Phosphoinositides (PI) control numerous cellular processes including cell signaling, growth, vesicular trafficking, and actin cytoskeletal arrangements (8, 43, 50, 54). In particular, phosphatidylinositol(4,5)-bisphosphate [PI(4,5)P$_2$] provides important regulatory roles at the cell surface, as a precursor of the signaling molecules DAG and IP$_3$, and through direct interactions with downstream effector proteins (31, 34). During endocytic internalization, PI(4,5)P$_2$ recruits or activates several proteins, including the clathrin adaptor complex AP-2, AP180, epsin, and the GTPase dynamin, leading to the formation and fission of clathrin-coated vesicles (13, 15, 16, 25, 40). PI(4,5)P$_2$ also recruits or controls several actin-regulatory proteins (54). Actin polymerization may provide a propulsive force to facilitate membrane invagination and fission events or to transport newly formed endocytic vesicles away from the plasma membrane. Thus, PI(4,5)P$_2$ synthesis plays several important roles in the formation and fission of clathrin-coated vesicles during endocytosis.

Likewise, previous studies implicate dephosphorylation of PI(4,5)P$_2$ by the polyphosphoinositide phosphatase (PPiPase) synaptojanin as an important step in endocytic internalization (7, 22, 27, 41, 42, 51). Synaptojanins contain two distinct PI phosphatase domains and a C-terminal proline-rich domain (PRD). The N-terminal Sac1 domain (PPiPase domain) acts on several positions of the inositol ring, while its central phosphatase domain specifically acts at the 5' position (5-Pase domain) (20, 37). The PRD region functions as a targeting domain via interactions with various SH3 domain-containing proteins, such as endophilin, amphiphysin, and CIN85 (5, 28, 42, 45, 51). Coated vesicles accumulate in cells bearing genetic disruptions of synaptojanin isoforms in the mouse, Drosophila melanogaster, and Caenorhabditis elegans, suggesting that turnover of PI(4,5)P$_2$ mediates release of coat proteins from membranes of newly formed vesicles (7, 22, 51). Since synaptojanins regulate PI(4,5)P$_2$-mediated actin polymerization, they may also control earlier vesicle formation and/or fission events, along with their role in postfission vesicle uncoating.

Three synaptojanin-like proteins are present in Saccharomyces cerevisiae: Sjl1, Sjl2, and Sjl3 (also named Inp51, Inp52, and Inp53) (46, 49). Like mammalian synaptojanin, Sjl2 and Sjl3 exhibit Sac1 PPIPase activities, and all three possess 5-Pase activity (Fig. 1) (20). Factors that target the yeast synaptojanin-like proteins via their PRDs have yet to be demonstrated in vivo. Interestingly, the C-terminal domains of Sjl1, Sjl2, and Sjl3 do not share extensive identity (Fig. 1). Thus, while synaptojanins display similar activities in vitro, each may provide specialized functions in vivo and may be uniquely recruited or activated at distinct membrane sites.

Previous studies have indicated that Sjl2 provides essential overlapping functions at both the plasma membrane and intracellular endosomal/late Golgi compartments (17, 44, 46, 48, 49). Moreover, we have shown that yeast cells expressing only a temperature-conditional Sjl2 allele inappropriately accumu-
late PI(4,5)P₂ in intracellular compartments (48). These findings have thus led to the question of whether SJL2 acts primarily at the plasma membrane or at endosomes in the steady-state distribution. To address this question, we have examined the localization of SJL2 in vivo. SJL2 was recruited to cortical actin patches in a manner dependent on filamentous actin and the PRD of SJL2. We further addressed how SJL2 is recruited to its site of action at the plasma membrane or at endosomes in the control of endocytic activities shown for Sjl1, Sjl2, and Sjl3. Relevant PI metabolism activities are shown for these PI phosphatase domains. The light gray boxes between Sjl1, Sjl2, and Sjl3 show the percentages of identity between their PPIPase domains. The C-terminal PRDs (black bars) of the yeast synaptojanins do not share extensive identity. CB, clathrin box motifs in Sjl2 and Sjl3. NPF, a putative EH or Sla1 domain binding site in Sjl1.

**FIG. 1.** Yeast synaptojanin-like proteins possess unique C-terminal domains. The Sac1 domains in Sjl1p, Sjl2p, and Sjl3p are shown by open ovals. The asterisks in the Sac1-like domain of Sjl1 indicate that it does not possess PPIPase activity. Dark gray bars, 5-Pase domains in Sjl1, Sjl2, and Sjl3. Relevant PI metabolism activities are shown for these PI phosphatase domains. The light gray boxes between Sjl1, Sjl2, and Sjl3 show the percentages of identity between their PPIPase domains and between their 5-Pase domains. The C-terminal PRDs (black bars) of the yeast synaptojanins do not share extensive identity. CB, clathrin box motifs in Sjl2 and Sjl3. NPF, a putative EH or Slal domain binding site in Sjl1.

**TABLE 1.** Strains used in this study

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**MATERIALS AND METHODS**

**Yeast strains and plasmid constructions.** Standard yeast genetic methods to create integrated deletions, truncations, and fusions were used throughout (33). Saccharomyces cerevisiae strains used in this study are listed in Table 1. Primers used in this study are available upon request.

To generate the SJL2Δ75C-green fluorescent protein (GFP) fusion used in these studies, the 0.6-kb EcoRI fragment (encoding the 39 C-terminal residues of SJL2) was removed from pRS416/SJL2 (48) to create pRS416/SJL2ΔK1120V, which then had a unique EcoRI site. PCR was then used to amplify GFP(65T)-encoding sequences. The resulting product was digested with EcoRI and subcloned in frame and in the appropriate orientation into pRS416/SJL2ΔK1120V, which was cleaved with EcoRI to create pRS416/SJL2Δ75C-GFP. To generate the SJL2ΔP1121D-GFP fusion, the 3.7-kb Sall-XhoI fragment from pRS416/SJL2ΔK1120V was subcloned into pRS416 cleaved with XhoI to create pRS416/SJL2Δ75C, which then had a unique XhoI site and thus excluded 190 C-terminal residues encoding the PRD of SJL2. PCR was then used to amplify GFP(65T)-encoding sequences. The resulting product was digested with XhoI-EcoRI and subcloned in frame into pRS416/SJL2Δ75C, which was cleaved with XhoI-EcoRI. The K1120V and P1121D mutations were introduced into pRS416/SJL2-GFP by QuikChange mutagenesis (Stratagene, La Jolla, Calif.). In order to overexpress SLA2 from a multicopy plasmid, a 4.3-kb EcoRI fragment encompassing the SLA2 locus (53) was subcloned into pRS426, which had been cleaved with EcoRI.

**Generation of SJL2-specific antisera, immunofluorescence, and microscopy on intact cells.** A 0.7-kb HindIII fragment of SJL2 encompassing codons 893 to 1129 was subcloned into pGEX KG, and the resulting glutathione S-transferase fusion protein was inducibly expressed in Escherichia coli, purified from bacterial extracts, and then used to immunize New Zealand White rabbits as described previously (2). Resulting antibodies were further purified by acetone extracts of sla2 cells. Immunofluorescence microscopy procedures were carried out essentially as described previously (2, 17), except that cells were stained with rhodamine phallidin according to the manufacturer’s recommendations (Molecular Probes, Eugene, Ore.).

To monitor endocytosis, cells were labeled with FM4-64 (Molecular Probes) as described elsewhere (48). Briefly, cells were grown to early-log phase in yeast extract-peptone-dextrose (YPD) and shifted to the appropriate temperature for 90 min. Cells (2 OD₅₀₀ [optical density at 600 nm] units) were harvested by centrifugation and labeled with 16 nM FM4-64 and 100 nM CMAC (Molecular Probes) in YPD prewarmed to the appropriate temperature, followed by a chase in YPD without the vital dyes at the appropriate temperature for 30 min. Cells were concentrated and visualized by fluorescence microscopy.

Live cells expressing SJL2Δ75C-GFP, SJL2ΔP1121D-GFP, SJL2ΔK1120V,P1121D,GFP, or SLa2-GFP were grown to mid-log phase and harvested for visualization.

Downloaded from http://mcb.asm.org/ on November 8, 2016 by guest
Where indicated, 200 µM latrunculin A (Calbiochem, La Jolla, Calif.) was added to cells 15 min prior to microscopy. For FM4-64 staining of the plasma membrane, cells were grown to mid-log phase at the appropriate temperature, incubated at the restrictive temperature for 90 min when indicated, and then harvested. Metabolic inhibitors (10 mM NaN₃ and NaF) were applied, and nonviable cells were stained with FM4-64 (Molecular Probes) at 0°C, as described elsewhere (44, 48). Cells were visualized on a Zeiss Axiovert S1002TV fluorescence microscope equipped with fluorescein isothiocyanate and rhodamine filters, captured with a Photometric camera, and deconvolved by using Delta Vision software (Applied Precision, Issaquah, Wash.). Fields showing localization of GFP-tagged forms of Sjl2 were two-dimensional representations of several Z-sections using Delta Vision software. Results presented were based on observations of more than 100 cells. For live-cell two-color movies following Sjl2-GFP and Abp1-DsRed localization, cells were visualized on a spinning disk confocal microscope (Yokogawa) equipped with a 100× NA-1.4 objective and a cooled charge-coupled device camera (Hamamatsu) controlled by Metamorph software (Universal Imaging). Image pairs from single optical sections scanned at 488 and 568 nm were collected every 1.8 s. Throughout, representative fields were shown and scales were constant within each figure.

In vivo biochemical analyses. For the coisolation of Sjl2 or Sjl2-myc with GFP-Abp1, 20 OD₆₀₀ equivalents of cells were harvested, washed, spheroplasted, and lysed in phosphate-buffered saline lysis buffer, consisting of 8 g of NaCl/liter, 0.2 g of KCl/liter, 1.44 g of Na₂HPO₄/liter, 0.24 g of KH₂PO₄/liter (pH 7.2), 0.1 mM EDTA, the protease inhibitors aminochetyl-benzene sulfonyl fluoride (AEBSF; Calbiochem), benzamidine, and pepstatin A, and a protease inhibitor cocktail (Complete; Roche). Lysates were solubilized with 0.5% Tween 20 (v/v) by incubation on ice for 10 min. Samples were cleared by centrifugation at 13,000 × g, and supernatants were processed to immunosolubilize GFP or GFP-Abp1 by using anti-GFP antibodies (provided by C. Zuker) and protein A-Sepharose beads (Pharmacia). Material was then visualized by Western blotting with either anti-Sjl2 or anti-myc (Genosys, The Woodlands, Tex.) antibodies.

For sizing analysis of Sjl2 complexes in wild-type and abp1Δ cells, 100 OD₆₀₀ equivalents of cells were harvested, washed, and lysed in phosphate-buffered saline lysis buffer plus 1% Triton X-100. Solubilized lysates were cleared by centrifugation at 13,000 × g and subsequently loaded onto a linear 10 to 40% glycerol gradient equilibrated in lysis buffer plus 1% Triton X-100. Samples containing cleared lysates or molecular weight standards (aldolase, ferritin, and thyroglobulin) were then subjected to centrifugation at 100,000 × g for 4 h in an SW41 rotor. Fractions were then collected from the resulting gradients and analyzed by Western blotting with anti-Sjl2 antibodies. Sizing standards were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (adoglase), OD₂₈₀ absorbance (adoglase and thyroglobulin), and OD₂₂₀ absorbance (ferritin). The ESCRT-I 350-kDa complex was detected by using anti-Vps23 antibodies (2).

RESULTS

Sjl2 localizes to cortical actin patches via its PRD domain.

By virtue of its Sac1-like PPIPase and 5-Pase activities (Fig. 1), Sjl2 likely functions at multiple membrane sites in vivo in the control of PI isoforms [e.g., PI3P on endosomes, PI4P at the Golgi complex, and PI(4,5)P₂ at the plasma membrane]. To address its primary site of action, we examined the localization of Sjl2 by immunofluorescence experiments. To do this, we generated polyclonal antisera against the PRD of Sjl2. The antisera specifically recognized Sjl2; no signal was detected in sjl2Δ mutant cells (Fig. 2A). Sjl2 was detected on many peripheral or cortical punctate structures in wild-type cells (Fig. 2A). Several of these structures concentrated in the bud and contained with rhodamine-phalloidin, indicating that these Sjl2-positive structures were cortical actin patches (Fig. 2A, merge). Cortical actin patches have been implicated as sites of endocytic internalization in yeast (26), in agreement with the known role of Sjl2 in endocytosis (44, 48). Additional puncta were observed that did not overlap with cortical actin patches (Fig. 2A). Future studies will focus on the identification of these Sjl2-containing structures, such as the Golgi complex or endosomes, where Sjl2 function has also been implicated (4, 21, 48).

In order to rapidly detect Sjl2 localization in vivo, we fused GFP near the C terminus of Sjl2. (A) Wild-type (SEY6210.1) and sjl2Δ (YCS63) cells were observed by immunofluorescence microscopy using anti-Sjl2 antibodies. Cells were costained with rhodamine-phalloidin (actin) and aligned with Nomarski optics (merge). (B) Wild-type cells (SEY6210.1) expressing Sjl2Δ₃⁷C-GFP in the absence or presence of latrunculin A (top and center, respectively) or Sjl2Δ₃⁷CPRD-GFP (bottom) were observed by fluorescent microscopy and aligned with Nomarski optics. Bar, 5 µM.

FIG. 2. Sjl2 localizes to cortical actin patches in a manner that requires actin filaments and the PRD of Sjl2. (A) Wild-type (SEY6210.1) and sjl2Δ (YCS63) cells were observed by immunofluorescence microscopy using anti-Sjl2 antibodies. Cells were costained with rhodamine-phalloidin (actin) and aligned with Nomarski optics (merge). (B) Wild-type cells (SEY6210.1) expressing Sjl2Δ₃⁷C-GFP in the absence or presence of latrunculin A (top and center, respectively) or Sjl2Δ₃⁷CPRD-GFP (bottom) were observed by fluorescent microscopy and aligned with Nomarski optics. Bar, 5 µM.
Sjl2Δ37C-GFP-expressing cells treated with the actin-depolymerizing agent latrunculin A, Sjl2Δ37C-GFP fluorescence was redistributed throughout the cytoplasm (Fig. 2B), indicating that Sjl2Δ37C-GFP localization or recruitment was dependent on filamentous actin.

A previous study has shown that the PRD of Sjl2 is sufficient for recruitment to nonpolarized actin filaments under hyperosmotic stress conditions (39). We examined whether this region of Sjl2 was necessary for the recruitment of Sjl2 to cortical actin patches at the plasma membrane. We fused GFP to a truncated form of Sjl2 lacking 190 C-terminal residues (Sjl2ΔPRD-GFP). This mutant form of Sjl2 was expressed at wild-type levels and at the predicted molecular weight, as determined by immunoblotting experiments (data not shown). However, Sjl2ΔPRD-GFP localized diffusely throughout the cytoplasm rather than on punctate structures (Fig. 2B). Taken together, our results indicated that Sjl2 is recruited to cortical punctate structures and that this process requires filamentous actin and the PRD of Sjl2.

**Abp1 is necessary for Sjl2 recruitment in vivo.** Next, we searched for trans-acting factors that contribute to Sjl2 at cortical patches. Since the PRD was necessary for Sjl2 recruitment and SH3 domains bind proline-rich motifs, we examined roles for SH3-containing proteins in the localization of Sjl2 at cortical actin patches. We identified the actin-binding protein Abp1 as necessary for Sjl2Δ37C-GFP recruitment to cortical structures. In abp1Δ mutant cells, Sjl2Δ37C-GFP localized diffusely throughout the cytoplasm (Fig. 3A), a pattern similar to that of Sjl2ΔPRD-GFP expressed in wild-type cells. In contrast, the SH3-containing proteins Slal and Rvs167, which have recently been shown to interact biochemically with Sjl2 (47), were not essential for Sjl2Δ37C-GFP cortical recruitment (Fig. 3A). However, cytoplasmic Sjl2Δ37C-GFP fluorescence appeared higher in rvs167Δ and sla1Δ cells than in wild-type cells. Thus, while multiple factors may be involved in targeting Sjl2, our results have suggested an important role for Abp1.

A previous in vitro study used phase display to identify a proline-rich peptide (PPVVKKP) in the PRD of Sjl2 (residues 1115 to 1121) as a possible binding site for the Abp1 SH3 domain (14). To address whether these residues were involved in Sjl2 localization in vivo, we constructed a mutant form of Sjl2Δ37C-GFP that bore substitutions in the 1115PPVVKKP1121 motif (Fig. 3B). This mutant form of Sjl2 (Sjl2Δ1210v.P1121d,Δ37C-GFP) was mislocalized to the cytoplasm of wild-type cells (Fig. 3B), a pattern similar to the diffuse localization of wild-type Sjl2Δ37C-GFP in abp1Δ cells. Altogether, our results suggested that Abp1 acts as a recruitment factor for Sjl2 and identified specific residues within the Sjl2 PRD important for Sjl2 localization, likely by direct association with the Abp1 SH3 domain.

**Sjl2 and Abp1 colocalize and physically interact in vivo.** To examine whether Sjl2 colocalizes with Abp1 and to monitor the temporal relationship of these actin patch proteins, we coexpressed Sjl2Δ37C-GFP and a functional Abp1-DsRed fusion protein (1) and imaged cells in real time by spinning disk confocal microscopy. Due to differences in the relative intensities of Sjl2Δ37C-GFP and Abp1-DsRed fluorescence, Sjl2Δ37C-GFP was expressed from a high-copy-number plasmid in these experiments. In agreement with our results indicating that Abp1 was necessary for Sjl2 cortical recruitment, significant colocalization was observed between Sjl2Δ37C-GFP and Abp1-DsRed at cortical patches (in four of seven Sjl2Δ37C-GFP patches at the initial time point [Fig. 4A]). Closer examination indicated that two additional Sjl2Δ37C-GFP patches overlapped with faint Abp1-DsRed fluorescence (Fig. 4A). More importantly, this analysis revealed an ordered sequence for the recruitment and disappearance of Sjl2 and Abp1 at cortical patches. Based on observations of more than 30 patches from several cells, Abp1-DsRed appeared at patches prior to Sjl2Δ37C-GFP, as Sjl2Δ37C-GFP consistently concentrated at preexisting Abp1-DsRed patches. To demonstrate an example of this progression, a specific region in Fig. 4A was magnified (by 120%) and shown throughout the course of this experiment in Fig. 4B. Interestingly, the two proteins coexisted on patches only transiently, with Abp1-DsRed disappearing from the patch rapidly after Sjl2Δ37C-GFP recruitment (Fig. 4B). In addition, Sjl2Δ37C-GFP remained in patches following the disappearance of Abp1-DsRed. For example, each of the Sjl2Δ37C-GFP patches that initially colocalized with Abp1-DsRed (Fig. 4A) persisted following the disappearance of Abp1-DsRed. This process may explain the observation of Sjl2Δ37C-GFP patches that did not appear to display significant overlap with Abp1-DsRed at the start of this experiment. Alternatively, Sjl2 may exist in a separate complex that transiently interacts with Abp1 patches.

To further demonstrate a role for Abp1 in the recruitment of Sjl2 to cortical actin patches, we examined whether Abp1 and Sjl2 physically associate in vivo. Wild-type cells expressing GFP alone or functional GFP-Abp1 (9) were lysed. Next, GFP or GFP-Abp1 was immunoprecipitated from lysates solubilized under native conditions by using anti-GFP antibodies. Bound material was then probed for the presence of Sjl2 by using Sjl2-specific antisera. Sjl2 copurified with GFP-Abp1 but not with GFP alone (Fig. 5A). To address whether this interaction was mediated through the PRD of Sjl2, GFP-Abp1 was immunoprecipitated from cells expressing full-length Sjl2-myc or a mutant form that lacked the PRD, Sjl2ΔPRD-myc. These experiments employed myc-tagged fusions, because the anti-Sjl2 antisera were directed against the PRD of Sjl2. Sjl2-myc copurified with GFP-Abp1 but not with GFP alone (Fig. 5B), results similar to those obtained using native Sjl2. In contrast, Sjl2ΔPRD-myc did not copurify with GFP-Abp1. Taken together, these data suggested that Sjl2 and Abp1 associate in vivo and that this interaction occurs through the PRD of Sjl2, in agreement with our findings that the PRD of Sjl2 and Abp1 direct the recruitment of Sjl2 to cortical patches in vivo.

We further tested whether Abp1 and Sjl2 physically associate in vivo by determining the relative molecular size of Sjl2 in extracts from wild-type and abp1Δ cells by using velocity gradient sedimentation analysis. In wild-type cell extracts, Sjl2 was present in fractions corresponding to a range of 440 to 669 kDa (Fig. 6). In extracts from abp1Δ cells, the profile of Sjl2 was shifted to a lower-molecular-size range (approximately 350 to 440 kDa [Fig. 6]), consistent with our earlier result that Abp1 and Sjl2 interact. However, Sjl2 remained in a complex larger than its monomeric size (approximately 120 kDa) in abp1Δ cells, suggesting that additional factors associate with Sjl2. In support of this idea, dual-color imaging of Sjl2 and Abp1 fusions indicated that Sjl2-GFP persisted in cortical patches following the rapid movement or disappearance of Abp1-DsRed from these patches (Fig. 4). Further studies will be
necessary to define the precise composition of the Sjl2 complex(es). As a control, the size distribution of the 350-kDa ESCRT-I complex, consisting of Vps23, Vps28, and Vps37 (2), remained constant in extracts prepared from wild-type and abp1Δ/H9004 cells (Fig. 6).

Abp1 and Sjl2 share similar functions in vivo. To further examine the role of the Abp1-Sjl2 interaction, we investigated whether Abp1 was involved in Sjl2 function in vivo. Thus, we examined whether abp1Δ/H9004 mutant cells displayed phenotypes

FIG. 3. Recruitment of Sjl2Δ37C-GFP to cortical patches is dependent on Abp1. (A) Localization of Sjl2Δ37C-GFP in living wild-type (SEY6210.1), abp1Δ (YCS301), rvs167Δ (YCS192), and sla1Δ (YCS288) cells was observed by fluorescence (Sjl2Δ37C-GFP) and Nomarski (merge) microscopy. (B) Wild-type cells (SEY6210.1) expressing Sjl2K1120V,P1121D,Δ37C-GFP (Sjl2KV,PΔ37C-GFP) were observed by fluorescence and Nomarski microscopy. The diagram shows substitutions in the Sjl2 PRD used in this experiment. Bars, 5 μM.

FIG. 4. Sjl2Δ37C-GFP and Abp1-DsRed colocalize in an ordered, transient manner. (A) Sjl2Δ37C-GFP was coexpressed in wild-type cells expressing Abp1-DsRed (strain AAY1701). Frames from a single focal plane movie visualizing Abp1-DsRed (left) and Sjl2Δ37C-GFP (center) are shown along with merged images (right). The time to acquire one image pair was 1.8 s; every other time point has been shown. Arrowheads and asterisks indicate significant and faint colocalization of Sjl2Δ37C-GFP and Abp1-DsRed at cortical patches, respectively. (B) Time series showing Abp1-Sjl2 composition of a single patch (boxed region in panel A). Individual images showing Abp1-DsRed (top) and Sjl2Δ37C-GFP (middle) are aligned with the merged image pairs (bottom). The dashed line indicates the cell cortex.
similar to those of cells lacking Sjl2. Because abp1Δ and sjl2Δ single-mutant cells display no obvious phenotypes, we compared phenotypes exhibited by sjl1Δ sjl2Δ double-mutant cells with those of sjl1Δ abp1Δ double-mutant cells and found that they displayed similar properties. Cells lacking both Sjl1 and Sjl2 have been shown to accumulate abnormal, enlarged plasma membrane invaginations that fail to undergo fission and often fold around themselves to enclose portions of cytoplasm (44, 46, 48, 49). These structures can be readily visualized by staining the membrane surfaces of sjl1Δ sjl2Δ cells with the lipophilic dye FM4-64 under conditions that block endocytosis (0°C and metabolic inhibitors) (44). As expected, >90% of sjl1Δ sjl2Δ double-mutant cells displayed massive, furrow-like invaginations stained with FM4-64 that are continuous with the plasma membrane (Fig. 7). These invaginations displayed increased FM4-64 staining relative to the plasma membrane due to their double membrane structure and because FM4-64 did not rapidly photobleach in these structures. Addition of FM4-64 to wild-type, sjl1Δ, and sjl2Δ cells under these conditions did not reveal the presence of any such cell surface structures (Fig. 7 and data not shown). Likewise, abp1Δ mutant cells did not accumulate invaginations of the plasma membrane like those that form in sjl1Δ sjl2Δ double-mutant cells. Interestingly, deletion of SIL1 and ABP1 in combination resulted in the formation of deep, furrow-like invaginations of the plasma membrane that were stably labeled with FM4-64 in >50% of cells observed in these experiments (Fig. 7). Thus, sjl1Δ sjl2Δ double-mutant cells and sjl1Δ abp1Δ double-mutant cells displayed similar phenotypes. Notably, abp1Δ sjl2Δ mutant cells did not accumulate the furrow-like plasma membrane structures that are present in sjl1Δ sjl2Δ double-mutant cells (Fig. 7), suggesting that Sjl1 is recruited by a different mechanism.

Previous studies have shown that sjl1Δ sjl2Δ double mutants display defects in endocytic internalization, while sjl1Δ, sjl2Δ, or abp1Δ single-mutant cells do not (24, 44). Thus, we examined whether sjl1Δ abp1Δ double-mutant cells displayed endocytic defects similar to those of sjl1Δ sjl2Δ double-mutant cells. To do this, we monitored the internalization and transport of the lipophilic dye FM4-64 to the vacuole. Cells were pulse-labeled with FM4-64 and CMAC (a vital dye that accumulates in the vacuole lumen), washed, and chased in a medium that did not contain FM4-64 or CMAC. As expected, wild-type and abp1Δ mutant cells displayed FM4-64 fluorescence only in vacuole membranes or late endosomal compartments adjacent to vacuoles following a 30-minute chase (Fig. 8). Likewise, >80% of sjl1Δ sjl2Δ double-mutant cells expressing Sjl2K1120V,P1121D, GFP from a plasmid delivered FM4-64 to vacuole membranes or compartments adjacent to vacuoles within the chase period (Fig. 8). In contrast, sjl1Δ sjl2Δ double-mutant cells displayed defects in delivery of FM4-64 to the vacuole. FM4-64 fluorescence was observed in structures that were distinct from vacuoles, possibly corresponding to cell surface invaginations (Fig. 8). Interestingly, sjl1Δ abp1Δ double-mutant cells displayed a delay in endocytic trafficking to vacuoles; FM4-64 was found in peripheral structures in >50% of these cells (Fig. 8). We also expressed Sjl2K1120V,P1121D,F37C-GFP in cells lacking SIL1 and Sjl2 and found FM4-64 in peripheral structures in >40% of these cells (Fig. 8). Together, these results further implicate Abp1 in endocytosis and suggest that the predicted Abp1 binding site in Sjl2 is necessary for full Sjl2 function in the endocytic pathway.
activity. Actin polymerization has been proposed to mediate endocytic internalization and motility of actin patch components (26). Phosphoinositides, particularly PI(4,5)P₂, have been implicated in the organization of the actin cytoskeleton (54). Thus, we examined whether actin polymerization is required for the formation of extensive plasma membrane invaginations in Sjl-deficient cells. To perform these experiments, we utilized strains lacking Sjl and Sjl₃ that expressed either wild-type Sjl₂ or a temperature-conditional sjl₂ts allele. We have previously shown that sjl₂ts cells display endocytic defects and accumulate abnormal plasma membrane invaginations upon prolonged incubation at the restrictive temperature (48) (see Fig. S1 in the supplemental material). Sjl₂ and sjl₂ts cells were grown at the permissive temperature and then shifted to the nonpermissive temperature for 90 min in the absence or presence of latrunculin A. Cells were then labeled with FM4-64 under conditions that block endocytosis in order to visualize the plasma membrane. Upon extended incubation at the nonpermissive temperature, >80% of untreated sjl₂ts cells accumulated enlarged plasma membrane invaginations stained with FM4-64, consistent with our previous results (Fig. 9A). In contrast, cells expressing wild-type Sjl₂ displayed normal plasma membrane morphology, both in the absence and in the presence of latrunculin A (Fig. 9A and data not shown). To determine whether the abnormal cell surface morphology observed in sjl₂ts mutant cells was due to continued actin polymerization, we pretreated sjl₂ts cells with latrunculin A prior to the shift to the restrictive temperature. Interestingly, extensive, furrow-like plasma membrane structures did not form in >90% of sjl₂ts cells treated with latrunculin A at the nonpermissive temperature (Fig. 9A). We did observe regions of the plasma membrane with more-intense FM4-64 staining; however, these regions did not form the extensive invaginations that characteristically accumulate in sjl₂ts or sjl₁Δ sjl₂Δ mutant cells. Thus, actin polymerization was necessary for the formation of large invaginations of the plasma membrane in sjl₂ts mutant cells.

Sla2 is mislocalized in cells with impaired synaptojanin activity. We have previously reported that the inappropriate accumulation of PI(4,5)P₂ on intracellular compartments in sjl₂ts mutant cells at the restrictive temperature correlated with defects in actin organization, plasma membrane morphology, and endocytosis (48). We therefore wished to identify potential PI(4,5)P₂ downstream effector proteins that may become limiting at the plasma membrane in sjl₂ts mutant cells. Sla2, the yeast ortholog of mammalian Hip1-related protein (Hip1R), was a particularly interesting candidate. Sla2 and mammalian Hip1R possess an N-terminal ANTH domain, a central coiled region, and a C-terminal talin-like domain (Fig. 9B). ENTH/ANTH domains from several endocytic proteins, such as epsin and AP180, have been shown to bind PI(4,5)P₂ in vitro (15, 25). A previous study has shown that Sla2p can bind the clathrin light chain through its central region (23). Likewise, the Sla2p homologs Hip1 and Hip1R have been shown to bind clathrin, AP-2, and F-actin (10, 11, 30, 38). The C-terminal talin-like domain of Sla2 has been shown to bind F-actin in vitro (35, 36) and was sufficient for localization to actin patches in vivo (53). Accordingly, Sla2/Hip1R proteins have been proposed to link actin and clathrin coat components during endocytosis.
FIG. 8. Deletion of ABP1 and SJL1 results in synthetic endocytic defects, similar to those caused by sjl1 and sjl2 mutations. Wild-type (SEY6210.1), sjl1Δ sjl2Δ (YCS66), abp1Δ (YCS301), and sjl1Δ abp1Δ (YCS323) cells, or strain YCS66 expressing sjl2Δ37C-GFP or Sjl2K1120V,P1121D,Δ37C-GFP (Sjl2KV,PD,Δ37C-GFP) from a plasmid, were labeled with the vital dyes FM4-64 and CMAC for 15 min. After labeling, cells were chased for 30 min. (Left panels) Cells under fluorescent illumination in the rhodamine channel (FM4-64). (Center panels) Cells as observed by CMAC fluorescence to visualize the lumens of vacuoles. (Right panels) Fluorescent images were merged with corresponding DIC images (merge). Arrowheads mark examples of delayed endocytic intermediates containing FM4-64. Bar, 5 μM.
First, we addressed whether overexpression of Sla2 might restore normal plasma membrane morphology in sjl2<sup>ts</sup> cells. Thus, SLA2 was expressed from a high-copy-number plasmid in SJL2 and sjl2<sup>ts</sup> cells grown at the permissive temperature and subsequently labeled with FM4-64 at 0°C to visualize the plasma membrane. Expression of SLA2 from a high-copy-number plasmid had no effect on plasma membrane morphology in SJL2 cells (data not shown). As expected, >80% of sjl2<sup>ts</sup> cells carrying vector alone accumulated enlarged plasma membrane invaginations stained with FM4-64 upon extended incubation at the nonpermissive temperature (Fig. 9B). Notably, extended, furrow-like plasma membrane structures did not form in >70% of sjl2<sup>ts</sup> cells overexpressing SLA2 (Fig. 9B). We did not observe punctate structures at the plasma membrane with FM4-64 staining; however, these regions did not develop into the deep invaginations that form in sjl2<sup>ts</sup> cells.

Next, we addressed whether overexpression of Sla2 might rescue endocytic trafficking defects in sjl2<sup>ts</sup> cells. To do this, we monitored the transport of FM4-64 to the vacuole in SJL2 and sjl2<sup>ts</sup> cells expressing SLA2 from a high-copy-number plasmid following incubation at the restrictive temperature. After a 30-min chase, FM4-64 fluorescence was efficiently delivered to the vacuole membrane in SJL2 cells carrying either vector alone or the SLA2 plasmid (Fig. 10). As expected, sjl2<sup>ts</sup> cells displayed defects in delivery of FM4-64 to the vacuole, since FM4-64 fluorescence was observed in punctate structures that were distinct from vacuoles (Fig. 10). Interestingly, expression of SLA2 from a high-copy-number plasmid increased the rate of endocytic trafficking to the vacuole in sjl2<sup>ts</sup> cells, as FM4-64 efficiently labeled the vacuole membrane in >75% of these cells (Fig. 10). Together, these results suggested that Sla2 function was limiting or misregulated in sjl2<sup>ts</sup> cells at the nonpermissive temperature.

Thus, we examined the localization of a functional Sla2-GFP fusion in wild-type and sjl1<sup>Δ</sup> sjl2<sup>Δ</sup> double-mutant cells. Cells lacking SJL1 and SJL2 have been reported to display defects in actin organization and endocytic internalization (44, 46). In wild-type cells, Sla2-GFP was observed at cortical patches concentrated in the bud, a pattern similar to previously published patterns of Sla2 localization at cortical actin patches (53) (Fig. 11A). However, in sjl1<sup>Δ</sup> sjl2<sup>Δ</sup> mutant cells, Sla2-GFP displayed random distribution in both mother and daughter cells and was no longer strictly cortical (Fig. 11A). We also addressed whether Sla2 localized to the plasma membrane furrows that accumulate in sjl1<sup>Δ</sup> sjl2<sup>Δ</sup> cells. To do this, we labeled the plasma membranes of sjl1<sup>Δ</sup> sjl2<sup>Δ</sup> double-mutant cells expressing Sla2-GFP with FM4-64 under conditions that block endocytosis (0°C and metabolic inhibitors). We did not observe significant overlap between FM4-64 and Sla2-GFP fluorescence in these cells (see Fig. S2 in the supplemental material), consistent with the idea that Sla2 might be limiting at sites of endocytic internalization in sjl1<sup>Δ</sup> sjl2<sup>Δ</sup> mutant cells.

Yeast cells that lack Sla2 and mammalian cells depleted of Hip1R have been shown to accumulate comet-like actin tails at sites of endocytic internalization (12, 26). We therefore examined whether sla2Δ mutant cells form plasma membrane invaginations similar to those of sjl2<sup>ts</sup> or sjl1<sup>Δ</sup> sjl2<sup>Δ</sup> mutant cells. Thus, wild-type and sla2Δ cells were stained with FM4-64 under conditions that block endocytosis, as before. As expected, wild-type cells displayed normal plasma membrane morphology (Fig. 11B). Interestingly, >75% of sla2Δ cells displayed multiple punctate plasma membrane structures stained with FM4-64 (Fig. 11B). While these structures did not appear to be as extensive as the invaginations that form in sjl2<sup>ts</sup> or sjl1<sup>Δ</sup> sjl2<sup>Δ</sup> cells, these results suggested that sla2Δ mutant cells accumu-
late plasma membrane invaginations. Taken together, our results have suggested that Sla2 becomes misregulated or limiting at the plasma membrane in cells with impaired SJL-encoded PI Pase activity, which may subsequently result in inefficient membrane fission or scission during endocytic internalization.

**DISCUSSION**

We have shown that Sjl2 localizes to cortical actin patches and that this localization depended on the PRD of Sjl2, actin filaments, and the actin patch component Abp1. Previous studies have indicated that elevated PI(4,5)P2 levels in cells lacking SJL-encoded PI Pase activity cause defects in actin filament organization (39, 48). Here, we have shown that actin filaments were necessary for the formation of extensive plasma membrane invaginations in sjl2 cells, suggesting that misregulated actin polymerization may lead to defects in proper fission and release of endocytic vesicle intermediates. Finally, our studies have indicated that overexpression of the actin patch protein Sla2 partially rescued the endocytic and plasma membrane morphology defects observed in sjl2 mutant cells.

**Sjl2 colocalizes with Abp1.** Several lines of evidence indicate that Sjl2 and Abp1 function at the same site in the control of endocytosis. Both Abp1 and Sjl2Δ37C-GFP are recruited to actin patches in a latrunculin-sensitive manner (Fig. 2B) (26). In abp1Δ mutant cells, Sjl2Δ37C-GFP localizes diffusely throughout the cytoplasm (Fig. 3A). The PRD of Sjl2 is necessary for the recruitment of Sjl2 to cortical actin patches and for physical association with Abp1 (Fig. 2B and 5). Thus, our results provide in vivo evidence that Abp1 acts as an adaptor for Sjl2 at actin patches via the PRD of Sjl2. Deletion of SJL2
Thus, Abp1 appears to specifically recruit Sjl2 at cortical actin
patches, but not Sjl1 or Sjl3. Further studies are needed to identify Sjl1- and Sjl3-specific targeting factors.

Abp1 is recruited to actin patches at a late step during endocytic internalization and then rapidly moves away from the plasma membrane (26). Consistent with a role for Sjl2 during late stages of vesicle fission and uncoating, Sjl2Δ[^37C]-
GFP concentrates at preexisting Abp1 cortical patches, and Abp1 disappears from patches shortly following Sjl2 recruit-
ment (Fig. 4). Previous studies indicate that Abp1 also inter-
acts with several other components of actin patches, including the Arp2/3 complex, the yeast amphiphysin ortholog Rvs167
(see below), the type I myosin Myo3, and the kinases Ark1/
Prk1 (6, 14, 19, 32). It is not likely that Abp1 binds to these
factors simultaneously. So then, how are multiple Abp1 inter-
actions selectively coordinated at actin patches? One possible
explanation comes from previous studies indicating that mam-
alian synaptotagmin is a member of the dephosphin family of
endocytic proteins, which also includes dynamin, AP180, end-
dophilin, and amphiphysin; these proteins associate with each
other only following Ca^2+ -stimulated dephosphorylation (29,
45). Thus, a complex series of phosphorylation and dephos-
phorylation events may regulate the recognition by Abp1 of
various targets during late events in endocytic internalization.
Sjl2 might be dephosphorylated temporally later than other
Abp1 targets, thus avoiding premature termination of PI(4,5)P_2-mediated vesicle formation events. Further studies
are needed to examine whether Sjl2 is regulated by phosphor-
ylation-dephosphorylation and, if so, to identify the relevant
kinases and phosphatases.

Our studies also indicated that multiple Sjl2-interacting fac-
tors exist, analogous to the complex interactions that control
the localization and activity of mammalian synaptotagmins. First,
we found that Sjl2Δ[^37C]-GFP persisted in cortical patches fol-
lowing the disappearance of Abp1-DsRed (Fig. 4). In addition,
our sizing analysis indicated that Sjl2 remained in a complex
larger than its predicted monomeric size in lysates from abp1Δ
cells (Fig. 6). Recent studies have shown that Sjl2 physically
associates with two other SH3 domain-containing proteins,
Rvs167 and Sla1, and another protein, Bsp1 (47, 52). Rvs167 is
the yeast amphiphysin ortholog; thus, the association of Sjl2
with Rvs167 is consistent with the known interaction between
amphiphysin and mammalian synaptotagmin (5, 45). Likewise,
a recent study has indicated that the mammalian homolog of
Sla1, CIN85, physically associates with synaptotagmin 2 (28).
Bsp1 was identified as a cortical actin patch protein that inter-
acts with the Sac1 domains of Sjl2 and Sjl3 (47). Sla1, Rvs167,
and Bsp1 may act as additional recruitment or specificity fac-
tors for Sjl2 localization or regulation (see Fig. 12), consistent
with our genetic results indicating that sjl1Δ abp1Δ mutant
cells did not fully phenocopy sjl1Δ sjl2Δ mutant cells. While
our initial Sjl2Δ[^37C]-GFP localization experiments suggested
that Sla1, Rvs167, and Bsp1 were not essential for Sjl2 locali-
zation at cortical patches (Fig. 3 and data not shown), these
proteins may influence the stability or lifetime of Sjl2 at cor-
tical patches. Our study has not excluded the possibility that residues deleted in Sjl2Δ[^37C]-GFP provide important inter-
actions with Sla1 or Rvs167, for example, that direct Sjl2 locali-
zation along with Abp1. Alternatively, Sjl2 may exist in a
stable subcomplex that transiently interacts with Abp1 patches.

and deletion of ABP1 have similar effects; sjl1Δ abp1Δ double-
mutant cells display furrow-like plasma membrane invagina-
tions and endocytic defects similar to those observed in sjl1Δ
sjl2Δ double-mutant cells (Fig. 7). Notably, abp1Δ sjl2Δ mutant
cells do not display defects in plasma membrane morphology
or actin cytoskeletal organization (Fig. 7 and our unpublished
data), unlike sjl1Δ sjl2Δ or sjl2Δ sjl3Δ mutant cells (44, 46).
Thus, Abp1 appears to specifically recruit Sjl2 at cortical actin

FIG. 11. (A) Proper localization of Sla2 at cortical patches is de-
pendent on Sjl1 and Sjl2. Localization of Sla2-GFP in living wild-type
(YCS284) and sjl1Δ sjl2Δ (YCS374) cells was observed by fluorescence
(Sla2-GFP) and Nomarski (merge) microscopy. (B) Deletion of SLA2
confers abnormal plasma membrane dynamics. Wild-type
(SEY6210.1) and sla2Δ (YCS388) cells were made nonviable by the
addition of NaN_3 and NaF to inhibit internalization from the plasma
membrane. Cells were then stained with FM4-64 at 0°C as described
previously (48) and observed by fluorescent microscopy (FM4-64) and
Nomarski optics (merge). Bars, 5 μM.
In cells lacking Abp1, this complex may become destabilized. Thus, further experiments will be necessary to examine the temporal and spatial recruitment of Sjl2 relative to additional actin patch components such as Sla1, Rvs167, and Bsp1. PI(4,5)P2 turnover regulates the timing of plasma membrane invagination and fission events during endocytic internalization. Previous studies have shown that synaptojanins are necessary for the postfission uncoating of adaptor proteins from newly formed endocytic vesicles (7, 22, 51). Consistent with this, sjl2ts cells have been shown to accumulate PI(4,5)P2 in intracellular compartments at the restrictive temperature (48). However, if the single function for synaptojanins during endocytosis is to recycle PI(4,5)P2-regulated vesicle formation factors from endosomes, subsequent rounds of internalization may not proceed in synaptojanin-deficient cells. Strikingly, our studies and previous studies have indicated that sjl2ts and sjl1/H9004sjl2/H9004 cells accumulate numerous, extensive plasma membrane invaginations (44, 48). At the ultrastructural level, the cytoplasmic face of these invaginations appeared to be lined by an electron-dense “coat”-like material. In addition, a clearing or electron-transparent region was often observed at the tips of the invaginations (see Fig. S1B in the supplemental material). Further experiments are needed to determine whether these are components of clathrin coats and the actin polymerization machinery. Regardless, these examinations have suggested that vesicle formation factors may not become limiting in Sjl-deficient cells and that multiple internalization events can initiate. Rather, these findings have indicated that reduced SJL-encoded PI Pase function leads to increased growth of plasma membrane invaginations. Thus, factors important for promoting vesicle scission may be limiting or impaired in cells deficient in SJL-encoded PI Pase activity.

Several lines of evidence suggested that Sla2, the Hip1R homolog shown to associate with the actin cytoskeleton and components of clathrin coats, becomes limiting at the plasma membrane in sjl mutant cells. First, Sla2-GFP was mislocalized in sjl2 mutan cells. More importantly, SLA2 overexpression attenuated the formation of the large plasma membrane invaginations that accumulate in sjl2ts cells (Fig. 9). We found that overexpression of another ANTH domain-containing endocytic protein, yAP180a, or the PH domain from PLCγ2, as a control, did not rescue the plasma membrane morphology defects in sjl2ts cells (data not shown). Thus, suppression was not simply due to sequestration of excess cellular PI(4,5)P2. Furthermore, SLA2 overexpression rescued the endocytic defects displayed by sjl2ts cells, as assessed by monitoring of the internalization and delivery of FM4-64 to the vacuole (Fig. 10).

Previous studies have shown that deletion of SLA2 in yeast and depletion of Hip1R in mammalian cells resulted in the formation of comet-like actin tails associated with endocytic

FIG. 12. Model for Sjl2 recruitment and function during endocytic internalization. PI(4,5)P2 synthesis by the PI(4)P 5-kinase Mss4 recruits effector proteins that promote actin polymerization (red cables), clathrin coat formation (brown ovals), and vesicle budding (step 1). The SH3 domain-containing protein Abp1 is recruited at a late stage during vesicle formation (26) and subsequently recruits Sjl2. Additional Sjl2-interacting factors, such as the SH3 domain-containing proteins Rvs167 and Sla1 (47), may also target Sjl2 to actin patches. Following Abp1 recruitment, certain actin patch components, such as the ANTH domain-containing protein Sla2, disassociate from patches (indicated by dashed arrows) (26). Concentration of Sjl2 at patches by Abp1 may serve an important role in this process. Turnover of PI(4,5)P2 at the plasma membrane by Sjl1 and Sjl2 provides an important step in coordinating actin polymerization and vesicle fission (step 2); cells lacking SJL1 and SJL2 accumulate extensive endocytic intermediates. Synaptojanins, such as Sjl2 and Sjl3, also dephosphorylate PI(4,5)P2 on newly formed vesicles to recycle or uncoat PI(4,5)P2-binding factors, such as actin patch components and clathrin coat proteins (step 3).
cargo and machinery (12, 26). In addition, sla2 mutations have been shown to cause the formation of large, aggregated actin patches or clumps (3, 53). These studies have indicated that Sas2 regulates actin cytoskeletal dynamics at cortical actin patches by destabilizing actin filaments or by negatively regulating the Arp2/3 actin polymerization complex or its activators. Thus, mislocalization of Sas2 in SJL-deficient cells may cause increased actin polymerization and defects in productive clathrin coat formation, ultimately leading to continued or extended growth of plasma membrane invaginations. Consistent with this idea, actin polymerization was necessary for the formation of extensive plasma membrane invaginations in sjl2Δ cells (Fig. 9). However, the punctate structures at the plasma membrane in sla2Δ cells (Fig. 11) were not nearly as extensive as the furrow-like invaginations that form in cells lacking SJL and SJL2. Thus, additional PI(4,5)P2 effector proteins are likely to be misregulated and to contribute to the dramatic effects on plasma membrane and actin cytoskeleton dynamics observed in SJL-deficient cells. Consistent with this, overexpression of SJL2 was not sufficient to bypass the growth defect of sjl2Δ cells at the restrictive temperature (data not shown).

PI(4,5)P2 has been shown to stimulate actin polymerization by binding several actin-regulatory proteins, such as WASP, cofillin, gelsolin, and capping proteins (54). Thus, increased PI(4,5)P2 levels at the plasma membrane in SJL1/2-deficient cells may also enhance cortical actin filament growth and stability, leading in turn to additive effects on proper fission and release of endocytic vesicles (see Fig. 12). Consistent with this idea, we have previously shown that increased cellular PI(4,5)P2 levels directly affect plasma membrane dynamics and actin organization, since sjl1Δ sjl2Δ mss4Δ triple-mutant cells, which synthesize normal PI(4,5)P2 levels, do not display abnormal plasma membrane furrows or depolarized actin patches such as those seen in sjl1Δ sjl2Δ double-mutant cells (48). In yeast, at least three actin patch components, Las17 (a WASP ortholog), Pan1 (an Eps15 ortholog), and Abp1, have been shown to be activators of the Arp2/3 complex that mediates actin polymerization (18). Interestingly, Abp1 is recruited to actin patches temporally later than Las17 and Pan1 (26). Furthermore, Las17 and Pan1 were shown to disassociate or disappear from actin patches upon Abp1 recruitment (26). Thus, the role of Abp1 in recruiting SJL2 to endocytic vesicles may be to terminate earlier PI(4,5)P2-dependent stages of vesicle formation mediated by Las17 or Pan1. Alternatively, SJL2 may be recruited to actin patches by Abp1 to regulate Abp1 function itself. Interestingly, disappearance or dissociation of cortical Abp1 consistently occurred following SJL2 recruitment (Fig. 4). Further experiments that examine the spatial and temporal relationships of known actin patch proteins in cells with impaired SJL1, SJL2, and SJL3 activity may provide further insights that allow us to distinguish between these potential models.

Likewise, further studies are necessary to identify additional regulatory factors for SJL1, SJL2, and SJL3. Each contains unique targeting signals, and thus, specific recruitment and activation mechanisms for these proteins must exist. Further studies may uncover specialized roles for these PI phosphatases within the ordered processes of membrane invagination, actin cytoskeletal dynamics, vesicle scission, and subsequent uncoating events that occur during endocytosis.

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