictive temperature (2.2-fold; see Figure 2), consistent with our initial results demonstrating a requirement for PI 5-Pase activity for viability.

The physiological consequences of inactivating the yeast synaptojanins observed in this study were specifically due to increases in PtdIns(4,5)P₂. Thus, an interesting question arises as to why Sjl2p, Sjl3p, and mammalian synaptojanin possess both PIPase and 5-Pase activities. Previous in vitro studies have indicated that the 5-Pase and Sac1 domains of yeast and mammalian synaptojanins act sequentially to dephosphorylate PtdIns(4,5)P₂ to PtdIns(4)P and PtdIns, respectively (Guo *et al.*, 1999). However, our results suggested that the Sac1 and 5-Pase domains of Sjl2p may have separate functions as well, because *sjl2^{ts}* cells displayed increases in both PtdIns(3,5)P₂ and PtdIns(4,5)P₂ cellular levels. Consistent with this, previous work has indicated overlapping roles for the Sac1 domain-containing proteins Sjl2p, Sjl3p, and Sac1p in the control of PtdIns(4)P cellular levels (Foti *et al.*, 2001).

Coordinate Control of PtdIns(4,5)P₂, Actin, and Cell Surface Morphology by PI 5-Pases

Previous studies have implicated PIs in the regulation of the actin cytoskeleton (Janmey, 1994). Our studies confirmed that synaptojanin activity is required for proper actin organization in yeast. Consistent with this, previous work has shown that Sjl2p and Sjl3p partially colocalize with actin patches (Ooms *et al.*, 2000). The defects in actin cytoskeletal organization observed in synaptojanin-deficient cells specifically correlated with increases in PtdIns(4,5)P₂ levels, because *sjl2^{ts}* cells displayed defects in the actin cytoskeleton only at the nonpermissive temperature (Figure 3A). Others have shown that Mss4p, the major yeast PtdIns(4)P 5-kinase, was required for proper actin organization (Desrivieres *et al.*, 1998). Thus, both increases and decreases in cellular PtdIns(4,5)P₂ levels affected actin organization in yeast. Interestingly, we found that restoration of normal cellular PtdIns(4,5)P₂ levels in *sjl1Δ sjl2Δ mss4^{ts}* cells rescued the actin cytoskeletal defects observed in *sjl1Δ sjl2Δ* cells (see Figure 3, B and C), indicating that cellular PtdIns(4,5)P₂ levels directly affected organization of the actin cytoskele-

ton. Consistent with this, a previous study showed that overexpression of a mammalian PI 5-Pase corrected the lipid and actin defects observed in cells lacking Sjl1p, Sjl2p, and Sjl3p (O'Malley *et al.*, 2001).

Thus, in a simple model, Mss4p and the yeast synaptojanins control cellular PtdIns(4,5)P₂ levels, which in turn govern the actin cytoskeleton by recruiting and/or regulating actin-binding proteins. Accordingly, PtdIns(4,5)P₂ has been shown to control actin polymerization by binding several actin regulatory proteins, such as N-WASP, profilin, cofilin, and capping proteins (Schafer *et al.*, 1996; Higgs and Pollard, 2000; Prehoda *et al.*, 2000; Rohatgi *et al.*, 2000). Thus, both increases and decreases in cellular PtdIns(4,5)P₂ levels may confer effects upon actin polymerization and organization in vivo. We were not able to detect significant changes in the relative amounts of filamentous actin in pellet fractions made from lysates of *SJL2* and *sjl2^{ts}* cells after incubation at the nonpermissive temperature (Stefan, Audhya, and Emr, unpublished results). Thus, the yeast synaptojanins may not regulate cellular levels of filamentous actin per se but rather control the organization of actin filaments in vivo, similar to a previously published study using various mutants and conditions known to affect the arrangement of yeast actin patches and cables (Karpova *et al.*, 1998). Additional biochemical experiments will be required to more directly examine the effects of inactivating the yeast synaptojanins on PtdIns(4,5)P₂-regulated actin regulatory proteins.

Several mammalian studies have shown that alterations in intracellular PtdIns(4,5)P₂ levels have varying effects on the actin cytoskeleton. Overexpression of PI 5-Pases has been shown to concurrently reduce PtdIns(4,5)P₂ levels, formation of actin stress fibers, and the attachment of existing filaments to the plasma membrane (Sakisaka *et al.*, 1997; Raucher *et al.*, 2000). Likewise, overexpression of type I PI 5-kinase induced dramatic changes in the actin cytoskeleton. These included stabilization of comet-like actin tails associated with vesicles (Rozelle *et al.*, 2000), either induction or inhibition of plasma membrane ruffling (Honda *et al.*, 1999; Yamamoto *et al.*, 2001), and stabilization of thick actin stress fibers (Yamamoto *et al.*, 2001). Thus, extensive evidence including our work has indicated that both increases and decreases in cellular PtdIns(4,5)P₂ levels can confer effects on actin cytoskeletal organization in vivo.

Previous studies have implicated PIs in the regulation of membrane dynamics and organelle morphology (Odorizzi *et al.*, 2000). Accordingly, a previous study has demonstrated that PtdIns(4,5)P₂ controls tension between the plasma membrane and actin cytoskeleton (Raucher *et al.*, 2000). Our studies demonstrated that the yeast synaptojanins control plasma membrane dynamics by regulating cellular PtdIns(4,5)P₂ levels. Importantly, we found that restoration of normal cellular PtdIns(4,5)P₂ levels in *sjl1Δ sjl2Δ mss4^{ts}* cells rescued the cell surface morphology effects observed in *sjl1Δ sjl2Δ* cells (see Figure 4 C).

The cell surface morphology defects observed in yeast synaptojanin mutants are likely because of additive effects on PtdIns(4,5)P₂-mediated control of multiple pathways. Besides effects on actin and plasma membrane dynamics, inactivation of the yeast synaptojanins confers rapid effects on cell wall structure (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998a; Stefan, Audhya, and Emr, unpublished results). The thick-

ened cell wall phenotype observed in yeast synaptojanin mutants may be due to improper localization of chitin synthases at the plasma membrane (Ziman *et al.*, 1998). Alternatively, PtdIns(4,5)P₂ could regulate yeast cell surface morphology through the PKC1 protein kinase pathway, which controls the actin cytoskeleton and cell wall biosynthetic enzymes after heat shock or other extracellular stimuli (Helliwell *et al.*, 1998; Delley and Hall, 1999). Previous studies implicated PtdIns(4,5)P₂ in the yeast PKC1 protein kinase pathway, because *mss4^{ts}* cells are unable to repolarize their actin cytoskeletons and undergo cell lysis at restrictive temperatures (Desrivieres *et al.*, 1998). Accordingly, the yeast synaptojanins may act as regulators of the PKC1 pathway, because *sjl2^{ts}* cells display actin defects and cell wall thickening at the nonpermissive temperature. Additional experiments will be needed to examine the precise roles of the yeast synaptojanins in each of these pathways. Nonetheless, our studies suggest that the yeast synaptojanins coordinately control actin cytoskeletal organization, endocytosis, and cell wall synthesis by regulating PtdIns(4,5)P₂ levels at the plasma membrane.

The Role of PI 5-Pases in Clathrin Function

PIs have been implicated in intracellular trafficking (Simonsen *et al.*, 2001). Accordingly, previous work has suggested that mammalian synaptojanin regulates the association of clathrin with membranes (Hill *et al.*, 2001). Mammalian synaptojanin has been proposed to act in the uncoating step of endocytic clathrin-coated vesicles (Cremona *et al.*, 1999) and has been demonstrated to physically interact with clathrin coats (Haffner *et al.*, 2000). Likewise, the yeast synaptojanins have been implicated in endocytosis (Singer-Kruger *et al.*, 1998) and have been shown to genetically interact with clathrin (Bensen *et al.*, 2000). Our results indicated that regulation of PtdIns(4,5)P₂ by the yeast synaptojanins controls two clathrin-mediated transport pathways, endocytosis (Figure 4A) and sorting between the TGN and endosomes (Figure 5). Although α -factor processing was affected in synaptojanin-deficient cells, we did not observe effects on additional transport pathways from the TGN. Thus, improper accumulation of PtdIns(4,5)P₂ may specifically affect clathrin-mediated trafficking between the TGN and endosomes rather than conferring general effects on transport from the Golgi.

Spatial Control of PtdIns(4,5)P₂ by the Yeast Synaptojanins

Because the accumulation of PtdIns(4,5)P₂ was detrimental to several cellular processes, we developed a convenient and reliable method to visualize this PI in yeast. For this purpose, we used the PH domain from PLC δ that specifically binds PtdIns(4,5)P₂ in vitro (Kavran *et al.*, 1998). The PtdIns(4,5)P₂-specific FLARE used in this study, GFP-2 $\times$ PH(PLC δ), localized to the plasma membrane in a manner dependent on Mss4p activity but not on intracellular compartments (Figure 6A). Likewise, GFP-2 $\times$ PH(PLC δ) was restricted to the plasma membrane in *sjl2^{ts}* cells at the permissive temperature (Figure 6B). This result indicated that Sjl2p 5-Pase activity was sufficient to maintain the steady-state distribution of PtdIns(4,5)P₂ at the plasma membrane and not in intracellular compartments. However, GFP-

2 $\times$ PH(PLC δ) was observed in intracellular compartments as well as the plasma membrane in *sjl2^{ts}* cells at the nonpermissive temperature (Figure 6, B and C), indicating that the yeast synaptojanins were essential to restrict the steady-state accumulation of PtdIns(4,5)P₂ to the plasma membrane. Although these experiments did not address whether the PtdIns(4,5)P₂ that accumulated intracellularly in *sjl2^{ts}* cells was synthesized at the plasma membrane or in intracellular compartments, previous work demonstrated that Mss4p localized primarily to the plasma membrane and not on internal structures (Homma *et al.*, 1998).

Whereas our initial studies indicate that the phenotypes observed in synaptojanin-deficient cells correlate with an increase in total cellular PtdIns(4,5)P₂ levels, our studies using GFP-2 $\times$ PH(PLC δ) demonstrate that the yeast synaptojanins are critical for the spatial control of PtdIns(4,5)P₂. Thus, certain phenotypes observed in synaptojanin-deficient cells may be due to the inappropriate localization of PtdIns(4,5)P₂ rather than slight increases of this lipid at the plasma membrane. Although overall PtdIns(4,5)P₂ levels are increased only twofold in synaptojanin-deficient cells, the relative concentration of PtdIns(4,5)P₂ on certain intracellular membranes may be increased by more than an order of magnitude (see Figure 6). Consistent with this idea, although *sjl2 Δ* *sjl3 Δ* cells do not display an increase in total cellular PtdIns(4,5)P₂ levels (Stolz *et al.*, 1998a), we find that the GFP-2 $\times$ PH(PLC δ) reporter is present on intracellular structures in these cells (Stefan, Audhya, and Emr, unpublished results). Accordingly, PtdIns(4,5)P₂-interacting proteins may become mislocalized in synaptojanin-deficient cells, resulting in the titration of these PtdIns(4,5)P₂ effector molecules away from their normal site of function at the plasma membrane. Alternatively, mislocalization of PtdIns(4,5)P₂ may result in the recruitment of effectors to new membrane sites, where they could interfere with the normal function of these membranes. These effects could explain the lethality of cells lacking synaptojanin-like activity. The future identification of these effectors (e.g., various PH and/or ENTH domain-containing proteins) will allow us to directly test this hypothesis.

Previous studies using various PI-specific FLAREs have suggested that distinct PI isoforms are compartmentalized within cells (Balla *et al.*, 2000). Our studies using a PtdIns(4,5)P₂-specific FLARE indicated that PtdIns(4,5)P₂ accumulated on the plasma membrane in wild-type yeast cells. Other studies utilizing PtdIns(3)P-specific FLAREs have demonstrated that PtdIns(3)P was present in endosomal compartments (Burd and Emr, 1998; Gillooly *et al.*, 2000). Several recent reports have suggested that Pik1p, a PtdIns 4-kinase, regulates transport from the Golgi in yeast (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). Thus, we fused GFP to a PH domain that specifically binds PtdIns(4)P in vitro (Dowler *et al.*, 2000). We found that this PtdIns(4)P-specific FLARE, GFP-PH(FAPP1), localized to punctate, intracellular compartments in a manner dependent on Pik1p activity (Figure 7). Moreover, GFP-PH(FAPP1) localized to ring-like structures in *arf1 Δ* cells (Figure 7), perhaps corresponding to the abnormal ring-like Golgi compartments previously observed in *arf1 Δ* cells by electron microscopy and fluorescence experiments (Gaynor *et al.*, 1998). Taken together, these results suggested that PtdIns(4)P generated by the PtdIns 4-kinase Pik1p localized

to yeast Golgi compartments, consistent with a role for Pik1p in transport from the Golgi. Accordingly, Pik1p has previously been localized to Golgi structures (Walch-Soliman and Novick, 1999).

Overall, these studies using GFP-based FLAREs with distinct PI-binding specificities provide additional evidence for the compartmentalization of particular PI species at discrete membrane sites. More importantly, our studies provide the first evidence that the yeast synaptojanins not only control PtdIns(4,5)P₂ cellular levels but also are essential for the proper spatial control of this PI isoform. Future studies using synaptojanin-deficient cells will likely reveal various effects on the activity and/or localization of several downstream effector proteins that bind PtdIns(4,5)P₂.

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