**Scd5p Mediates Phosphoregulation of Actin and Endocytosis by the Type 1 Phosphatase Glc7p in Yeast**

Guisheng Zeng,* Bo Huang,* Suat Peng Neo, Junxia Wang, and Mingjie Cai

Institute of Molecular and Cell Biology, Singapore 138673, Republic of Singapore

Submitted June 26, 2007; Revised September 5, 2007; Accepted September 13, 2007

Monitoring Editor: Sandra Schmid

Pan1p plays essential roles in both actin and endocytosis in yeast. It interacts with, and regulates the function of, multiple endocytic proteins and actin assembly machinery. Phosphorylation of Pan1p by the kinase Prk1p down-regulates its activity, resulting in disassembly of the endocytic vesicle coat complex and termination of vesicle-associated actin polymerization. In this study, we focus on the mechanism that acts to release Pan1p from phosphorylation inhibition. We show that Pan1p is dephosphorylated by the phosphatase Glc7p, and the dephosphorylation is dependent on the Glc7p-targeting protein Scd5p, which itself is a phosphorylation target of Prk1p. Scd5p links Glc7p to Pan1p in two ways: directly by interacting with Pan1p and indirectly by interacting with the Pan1p-binding protein End3p. Depletion of Glc7p from the cells causes defects in cell growth, actin organization, and endocytosis, all of which can be partially suppressed by deletion of the PRK1 gene. These results suggest that Glc7p antagonizes the activity of the Prk1p kinase in regulating the functions of Pan1p and possibly other actin- and endocytosis-related proteins.

**INTRODUCTION**

Clathrin-mediated endocytosis involves elaborate spatial and temporal recruitment of endocytic adaptors, coat elements, and actin cytoskeletal factors in yeast and mammalian cells (Kaksonen et al., 2003; Merrifield, 2004; Kaksonen et al., 2005, 2006). Some four protein modules have been recently suggested to be responsible for driving distinct stages of endocytosis in yeast (Kaksonen et al., 2005). The coat module consists of proteins that assemble early at the endocytic sites to initiate coat formation. The WASP/Moysin module activates the Arp2/3-mediated actin polymerization to induce membrane invagination. The actin module organizes actin filaments into a branched meshwork to facilitate coat movement, and the amphiphysin module functions in vesicle scission. The released vesicles are thought to be propelled into the cytosol by actin polymerization, accompanied by the disassembly of the coat complex (Kaksonen et al., 2006).

The essential yeast protein Pan1p plays a central role in coat formation by interacting with multiple coat proteins, including End3p, Sla1/2p, Yip1801/2p, and Ent1/2p (Tang et al., 1997; Wendland and Emr, 1998; Wendland et al., 1999; Tang et al., 2000; Toshima et al., 2005). Pan1p also possesses the ability to activate the Arp2/3 complex, and may cooperate with other nucleation-promoting factors, such as Las17p and Myo5p, to initiate actin assembly at endocytic sites (Duncan et al., 2001; Sun et al., 2006). Furthermore, Pan1p can directly bind to actin filaments via its WH2-like region (Toshima et al., 2005), thereby providing an anchor point for actin meshwork to associate with the endocytic coat. The functions of Pan1p are under a negative regulation by the serine/threonine kinase Prk1p (Zeng and Cai, 1999; Zeng et al., 2001; Toshima et al., 2005). Phosphorylation of Pan1p by Prk1p on the LxxQxTG motifs disrupts the Pan1p–Sla1p complex (Zeng et al., 2001), and prevents Pan1p from associating with actin filaments and activating the Arp2/3 complex (Toshima et al., 2005). This regulation is presumably to allow vesicles to be uncoated and uncoupled from the actin meshwork after they are internalized. Compared with the progressing studies of the regulation of Pan1p by phosphorylation, very little is known about how Pan1p regains its activity after phosphoinhibition.

The type 1 protein phosphatase (PP1) of *Saccharomyces cerevisiae*, Glc7p, has been shown to be involved in a diversity of cellular processes such as glycolen metabolism (Feng et al., 1991), translational control (Wek et al., 1992), glucose repression (Tu and Carlson, 1994), cell cycle progression (Hisamoto et al., 1994), and chromosome segregation (Francisco et al., 1994). The ability of Glc7p to perform such diverse functions stems presumably from its interactions with various targeting factors that direct the phosphatase to different substrates and/or sites of activity. For example, Reg1p binds and targets Glc7p to its substrates in the glucose repression regulatory pathway (Tu and Carlson, 1995). A group of Glc7p-interacting proteins have been identified by Tu et al. (1996) using the two-hybrid protein interaction assay. One of them, Scd5p, has recently been confirmed to interact with Glc7p in vivo (Chang et al., 2002). Similar to Pan1p, Scd5p is also a phosphorylation target of Prk1p, and has been shown to function in cortical actin organization and endocytosis (Henry et al., 2002; Henry et al., 2003; Huang et al., 2003). There is a pressing need, therefore, to ascertain whether Glc7p is involved in the regulation of actin and endocytosis through its interaction with Scd5p.

In this study, we demonstrate that Glc7p functions antagonistically to Prk1p in modulating the phosphorylation status of Pan1p in vivo. Scd5p is a critical factor in determining
the phosphorylation status of Pan1p, as it serves not only as a Glc7p targeting factor but also as a switch in phosphorylation of Pan1p. These findings provide new insight into the mechanism that operates in the cycle of phosphoregulation of Pan1p. These findings provide new insight into the mechanism that operates in the cycle of phosphoregulation of Pan1p. These findings provide new insight into the mechanism that operates in the cycle of phosphoregulation of Pan1p. These findings provide new insight into the mechanism that operates in the cycle of phosphoregulation of Pan1p. These findings provide new insight into the mechanism that operates in the cycle of phosphoregulation of Pan1p. These findings provide new insight into the mechanism that operates in the cycle of phosphoregulation of Pan1p. These findings provide new insight into the mechanism that operates in the cycle of phosphoregulation of Pan1p. These findings provide new insight into the mechanism that operates in the cycle of phosphoregulation of Pan1p. These findings provide new insight into the mechanism that operates in the cycle of phosphoregulation of Pan1p.

<table>
<thead>
<tr>
<th>Table 1: Yeast strains used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
</tr>
<tr>
<td>W303-1A</td>
</tr>
<tr>
<td>W303-1B</td>
</tr>
<tr>
<td>SFY526</td>
</tr>
<tr>
<td>YMC422</td>
</tr>
<tr>
<td>YMC441</td>
</tr>
<tr>
<td>YMC446</td>
</tr>
<tr>
<td>YMC448</td>
</tr>
<tr>
<td>YMC449</td>
</tr>
<tr>
<td>YMC471</td>
</tr>
<tr>
<td>YMC472</td>
</tr>
<tr>
<td>YMC473</td>
</tr>
<tr>
<td>YMC474</td>
</tr>
<tr>
<td>YMC475</td>
</tr>
<tr>
<td>YMC476</td>
</tr>
<tr>
<td>YMC477</td>
</tr>
<tr>
<td>YMC478</td>
</tr>
<tr>
<td>YMC479</td>
</tr>
<tr>
<td>YMC480</td>
</tr>
<tr>
<td>YMC481</td>
</tr>
<tr>
<td>YMC482</td>
</tr>
<tr>
<td>YMC483</td>
</tr>
<tr>
<td>YMC484</td>
</tr>
<tr>
<td>YMC485</td>
</tr>
<tr>
<td>YMC486</td>
</tr>
<tr>
<td>YMC487</td>
</tr>
<tr>
<td>YMC488</td>
</tr>
<tr>
<td>YMC489</td>
</tr>
<tr>
<td>YMC494</td>
</tr>
<tr>
<td>YMC498</td>
</tr>
<tr>
<td>YMC499</td>
</tr>
<tr>
<td>YMC500</td>
</tr>
<tr>
<td>YMC491</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Strains, Plasmids, and General Methods**

Yeast strains and plasmids used in this study are listed in Table 1 and 2, respectively. Yeast cells were grown in standard yeast extract-peptone-dextrose (YPD) or synthetic complete (SC) medium lacking appropriate amino acids for plasmid maintenance. In experiments requiring the expression of genes under the GAL1 promoter, raffinose instead of dextrose was used as the carbon source and galactose was later added for GAL1 induction. To maintain the genomic copy of glc7-td, glc7-ntd, and prk1::PAN1-Myc-TPR1, plasmids pGEM-Myc and pSIL-Myc were linearized at the vector backbone and transformed into yeast cells. The C-terminal green fluorescent protein (GFP)-fused Scd5 in YMC496 and YMC498 were generated by the PCR-targeting method as described previously (Wach et al., 1997). Other strains were generated by integrating various linearized plasmids into corresponding host cells, respectively. Bacterial strains DH5α and BL21 were grown on standard medium supplemented with 100 µg/ml ampicillin to maintain plasmids. Genetic and recombinant DNA manipulations were performed according to standard techniques.

**Protein Extraction, Immunoprecipitation, and Immunoblotting**

Yeast extracts were prepared by either glass beads method or trichloroacetic acid (TCA) precipitation method as described previously (Tang et al., 1997; Huang et al., 2003). Immunoprecipitation and immunoblotting followed the published procedure (Tang et al., 1997) with slight modifications. The beads-conjugated anti-hemagglutinin (HA) or anti-Myc polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used to immunoprecipitate epitope-tagged proteins. For immunoblotting of precipitated proteins, monoclonal anti-HA or anti-Myc (clone 12CA5 and 9E10, respectively; Roche Molecular Biology of the Cell4886)

---

G. Zeng et al.
pGLC7c-HA-305 DNA fragment encoding Glc7p (60-312 aa) with its upstream 501 base pairs (bp) of the intron was cloned in pGEX-LR1 GST-LR1; DNA fragment encoding Pan1p (99-383 aa) was cloned into pGEX-4T-1 (Zeng and Cai, 1999)
pGLC7T152Kc-HA-305 T152K mutation was introduced into pGLC7c-HA-305 by PCR mutagenesis
pGLC7n-ntd-306 pUb-Arg-DHFR-HA-GLC7n-306; The Leu residue at position 66 in the DHFR moiety (Dohmen et al., 1994)
pGLC7T152Kc-Myc-305 DNA fragment encoding Glc7p (60-312 aa) with T152K mutation was cloned in frame with a C-terminal Myc epitope and placed under its own promoter control in pRS316
pMyc-END3C-314 DNA fragment encoding End3p (1-253 aa) was cloned in frame with a N-terminal Myc epitope and placed under its own promoter control in pRS314
Glutathione Transferase (GST) Fusion Proteins and In Vitro Kinase and Phosphatase Assays

To make GST-fusion proteins, DNA fragments were generated by PCR and cloned into a bacterial GST expression vector pGEX-4T-1 as indicated in Table 2. Expression and purification of GST-fusion proteins followed the published procedure (Zeng and Cai, 1999). Using VIVASPIN 500 columns (Vivascience, Hannover, Germany), GST-fusion proteins were washed and buffer exchanged into either binding buffer (100 mM NaCl, 20 mM HEPES, pH 7.3, 0.1% Triton X-100, and 1 mM dithiothreitol [DTT]) for in vitro binding assay or H2O for protein phosphatase assay. In vitro kinase assays were performed as described previously (Zeng and Cai, 1999) with slight modifications. Briefly, beads associated anti-HA antibodies were used to immunoprecipitate HA-Pan1p, GST-fusion proteins were mixed with the immunoprecipitated kinase, 60 µl of HBII buffer, 10 µl of 100 mM ATP, and 10 µl of 250 mM 3-(N-morpholino)propanesulfonic acid in a total volume of 200 µl and incubated at 25°C for 5 h, followed by gel electrophoresis and sequential immunoblotting with monoclonal anti-GST (BD Biosciences, Palo Alto, CA) and anti-PThr antibodies. For protein phosphatase assays, yeast extracts containing endogenously expressed Gic7-HA were prepared with phosphatase inhibitor-free lysate buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) five times. The immunoprecipitates, with or without the preincubation with 1 µl of PPI inhibitor 1-2 (New England Biolabs, Ipswich, MA) at 25°C for 15 min, were mixed with phosphorylated GST-fusion proteins and 10 µl of 10X PPI buffer (New England Biolabs) in a total volume of 100 µl. The mixture was incubated at 37°C, and 20 µl of supernatant was taken at each time point in 20-min intervals. Samples were then subjected to gel electrophoresis and sequential immunoblotting with anti-GST and anti-PThr antibodies.

Two-Hybrid Interaction and In Vitro Binding Assays

For yeast two-hybrid interaction assays, DNA fragments of SCD5, PAN1, EN3, and GLC7 were generated by PCR and cloned into pGBK7 or pGADT7 vectors as indicated in Table 2. Plasmids were cotransformed into either binding buffer (100 mM NaCl, 20 mM HEPES, pH 7.3, 0.1% Triton X-100, and 1 mM dithiothreitol [DTT]) for in vitro binding assay or H2O for protein phosphatase assay. In vitro kinase assays were performed as described previously (Zeng and Cai, 1999) with slight modifications. The beads associated anti-HA antibodies were used to immunoprecipitate HA-Pan1p, GST-fusion proteins were mixed with the immunoprecipitated kinase, 60 µl of HBII buffer, 10 µl of 100 mM ATP, and 10 µl of 250 mM 3-(N-morpholino)propanesulfonic acid in a total volume of 200 µl and incubated at 25°C for 5 h, followed by gel electrophoresis and sequential immunoblotting with monoclonal anti-GST (BD Biosciences, Palo Alto, CA) and anti-PThr antibodies. For protein phosphatase assays, yeast extracts containing endogenously expressed Gic7-HA were prepared with phosphatase inhibitor-free lysate buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) five times. The immunoprecipitates, with or without the preincubation with 1 µl of PPI inhibitor 1-2 (New England Biolabs, Ipswich, MA) at 25°C for 15 min, were mixed with phosphorylated GST-fusion proteins and 10 µl of 10X PPI buffer (New England Biolabs) in a total volume of 100 µl. The mixture was incubated at 37°C, and 20 µl of supernatant was taken at each time point in 20-min intervals. Samples were then subjected to gel electrophoresis and sequential immunoblotting with anti-GST and anti-PThr antibodies.

Actin Staining and Endocytosis Assay

Staining of actin filaments with rhodamine-phalloidin (Invitrogen, Carlsbad, CA) was performed as described previously (Huang et al., 2003). The uracil permease internalization assay was carried out according to Volland et al. (1999) with minor modifications. Yeast cells were transformed with a multicopy plasmid containing the URFA1 gene to increase the production of uracil permease. The transformants were grown in uracil dropout medium at 25°C to OD540 of 0.2–0.3, and then they were incubated at 37°C for 6 h. Cultures were shifted back to 25°C, and cycloheximide was immediately added to a final concentration of 100 µg/ml. On the addition of cycloheximide, samples were taken at 20-min intervals to assay the uracil permease activity. One milliliter of the culture at each time point was incubated with 5 µl of uracil solution containing 4.5 µl of 1 mM uracil and 0.5 µl of 1 mCi/ml [5-3H]uracil (GE Healthcare) for 20 s at 25°C. The mixture was then quickly filtered through a Whatman 25 mm GF/C filter, followed by washing twice with ice-cold water and counting for the retained radioactivity.

Live Cell Imaging

Yeast cells expressing GFP and/or cyan fluorescent protein (CFP)-tagged proteins were allowed to grow to early log phase at 30°C. Cells were harvested, resuspended in SC media, and adhered to the surface of a glass slide precoated with 2% agarose. The slide was then covered with a coverslip and sealed with petroleum jelly. Fluorescence microscopy was performed using a Zeiss Axivert 200 M microscope equipped with a Cool Snap HQ camera (Roper Scientific, Tucson, AZ). All imaging was done by keeping the slide within a closed chamber with a constant temperature of 30°C. Images were acquired continuously at 1 frame/2-5 s, depending on the signal intensity, with motorized GFP and CFP filters. To determine the patch lifetime, >30 patches of each protein were visually analyzed for their time courses between patch appearance and disappearance, and the lifetime was calculated as the average time course ± SD.

RESULTS

Interaction of Scd5p with Pan1p and End3p

To establish the link between Scd5p and Pan1p, we first tested the interaction between the two proteins by yeast two-hybrid assays. As shown in Figure 1A (top right), the N-terminal half of Scd5p displayed a clear interacting activity with the LR2 region of Pan1p. Dissection of SCD5-N into two smaller fragments yielded one with no LR2-binding activity (SCD5-N1) and another with an autoactivity (SCD5-N2). To clarify this issue, we carried out in vitro binding assays using His-tagged Scd5p fragments and GST-LR2. The results showed that SCD5-N2, which contains three Prk1p phosphorylation sites (LxxTxTG), could efficiently pull down GST-LR2 (Figure 1B, lane 6). The interaction between Scd5p and Pan1p was further confirmed by coimmunoprecipitation. Endogenously expressed Scd5-HA could be pulled down by the anti-Myc antibody when Pan1-Myc was coexpressed (Figure 1C, lane 4), indicating that Scd5p interacts with Pan1p in vivo.

Concurrently, we also tested the possibility of an interaction between Scd5p and End3p, which is known to form a complex with Pan1p in vivo (Tang et al., 1997). Two-hybrid assays revealed that SCD5-N could bind to the N terminus of End3p (Figure 1A, bottom right), which is noteworthy not involved in the interaction with Pan1p (Tang et al., 1997). This result was again supported by coimmunoprecipitation experiments (Figure 1D). We could not, however, detect an interaction between Scd5p and Sla1p, another endocytic protein that associates with Pan1p and End3p in vivo (Tang et al., 2000), by the two-hybrid assay (data not shown). In support of the finding that Scd5p interacts with both Pan1p and End3p, a temperature-sensitive mutant of scd5 defective in endocytosis and actin organization, scd5-1 (Chang et al., 2002; Huang et al., 2003), exhibited synthetic lethality with either pan1-4 (Tang and Cai, 1996), or end3Δ (Figure 1E), indicating that these genes have functions in common. Together, these experiments confirm a physical and functional association between Scd5p and the Pan1p–End3p complex, thus making Scd5p a possible link between Glc7p and Pan1p for the dephosphorylation purpose.

Elevation of Pan1p Phosphorylation Level in scd5 and end3 Mutants

Pan1p is known to be phosphorylated by Prk1p on potentially a large number of threonine residues present as the LxxTxTG motifs and clustered in the LR1 and LR2 regions (Zeng and Cai, 1999). The steady-state phosphorylation level of endogenously expressed Pan1p could be revealed by a phosphothreonine-specific antibody (anti-PThr), as shown in Figure 2A (left, lane 2). As expected, it was drastically reduced in the prk1Δ mutant (to ~15% of the wild-type control). In contrast, the Pan1p phosphorylation level was found to be significantly increased in the scd5-1 mutant at the nonpermissive temperature of 37°C (Figure 2B). This result shows that the phosphorylation status of Pan1p in vivo is dependent on the activity of Scd5p. As the scd5-1 allele is impaired in binding to Glc7p (Chang et al., 2002;
Figure 1. Interactions of Scd5p with Pan1p and End3p. (A) Two-hybrid interactions of Scd5p with Pan1p and End3p. The regions of Scd5p, Pan1p, and End3p used for two-hybrid assays were schematically shown on left. Domains and motifs important for each protein are also indicated. PBM, phosphatase binding motif; LR, long repeat; EH, Eps15 homology; C.C., coiled-coil; A, acidic motif; P.P., polyproline; PIP2, phosphatidylinositol 4,5-bisphosphate binding motif; ER, End3 repeat. Two-hybrid interactions were quantified by measuring β-galactosidase activity and expressed in Miller units with SD. Results are the average of three independent transformants. auto, autoactivity; n.d., not determined. (B) In vitro binding assays between Scd5p and Pan1p. Different fragments of His-tagged Scd5p (arrows) were bead immobilized and incubated with GST-LR2 (arrowhead) or GST, respectively. The precipitates, as well as portions of GST and GST-LR2 (loaded on lanes 1–2 as positive controls), were separated by gel electrophoresis and sequentially immunoblotted with anti-GST and anti-His antibodies. A cross-reaction between anti-GST antibody and His-SCD5-C was observed (lanes 7 and 8). (C) Coimmunoprecipitation of Scd5p with Pan1p. Yeast extracts prepared from YMC448 (lanes 1, 5, and 7), YMC441 (lanes 2, 6, and 8), or YMC471 (lanes 3–4 and 9), were subjected to anti-Myc or anti-HA immunoprecipitation followed by gel electrophoresis, and then they were immunoblotted with anti-Myc and anti-HA antibodies, as indicated. (D) Coimmunoprecipitation of Scd5p with End3p. Yeast extracts prepared from YMC448 (lanes 8 and 13), YMC474 containing pMyc-End3-314 (lane 2), YMC474 containing pMyc-End3ΔN-314 (lane 3), YMC474 containing pMyc-End3ΔC-314 (lane 4), YMC476 containing pMyc-End3ΔN-314 (lanes 5, 9, and 14), YMC476 containing pMyc-End3ΔC-314 (lanes 7, 11, and 16), and YMC476 containing pMyc-End3ΔNΔC-314 (lanes 6, 10, and 15), and YMC476 containing pMyc-End3ΔNΔC-314 (lanes 6, 10, and 15), were subjected to anti-Myc or anti-HA immunoprecipitation followed by gel electrophoresis, and then they were immunoblotted with anti-Myc and anti-HA antibodies, as indicated. (E) Genetic interactions of Scd5p with Pan1p and End3p. Yeast strains transformed with pRS314 or pPAN1-314 (top) and YMC472 transformed with pRS314 or pEND3-314 (bottom) were tested for growth on synthetic complete medium containing 1 mg/ml 5-fluoroorotic acid at 25°C for 5 d.
Huang et al., 2003), the elevation of Pan1p phosphorylation level in this mutant is likely resulted from a deficient access of Glc7p to Pan1p.

End3p may also have a crucial role in Pan1p dephosphorylation, because it has a stronger Scd5p-interacting activity than Pan1p in two-hybrid assays (Figure 1A). More importantly, the interaction between End3p and Pan1p has recently been found to be unaffected by the presence of the Prk1 kinase (Toshima et al., 2007). This is consistent with our domain mapping data showing the region of Pan1p involved in binding with End3p to be located immediately after the second EH domain, and hence outside of the Prk1 phosphorylation region (data not shown). End3p, therefore, could be a preferred entry point for Scd5p-Glc7p to gain access to phosphorylated Pan1p. The level of Pan1p phosphorylation in the end3 mutant was indeed markedly elevated (Figure 2C). However, it remained unclear whether this was due to a loss of access for Scd5p-Glc7p, or an overphosphorylation by Prk1p, as the binding of End3p could help shield Pan1p from Prk1p phosphorylation (Zeng and Cai, 1999), or both. To answer this question, we examined different truncation mutants of end3. End3p interacts with Scd5p and Pan1p through its N- and C-terminal regions respectively, and removal of either will disable its function as a bridging agent between Scd5p and Pan1p, resulting in an increase in the level of Pan1p phosphorylation. This was indeed the case (Figure 2D). Both End3ΔN and End3ΔC mutant proteins complemented the temperature sensitivity of the end3 null mutant (data not shown), indicating that they were expressed functionally in the cells. Since the C-terminal region of End3p was still capable of binding Pan1p (Tang et al., 1997), the role of End3p is probably more in promoting Pan1p dephosphorylation than protecting it from phosphorylation.

Dephosphorylation of Pan1p by Glc7p In Vitro and In Vivo

Next, we sought to test whether Glc7p could directly dephosphorylate Pan1p in vitro. To obtain the phosphorylated

G. Zeng et al.
Figure 3. Dephosphorylation of Pan1p and Scd5p by Glc7p in vitro and in vivo. (A) In vitro phosphorylation of Pan1p by Prk1p. Purified GST-LR1 was incubated with immunoprecipitated HA-Prk1p (lanes 3 and 4) or HA-Prk1<sup>D158Y</sup>p (lane 2) in the presence (lanes 2 and 4) or absence (lane 3) of nonradioactive ATP at 25°C for 3 h. The mixture was separated by gel electrophoresis and immunoblotted with anti-PThr and then anti-GST antibodies. (B and F) In vitro dephosphorylation of Pan1p (B) and Scd5p (F) by Glc7p. Immunoprecipitated Glc7-HA with or without the I-2 treatment was incubated with in vitro phosphorylated GST-LR1 or GST-SCD5 at 37°C. Samples were taken at 20-min intervals, electrophoresed, and immunoblotted with anti-PThr and anti-GST antibodies. (C and G) Measurement of Glc7p phosphatase activity on Pan1p (C) and Scd5p (G). The phosphorylation level of GST-LR1 (Figure 3B) and GST-SCD5 (Figure 3F) at each time point was measured by densitometer and normalized against its protein amount. (D) Pan1p phosphorylation level in <i>glc7-td</i> mutant. YMC484 (lanes 4–6) and YMC485 (lanes 1–3) cells were allowed to grow to the log phase at 25°C and then shifted to 37°C. Samples were taken at 0, 30, and 60 min, and Pan1p-Myc was immunoprecipitated, electrophoresed, and immunoblotted with anti-PThr and then anti-Myc antibodies. Total proteins of each sample also were extracted by TCA precipitation and subjected to Western analysis to assay the expression level of Glc7p. (E) Pan1p phosphorylation level in <i>glc7-T152K</i> mutant. Pan1-Myc was immunoprecipitated from YMC441 (lane 1) and YMC482 (lanes 2–3) cells grown at 30°C. The immunoprecipitates in lane 3 were preincubated with CIP for 30 min before loading. (H) Phosphorylation of Scd5p...
form of Pan1p, GST-LR1, which contains multiple Prk1p phosphorylation sites (Zeng and Cai, 1999), was subjected to phosphorylation by Prk1p in the presence of nonradioactive ATP. As shown in Figure 3A, GST-LR1 could be detected by the anti-PThr antibody after an incubation with immunoprecipitated HA-Prk1p, and no signal was detected if a kinase-dead mutant, Prk1D105R (Zeng and Cai, 1999), was used. The phosphorylated GST-LR1 thus obtained gradually reduced its phosphorylation level during incubation with immunoprecipitated Glc7-HA to, for example, ~20% after 80 min (Figure 3, B and C). This reduction in GST-LR1 phosphorylation was effectively blocked by I-2 (Figure 3, B and C), a phosphatase inhibitor previously demonstrated to be specific for type I protein phosphatases (Cohen et al., 1989). Similar results were also obtained using phosphorylated GST-LR2 as the substrate in the assay (data not shown). These experiments confirmed that Glc7p could execute dephosphorylation of Pan1p in vitro.

We further examined the effect of Glc7p on the phosphorylation level of Pan1p in vivo. To create a conditional mutant of Glc7p, a GLC7 construct containing a heat-inducible degradation signal fused to the N terminus (He and Moore, 2005), along with an HA epitope, was obtained and used to replace the wild type locus in our test strain. The mutant thus created, glc7-tld, displayed a temperature-dependent decline in the amount of Glc7p protein (Figure 3D). The depletion of Glc7p was not observed if the degradation signal in the mutant was disrupted (Figure 3D, glc7-ntd). It is evident that the phosphorylation level of Pan1p in glc7-tld cells increased in accordance with the depletion of Glc7p, while remained unchanged if Glc7p was not degraded (Figure 3D and Supplemental Figure S1A). Moreover, another mutant, glc7-T152K, previously characterized to be defective in association with Scd5p (Tu et al., 1996), also exhibited an elevated level of Pan1p phosphorylation (Figure 3E and Supplemental Figure S1B). These results allowed us to conclude that Glc7p, through its binding to Scd5p, is required for dephosphorylation of Pan1p in vivo.

Dephosphorylation of Scd5p by Glc7p In Vitro and In Vivo

Similar to Pan1p, Scd5p is also phosphorylated by Prk1p, and the phosphorylation sites are adjacent to the Glc7p binding motif (Henry et al., 2003; Huang et al., 2003). Therefore, Scd5p may also undergo dephosphorylation by Glc7p. To evaluate the effect of Glc7p on the phosphorylation status of Scd5p, we applied the similar assays as described above to Scd5p. First, purified GST-SCD5 was made suitable for in vitro phosphatase assay by incubation with nonradioactive ATP and Prk1p, and the resultant phosphorylated GST-SCD5 was assayed with immunoprecipitated Glc7-HA. As shown in Figure 3, Glc7p could efficiently dephosphorylate GST-SCD5 in vitro, and again, a preincubation with I-2 completely inhibited the Glc7p activity (Figure 3, F and G). Next, the phosphorylation status of Scd5p in vivo was analyzed. The steady-state phosphorylation level of Scd5p in wild-type cells turned out to be below detection, owing likely to the complex formation with Glc7p in vivo (Chang et al., 2002). It was possible to increase the basal phosphorylation level of Scd5p by adding phosphatase inhibitors to the culture medium (Figure 3H). Deletion of PRK1 reduced Scd5p phosphorylation to 36% of the wild-type level under the same condition, whereas disruption of Prk1p phosphorylation sites (SCD5AAA) extinguished the signal (Figure 3H and Supplemental SIC), indicating that Scd5p was phosphorylated mainly by Prk1p, although other Prk1p-like kinases (Ark1p or Akl1p) could also phosphorylate Scd5p in the absence of Prk1p (Henry et al., 2003). Similarly to Pan1p, the level of phosphorylated Scd5p was substantially increased in the glc7-tld mutant after the phosphatase was depleted by temperature shift (Figure 3I and Supplemental Figure S1D). Moreover, the increase in the Scd5p phosphorylation level was also observed in cells containing either the T152K mutation on Glc7p or PBM2Δ on Scd5p (Figure 3J and Supplemental SIE), both of which are known to impair the interaction between Glc7p and Scd5p (Tu et al., 1996; Chang et al., 2002; Huang et al., 2003). These results demonstrated that the activity of Glc7p and its interaction with Scd5p are required for maintaining Scd5p in an un- or underphosphorylated state in vivo.

Partial Suppression of glc7-tld by prk1Δ

The involvement of Glc7p in dephosphorylation of Pan1p and Scd5p in vivo suggests that it plays an antagonistic role to the Prk1p kinase in the regulation of actin and endocytosis. To investigate the functional relationship between the phosphatase and the kinase, we examined the actin cytoskeleton organization in the respective mutants. As shown in Figure 4A, the glc7-tld mutant exhibited a grossly distorted actin cytoskeleton after a prolonged incubation at 37°C (6 h) to diminish the Glc7p protein content (Figure 4A, center). The majority of glc7-tld cells contained large and aberrant actin aggregates similar to those found in the pan1-4, scd5-1, and end3Δ mutants (Benedetti et al., 1994; Tang and Cai, 1996; Huang et al., 2003). In contrast, the control cells (glc7-ntd) maintained a normal pattern of actin cytoskeleton after the same treatment (Figure 4A, top), suggesting that the actin abnormalities in glc7-tld cells were caused by the phosphatase depletion. Remarkably, the actin defects in glc7-tld cells could be largely reversed by deletion of PRK1, because the prk1Δ glc7-tld cells generally showed normal-looking cortical patches, although some aberrant actin aggregates were still visible in a minority of the double mutant cells (Figure 4A, bottom). This result is consistent with the proposed functional antagonism between Glc7p and Prk1p in the regulation of actin cytoskeleton organization.

To see whether depletion of Glc7p also results in an endocytic defect, we carried out the uracil permease internalization assay, by which the endocytic defect could be measured quantitatively. The yeast uracil permease is a plasma membrane protein that is internalized for degradation via the endocytic pathway (Volland et al., 1994). As shown in Figure 4B, after 6 h of incubation at 37°C, the control cells of glc7-ntd internalized uracil permease normally and exhibited a rapid decrease in uracil uptake upon addition of
Suppression of Transport of \([3H]\)uracil into yeast cells was used as a measurement permease was initiated by the addition of cycloheximide (CHX). Cultures were brought back to 25°C, and the internalization of uracil in independent assays. (C) The growth of the initial time point. The results shown are the averages of two surface. Uracil uptake is plotted as the percentage activity relative to for the relative amount of uracil permease retained on the cell.

Figure 4A. Actin staining of glc7-ntd (YMC499), glc7-td (YMC500), and prk1Δ glc7-td (YMC501) cells. The strains were grown at 25°C to early log phase and incubated at 37°C for 6 h followed by fixation and staining with rhodamine-phalloidin. Bar, 4 μm. (B) Uracil permease internalization assay of glc7-ntd, glc7-td, prk1Δ glc7-td, and scd5-1 cells. The cells were transformed with pFUR4-424, and the transformants were grown at 25°C to OD₆₀₀ of 0.2-0.3. After incubated at 37°C for 6 h, cultures were brought back to 25°C, and the internalization of uracil permease was initiated by the addition of cycloheximide (CHX). Transport of \([H]\)uracil into yeast cells was used as a measurement for the relative amount of uracil permease retained on the cell surface. Uracil uptake is plotted as the percentage activity relative to the initial time point. The results shown are the averages of two independent assays. (C) The growth of glc7-ntd, glc7-td, and prk1Δ cell cultures were transformed with pFUR4-424, and the transformants were grown at 25°C to OD₆₀₀ of 0.2–0.3. After incubated at 37°C for 6 h, cultures were brought back to 25°C, and the internalization of uracil permease was initiated by the addition of cycloheximide (CHX).

The mild retardation in the loss of uracil uptake activity of glc7-ntd cells was clearly attributable to the diminished phosphatase activity, because it could be corrected by removal of the Prk1p kinase. The prk1Δ glc7-td double mutant cells internalized uracil permease as efficiently as glc7-ntd cells (Figure 4B). The mild rather than severe defect in endocytosis in the glc7-td cells, as opposed to the actin defects described above, is likely due to the fact that the deletion of the phosphatase under these conditions was hardly complete, and the residual amount of Glc7p remained detectable even after 6 h of incubation at 37°C (data not shown). These observations suggest that the aspect of function of the Pan1p-associated complex with respect to actin is more sensitive to the Prk1p phosphorylation than that with respect to endocytosis.

Furthermore, we found that the growth defect of glc7-td mutant could also be partially suppressed by prk1Δ. As shown in Figure 4C, the glc7-td cells grew well at 25°C but could not sustain the growth at either 33 or 37°C. Deletion of the PRK1 gene rendered the glc7-td cells capable of much better growth at these temperatures, even though they were eventually still inviable (Figure 4C). This partial suppression of glc7-td by prk1Δ was not due to any recovery of the Glc7p protein level, because the level of the phosphatase remained same in both glc7-td and prk1Δ glc7-td cells at 25°C, and it was diminished at a similar rate at 33 and 37°C (Figure 4D).

**Regulation of Scd5p-dependent Interactions by Prk1p Phosphorylation**

To gain more insight into the mechanism of dephosphorylation of Pan1p by Glc7p, we examined the interactions among Scd5p, Pan1p, End3p, and Glc7p in more detail. The conspicuously low in vivo phosphorylation level of Scd5p led us to consider the prospect that Scd5p must be present in an unphosphorylated form to fulfill its role as a phosphatase-targeting factor. It is worth noting that both Pan1p and End3p bind to the region of Scd5p where the three Prk1p phosphorylation sites are located (Figure 1). Prk1p phosphorylation has been shown to disfavor the multivalent interaction between Pan1p and other proteins (Zeng et al., 2001; Toshima et al., 2005). It is therefore important to find out whether the interactions of Scd5p with Pan1p, End3p, and Glc7p could be affected by Prk1p phosphorylation on Scd5p. Mutations were generated at the Prk1p phosphorylation sites of Scd5p to convert the three T residues into either A or E to mimic unphosphorylated and phosphorylated states of Scd5p, respectively. Two-hybrid assays revealed that these mutations affected the interactions of Scd5p with both Pan1p and End3p, but not with Glc7p (Figure 5, A–C). Compared with the wild-type, Scd5AAA

**glc7-td strains at different temperatures.** Cells were grown at 25°C to OD₆₀₀ of 0.5, and 10⁻⁶ serial dilutions of each culture were dropped onto CuSO₄-containing plates for incubation at the indicated temperatures for 2 d. (D) Comparison of the Glc7p protein level in glc7-td, and prk1Δ glc7-td strains. Cells were grown at 25°C to log phase and then shifted to either 33°C or 37°C for 1 h. Samples were taken at 0 and 60 min. Total proteins of each sample were prepared and subjected to Western analysis.
exhibited a stronger binding activity with both Pan1p and End3p (increased to 196% for Pan1p and 125% for End3p), whereas Scd5EEEp showed a considerably reduced binding, to 60%, with the two proteins (Figure 5, A and B). These results are of a good indication that the interactions of Scd5p with Pan1p and End3p are both negatively regulated by phosphorylation on Scd5p. The binding with phosphatase Glc7p, in contrast, remained at a similar level irrespective of mutations (Figure 5C), suggesting that the Scd5p–Glc7p interaction was not affected by Scd5p phosphorylation. We also examined the effect of Pan1p phosphorylation on its interaction with Scd5p using an in vitro binding assay. As shown in Figure 5D, the bead-bound His-SCD5-N2 was able to bind unphosphorylated GST-LR2 efficiently, but failed to do the same to phosphorylated GST-LR2P, whose association with Pan1p in vivo is not affected by phosphorylation of Pan1p (Toshima et al., 2007).

The observation that phosphorylation of Scd5p impairs its interaction with the Pan1p–End3p complex suggests that the dissociation of Scd5p from Pan1p-End3p is one of the consequences after Prk1p phosphorylation. If this is the case, it will be interesting to find out whether the phosphorylation and dissociation of Scd5p is required for, or independent of, the phosphorylation of Pan1p. To address this question, we overexpressed the PRK1 gene from the GAL1 promoter to enhance the Pan1p phosphorylation in vivo in the nonphosphorylatable scd5AAA mutant. As shown in Figure 5, E and F, the level of Pan1p phosphorylation in the wild-type cells was elevated 3.5-fold after 4 h of kinase overexpression. In contrast, the scd5AAA mutant, whose steady-state Pan1p phosphorylation level was already lower before the induction, only showed a slight increase under the same conditions (Figure 5, E and F). This result suggests that phosphorylation of Pan1p has to be preceded by the phosphorylation of Scd5p in vivo. Scd5p, therefore, may serve as a switch in the event of Pan1p phosphorylation in addition to its role as a phosphatase-targeting factor.

Live Cell Imaging of Pan1p, Scd5p, and Prk1p
To correlate the above-mentioned studies with the patterns of protein localization, we examined the real-time protein
dynamics of Pan1p, Scd5p, and Prk1p in live cells. In agreement with previous studies (Kaksonen et al., 2003, 2005; Sun et al., 2006), the Pan1-GFP–labeled cortical patches showed restricted movement and exhibited a patch lifetime of ~28 s (Figure 6, A and B, and Supplemental Movie 1). The Scd5-GFP patches were virtually of the same pattern of dynamics with a life span of ~27 s (Figure 6, A and B, and Supplemental Movie 2). The Prk1-GFP patches, on the other hand, had a shorter lifetime (~17 s) and higher motility (Figure 6, A and B, and Supplemental Movie 3). Two-color imaging revealed that Scd5-GFP was recruited to cortical patches at the same point as Pan1-CFP, and the two proteins colocalized with each other on most of the patches, and disappeared together in the end (Figure 6, C–E, and Supplemental Movie 4). In contrast, Prk1-GFP joined the Pan1-CFP patches at a much later stage, just a few seconds before Pan1-CFP began to fade. It coexisted with Pan1-CFP transiently, and vanished shortly after the disappearance of Pan1-CFP (Figure 6, F–H, and Supplemental Movie 5). These data support the earlier suggestion that the Pan1p and Scd5p are components of the same endocytic complex, and phosphorylation by Prk1p leads to their dissociation. The disappearance of Pan1p and Scd5p from cortical patches shortly after the

Figure 6. Live cell imaging of Pan1p, Scd5p, and Prk1p patches. (A) Single frames from movies of wild-type cells expressing Pan1-GFP (YMC494), Scd5-GFP (YMC496), and Prk1-GFP (YMC495). Movies were taken with the frame intervals of 2 s for Pan1-GFP and Prk1-GFP, and 4 s for Scd5-GFP. See Supplemental Movies S1–S3. (B) Lifetimes ± SD of Pan1-GFP, Scd5-GFP, and Prk1-GFP patches in wild-type cells. n ≥ 30 for each protein. (C and F) Single frames from two-color movies of live cells expressing Pan1-CFP and Scd5-GFP (YMC498; C) or Pan1-CFP and Prk1-GFP (YMC497; F). Movies were taken with frame intervals of 5 s for C and 3 s for F. See Supplemental Movies S4 and S5. (D and G) Time series from two-color movies showing the dynamic localization of Scd5-GFP (D) and Prk1-GFP (G) in reference to Pan1-CFP. More than 20 patches were examined in each case, and ~90% of them had similar behaviors as shown. (E and H) Plots of patch fluorescence intensities in Figure 5D (E) and Figure 5G (H) with time. (I) Lifetimes ± SD of Pan1-GFP patches in YMC494 cells transformed with pRS424, pEND3-424, or pSCD5-424. n ≥ 30 patches. (J) Lifetime of Pan1-GFP patches in wild-type (YMC494) and scd5 AAA mutant (YMC499) cells. n ≥ 30 patches. All scale bars are 4 μm.
arrival of Prk1p suggests that these two proteins are diffused into cytosol after they are phosphorylated by Prk1p. Consistently, overproduction of End3p and Scd5p, both of which caused marked reduction in phosphorylation of Pan1p by Prk1p (data not shown), dramatically prolonged the patch life of Pan1-GFP to 40 and 45 s, respectively (Figure 6I). The life span of Pan1-GFP patches was also significantly extended in the scd5AAA mutant (Figure 6I), in agreement with the above-mentioned observation that Pan1p phosphorylation was hindered in this mutant. In summary, the behaviors of the fluorescent Scd5p and Prk1p in live cells correlate very well with their roles in phosphorylation of Pan1p.

DISCUSSION

Phosphorylation and dephosphorylation are the most commonly occurring scheme of regulation in a wide variety of biological processes. Pathways like endocytosis, which require recurrent assembly and disassembly of multiprotein complexes at restricted cellular locations, are perhaps best suited to this type of regulation. Our current understanding of phosphoregulation of actin-coupled endocytosis in yeast has been limited to some of the actions of the Prk1p family of kinases. In this report, we reveal that the type 1 phosphatase Glc7p also takes part in this regulatory circuit, counteracting Prk1p in modifying the phosphorylation status, and hence the activity, of the important endocytic protein Pan1p.

Glc7p as the Phosphatase for Pan1p Dephosphorylation

Glc7p is an essential type 1 protein phosphatase localized in the nucleus and the cytosol, and known to participate in diverse cellular processes (Stark, 1996). Although it has recently been implicated in the regulation of actin organization and endocytosis by the finding that it interacts with Scd5p (Chang et al., 2002), its exact function in this aspect has remained unexplored. We demonstrated that Glc7p is responsible for modulating the in vivo phosphorylation status of at least two regulatory targets of the Prk1p kinase, i.e., Pan1p and Scd5p, both of which are essential for normal actin organization and endocytosis. The findings that pin-point Glc7p as the phosphatase to dephosphorylate Pan1p include the phosphorylation level of Pan1p being markedly elevated in Glc7p-depleted cells, the phosphatase being able to dephosphorylate Pan1p in vitro, and the interaction with Scd5p being critical for maintaining the normal steady-state phosphorylation level of Pan1p. Although Glc7p was found to interact with Pan1p directly in a report of a large-scale two-hybrid screen (Uetz et al., 2000), we have failed to confirm such interaction in our assays (data not shown).

The role of Glc7p in dephosphorylation of Pan1p is also supported by the functional antagonism between Glc7p and Prk1p. The defects resulted from phosphatase depletion in glc7-td cells including actin aggregation, mild delay in endocytosis of uracil permease, and cell lethality, could all be at least partially alleviated by deletion of the kinase gene. As Glc7p is empowered with multifarious cellular functions, it is expected that removal of just one of its antagonistic kinases with a specialized function in actin and endocytosis will not be able to fully compensate for the loss of the phosphatase activity. Nevertheless, the much improved survival of the prk1Δ glc7-td cells at high temperatures does indicate that the actin- and endocytosis-related dephosphorylation is an important part of the overall Glc7p cellular functions.

Glc7p should be the major protein phosphatase for Pan1p dephosphorylation, because the Pan1p phosphorylation level was not altered in the mutants defective in Ppz1p and Ppz2p (our unpublished data), two other phosphatases that share some overlapping functions with Glc7p (Venturi et al., 2000).

The Roles of Scd5p in Phosphoregulation of Pan1p

The key to the mechanism of Pan1p dephosphorylation by Glc7p is the Glc7p targeting factor Scd5p. On one hand, it links the phosphatase to Pan1p by interacting with both Pan1p and End3p. Our results are consistent with the conclusion that Scd5p is essential for maintaining the physiological phosphorylation level of Pan1p by acting as a phosphatase-targeting factor. Scd5p, on the other hand, may also act as a switch in the phosphoregulation of Pan1p, because the phosphorylation status of Pan1p in vivo is dependent on that of Scd5p. When phosphorylation takes place, Prk1p may be obliged to phosphorylate Scd5p first before it can phosphorylate Pan1p, as suggested from the experiment that the nonphosphorylatable Scd5p (Scd5AAA) blocked phosphorylation of Pan1p by overexpressed Prk1p. This mechanism can be interpreted as to ensure the release of Scd5p from the Pan1p–End3p complex in the event of phosphorylation, as the dissociation of Scd5p from the Pan1p–End3p complex is likely required for its binding with Glc7p in the cytosol. The free and phosphorylated Scd5p, in effect, serves as a feedback signal for the process of dephosphorylation.

Therefore, the role of Scd5p in the phosphoregulation of Pan1p during endocytosis is twofold: the patch-localized Scd5p, in complex with Pan1p–End3p, functions as a switch in the event of phosphorylation, whereas the cytoplasmic Scd5p, in complex with Glc7p, acts as a phosphatase-targeting factor in the event of dephosphorylation. Of the two roles of Scd5p, only the latter is essential. It has been reported that the cortical localization of Scd5p is not required for its act and endocytic functions, since these functions remained apparently normal in a cytoplasmically localized Scd5p mutant (Chang et al., 2006). This mutant, nevertheless, is expected to be functional as the phosphatase-targeting factor, because it is still able to interact with End3p in the cytosol. The scd5Δ338 mutant, on the other hand, is temperature sensitive and defective in endocytosis, because the truncated protein is predominantly localized to the nucleus (Henry et al., 2002; Chang et al., 2006). Consistent with our findings, this mutation could be suppressed by deletion of the kinase gene PRK1 (Henry et al., 2003).

Cycle of Phosphoregulation of Pan1p during Endocytosis

With the identification of the role of the Scd5p–Glc7p phosphatase complex in dephosphorylation of Pan1p, combined with previous discoveries made by other groups, it is now possible to propose a model of a complete cycle of the phosphoregulation of Pan1p during endocytosis (Figure 7): 1) Assembly of the endocytic complex at the site of endocytosis, in which Pan1p is complexed with End3p and Scd5p, together with other coat proteins and present in an unphosphorylated state. 2) Initiation of actin polymerization by the Arp2/3p complex to induce membrane invagination. 3) Vesicle scission and inward movement propelled by actin polymerization. 4) Phosphorylation of Scd5p and Pan1p by Prk1p to disassemble the coat complex. Incorporation of the kinase into the complex at this stage involves Abp1p (Fazi et al., 2002), which appears on the Pan1p patches at about the same time as Prk1p (Kaksonen et al., 2003). The kinase starts by phosphorylating and dissociating Scd5p from Pan1p and End3p, followed by extensive phosphorylation of Pan1p. This presumably will cause a distortion/disrup-
Dephosphorylation in Actin and Endocytosis

Sla1p has also been reported to interact with Glc7p (Tu et al., 1996; Venturi et al., 2000), although we failed to confirm such interaction in our yeast two-hybrid assays. Whether Sla1p could act as a phosphatase-targeting factor and at the same time a substrate, similarly to Scd5p, remains to be clarified.

In mammalian cells, kinases with similar function and property as that of Prk1p have also been identified. The adaptor-associated kinase AAK1 negatively regulates clathrin-mediated endocytosis by phosphorylating the µ2 subunit of the AP2 adaptor protein complex (Conner and Schmid, 2002; Ricotta et al., 2002). Interestingly, AAK1 shares with Prk1p not only the extensive kinase sequence homology but also the similar recognition motif (Conner and Schmid, 2002; Ricotta et al., 2002). Another mammalian homologue of Prk1p, GAK, also exhibits a similar specificity on the µ2 subunit and plays a regulatory role in endocytosis (Zhang et al., 2005). AP2 is known to associate with Eps15, the mammalian counterpart of Pan1p, and such interaction is required for endocytosis (Benmerah et al., 1995, 1998). Unlike Pan1p, however, Eps15 contains no AAK1/GAK recognition motifs, and it has yet to be ascertained whether it is subjected to regulation by these kinases. It also remains unknown how the dephosphorylation of AP2 is carried out. The mammalian PP1 has not been demonstrated to be involved in clathrin-mediated endocytosis, despite its vast variety of other known cellular functions through >50 established or putative binding subunits (Cohen, 2002).

Therefore, it will be of great interest to identify novel targeting factor(s) of PP1, or other type of phosphatases, with a specific function in clathrin-mediated endocytosis in mammalian cells.

ACKNOWLEDGMENTS

We are grateful to Claire Moore (Tufts University) for providing the pGlc7-degron plasmid (pGlc7n-td-306) and to David Drubin (University of California, Berkeley) for the Pan1-GFP harboring strain DDDY3063. We also thank Desmond Uvarajoo and Yongjia Xiao for helping microscopy and imaging data analysis. We thank Jun Wang and Chong Yun Won for general technical assistance. This work was supported by the Agency for Science, Technology & Research of Singapore. M.C. holds an adjunct faculty appointment from the Department of Biochemistry, Faculty of Medicine, National University of Singapore.

REFERENCES


