Protein Phosphatase-1 Binding to Scd5p is Important for Regulation of Actin Organization and Endocytosis in Yeast

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Running title:
Scd5p-PP1 binding regulates actin function and endocytosis
SUMMARY

SCD5, an essential gene, encodes a protein important for endocytosis and actin organization in yeast. Previous two hybrid screens showed that Scd5p interacts with Glc7p, a yeast Ser/Thr-specific protein phosphatase-1 (PP1) which participates in a variety of cellular processes. PP1 substrate specificity in vivo is regulated by association with different regulatory or targeting subunits, many of which have a consensus PP1 binding site (V/I-x-F, with a basic residue at the –1 or –2 position). Scd5p contains two of these potential PP1 binding motifs: KVDF (amino acids 240-243) and KKVRF (amino acids 272-276). Deletion analysis mapped the PP1-binding domain to a region of Scd5p containing these motifs. Therefore, the consequence of mutating these two potential PP1-binding sites was examined. While mutation of KVDF had no effect, alteration of KKVRF dramatically reduced Scd5p interaction with Glc7p and resulted in temperature sensitive growth. Furthermore, this mutation caused defects in fluid-phase and receptor-mediated endocytosis and actin organization. Overexpression of GLC7 suppressed the temperature sensitive growth of the KKVRF mutant and partially rescued the actin organization phenotype. These results provide evidence that Scd5p is a PP1 targeting subunit for regulation of actin organization and endocytosis or that Scd5p is a PP1 substrate, which regulates Scd5p’s function in these processes.
INTRODUCTION

Protein phosphatase-1 (PP1\(^1\)) is one of the major Ser/Thr protein phosphatases of eukaryotic cells (1). It is highly conserved and is involved in a wide variety of cellular processes, including glycogen and protein synthesis, cell cycle regulation, muscle contraction, and calcium transport (1,2). While the catalytic enzyme has broad substrate specificities in vitro, many lines of evidence have shown that regulatory or targeting subunits direct PP1 to physiological substrates or subcellular locations to perform specific dephosphorylation in vivo (2,3).

In *Saccharomyces cerevisiae* the PP1 catalytic subunit is encoded by GLC7, and, not surprisingly because of its diverse functions, GLC7 is an essential gene (4,5). Many Glc7p-regulatory proteins have been identified in yeast. These include: Gac1p, a homologue of the mammalian G subunit involved in activation of glycogen synthase for glycogen accumulation (6,7); Reg1p, which binds PP1 to regulate glucose repression (8,9); Red1p and Gip1p, which play roles in meiosis and sporulation (10-12); Egp1p/Sds22p, which targets PP1 to substrates whose dephosphorylation is required for completion of mitosis (13-15); and Glc8p, a homologue of the mammalian PP1 inhibitor-2 (16,17).

Several mammalian and yeast PP1-binding proteins contain a consensus PP1-binding motif (V/I-x-F) with a basic residue at the −1 or −2 position (R/K-V/I-x-F or R/K-x-V/I-x-F) through which the regulatory subunits interact with the PP1 catalytic subunit (18,19). This structural motif forms an extended conformation and binds to a hydrophobic groove (a regulatory subunit-binding site) on the PP1 protein surface, which is on the opposite side from the catalytic site (18). The presence of a consensus PP1-binding motif also implies that the interaction of many different regulatory subunits with PP1 is mutually exclusive and
competitive. However, the V/I-x-F motif exists in more than 10% of all known proteins. Most of these are unlikely to interact with PP1, so the importance of this motif for PP1 binding and function has required confirmation by mutational analysis of the V/I-x-F sequence, such as has been done for yeast Gac1p (20) and Reg1p (21), and mammalian PTG (22), NIPP1 (23), and Nrb I (24).

A number of protein interaction screens have identified additional PP1 binding proteins in yeast (10,25,26). Among these is Scd5p, an 872 amino acid protein, which we have recently shown plays a critical role in actin cytoskeleton organization and endocytosis (27). Interestingly, Scd5p contains two potential PP1-binding motifs: KVDF and KKVRF (amino acids 240-243 and 272-276, respectively). In this report we map the Glc7p/PP1 binding site to a region of Scd5p containing these motifs. Mutational analysis indicates that the second putative PP1-binding site (KKVRF) is crucial for Glc7p interaction. Furthermore, this PP1-binding site mutation causes temperature sensitive growth and defects in actin cytoskeleton organization and endocytosis. These studies indicate that PP1-binding is necessary for the function of Scd5p and suggest that Scd5p may target PP1 to substrates that must be dephosphorylated for regulation of actin organization and endocytosis.
EXPERIMENTAL PROCEDURES

Strains, media, and growth conditions—Strains used in this study are listed in Table 1. Yeast were grown in 1% yeast extract, 2% peptone with 2% glucose (YEPD) or other sugars as indicated or selective dropout medium as described previously (28). Synthetic medium containing 5-fluoroorotic acid (5-FOA) was prepared as described in (29). Yeast transformation was performed using the lithium acetate method (30).

Plasmid constructions—pJSC2 (pGAL1:GST-SCD5) and pJSC12 (pGAL1:GST-scd5-Δ338) were generated in two steps. First a 0.68 kb BamH1-Sal1 fragment (containing the SCD5 start codon at the BamH1 site) was moved from pKRH20 (pGBD-scd5-Δ645; (27)) into the yeast glutathione S-transferase (GST) expression vector, pTB338 (CEN, LEU2, GAL1-GST from Michael Hall), to generate an in frame fusion between GST and SCD5 codons 1-227 under control of the GAL1 promoter (pJSC1). Then the remainder of SCD5 or scd5-Δ338 was reconstituted by inserting a 5.4 kb Xba1-Sph1 fragment (contains codons 188 through the end of the ORF) into pJSC1 to generate pJSC2 and pJSC12, respectively. A 516 bp BamH1-Pst1 PCR fragment (codons 828-1000 from BMS1) was amplified with primers 5’-CCAGGATCCGAAGACATCGTTGG-3’ and 5’-GAACTGCAGTATCACTCATTAGGATTTTATCC-3’. This was subcloned in frame with GST coding sequences in pTB338 to generate pDG101 (pGAL:GST-bms1(828-1000)). PP1 binding site mutant plasmids, pJSC8 (scd5-PP1∆1), pJSC9 (scd5-PP1∆2) and pJSC10 (scd5-PP1∆1∆2) were constructed by megaprimer mutagenesis (31). Primer 5’-ACTGGTGATCAAAAGGCCGCTGCTGACTCATTTGCTTCA-3’ was used for changing codons for KVDF (residues 240-243) to KAAA (∆1). Codons for KKVR (residues 272-276) were changed to AKAAA (∆2) using the primer 5’-ACTGGTGATCAAAAGGCCGCTGCTGACTCATTTGCTTCA-3’.
GTTATATGCTCTGAAGCGGCCGCCTTCGCACTCTTAAAATTC-3’. PCR fragments containing mutations were then gap-repaired into pCC545 (from Clarence Chan), which contains \( SCD5 \) in pRS315 (\( CEN \), \( LEU2 \)) (32). Regions amplified were verified by DNA sequencing. For two-hybrid analysis, pJSC18 (pGBD-\( scd5-\Delta 338-PP1\Delta 1 \)), pJSC19 (pGBD-\( scd5-\Delta 338-PP1\Delta 2 \)), and pJSC20 (pGBD-\( scd5-\Delta 338-PP1\Delta 1\Delta 2 \)) were generated by gap-repair of the Xba1-SexA1 cut pNT1 (pGBD-\( scd5-\Delta 338 \); (27)) with fragments containing the mutant sequences from pJSC8, pJSC9, and pJSC10, respectively. pJSC6 (pGBD-\( scd5-\Delta 523 \)) and pKRH20 (pGBD-\( scd5-\Delta 645 \); (27)) contain mutant alleles of Scd5p with stop codons at codons 350 and 228, respectively. All two hybrid Gal4 DNA binding domain (GBD) clones were made in pGBDU (2\( \mu \), \( URA3 \)) (33). pGAD is a 2\( \mu \), \( LEU2 \) two hybrid activation domain plasmid (33). pGAD-\( GLC7 \) (9) and pGAD-\( GAC1 \) (6) contain Glc7p and Gac1p fused to the Gal4 activation domain in a \( LEU2 \) prey plasmid, respectively. YCp-HA-\( GLC7 \) is described in (34). YE24 (35), YEps\( C D5 \) (36), and YEps\( GLC7 \) (from Michael Stark) are 2\( \mu \), \( URA3 \) multicopy plasmids.

**Affinity isolation of GST-Scd5p and GST-\( scd5-\Delta 338 \) fusion proteins**—pJSC2 (GST-\( SCD5 \)), pJSC12 (GST-\( scd5-\Delta 338 \)), or pDG101 (GST-\( bmsl-898-100 \)) were transformed into a protease-deficient strain, BJ2168, containing YCp-HA-\( GLC7 \). Transformants were grown in 5 ml synthetic complete (SC) medium lacking uracil and leucine and containing 1.95% galactose and 0.05% glucose at 30˚C overnight and then diluted and grown in 50 ml of the same medium until cultures reached 1.0 x 10^7 cells/ml. Approximately 50 x 10^7 cells were harvested, resuspended in 1.2 ml cold lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1% NP40, 1 mM PMSF plus a protease inhibitor cocktail (37)) and lysed by glass beads in a Braun homogenizer for 3 min. Cell extracts were transferred to an ice-cold centrifuge tube and spun down at 20,800 x g for 30 min at 4˚C. The supernatant was saved and protein concentration was
determined using the Bio-Rad protein determination kit. Extracts (420 µg) diluted to 5 ml with lysis buffer were incubated with 100 µl of a 75% (vol/vol) slurry of glutathione-Sepharose 4B beads (Pharmacia) for 3 h at 4°C with gentle mixing. The Sepharose beads were pelleted and washed four times with 1 ml lysis buffer and one time with 1 ml lysis buffer without detergent. The final bead pellets were resuspended in 100 µl 2x SDS-PAGE sample buffer and boiled for 5 min. Samples (30 µg) of crude extract were also diluted to 100 µl with sample buffer and boiled for 5 min. Crude extract and equal volumes of GST affinity-purified samples were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. Western blots were probed with affinity-purified rabbit antibodies to GST (1:1500, Santa Cruz Biotechnology) or anti-HA rat monoclonal antibodies 3F10 (1:750, Boehringer Mannheim). These were detected by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000, Sigma) or rabbit anti-rat IgG (1:10,000, Sigma), respectively, and enhanced chemiluminescence (Amersham).

**Immunoblotting of Scd5p**—Yeast cells were grown to log phase in 5 ml synthetic selective medium at 25°C. Approximately 2 x 10^7 cells were harvested, washed with dH2O, and resuspended in 0.1 ml 2x SDS-PAGE sample buffer containing 1 mM PMSF plus a protease inhibitor cocktail (37). After lysis with glass beads, extracts (22 µl) were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. Equal loading of protein was confirmed by amido black staining of transfers. Blots were probed with rabbit anti-Scd5p antibodies (1:6,000, from Clarence Chan) and developed as described above for GST-pulldowns.

**Two-hybrid analysis**—Bait plasmids (URA3, pGBD-fusions) were transformed into YPJ96-4A and prey plasmids (LEU2, pGAD-fusions) were transformed into SL3004. YPJ96-4A and SL3004 containing these plasmids were mated pairwise on a YEPD plate for 2 days and the diploids containing both plasmids were selected on C-LEU-URA. Cells were grown in liquid C-
LEU-URA medium to a concentration of $0.5 \times 10^7$ cells/ml and equal numbers of cells were spotted on C-LEU-URA and C-LEU-URA-ADE to monitor expression of the $GAL2-ADE2$ reporter gene. β-galactosidase assays were performed as described previously (38,39). Miller units of β-galactosidase activity were calculated from three independent cultures (40).

*Endocytosis assays*—The lucifer yellow (LY) uptake assay was performed essentially as described previously (41). Cells were grown at 25°C in YEPD to early log phase and cultures were kept at 25°C or pre-shifted to 37°C for 15 min before addition of LY (Sigma). After incubation for 1 h at 25°C or 37°C, cells were washed and observed immediately with a Zeiss Axioplan-2 fluorescence microscope equipped with DIC optics as described by (27). The $^{35}$S-alpha-factor internalization assay was performed as described previously (42).

*Actin staining*—Yeast cells were grown in YEPD to early log phase, fixed with formaldehyde, and stained with Alexa-568-phalloidin (Molecular Probes) as described previously (43). Immunofluorescence was performed as described in (44). Cells were fixed using a methanol/acetone dehydration method and stained with anti-actin guinea pig antibodies (1:2,000; (45)), followed by incubation with Alexa-594-conjugated goat anti-guinea pig IgG (1:800; Molecular Probes). Microscopy was performed using a LSM 410 confocal microscope (Fig. 6, phalloidin staining) or a Zeiss Axioplan-2 microscope (Fig. 6, anti-actin staining; Fig. 7) as described previously (27).
RESULTS

Scd5p Interacts with Glc7p In Vivo—Previous two-hybrid screens using Glc7p as a bait identified Scd5p as an interacting protein (10,25), suggesting that Scd5p might be a regulatory subunit of yeast PP1. To test whether Scd5p interacts with Glc7p in vivo, we expressed GST fusions of Scd5p and Scd5p-∆338 (a C-terminal truncation of 338 amino acids) in wild type yeast also expressing an HA-tagged version of Glc7p. Cells were grown on galactose to induce expression of the GST fusion proteins, which were then affinity-purified from protein extracts using glutathione-Sepharose (Fig. 1). HA-tagged Glc7p corresponding to a band of 37 kDa co-purified with both GST-Scd5p and GST-Scd5p-∆338 (Fig. 1, middle panel, lanes 2 and 3), but not with a nonrelevant control GST fusion (Fig. 1, middle panel, lane 1). These data confirm the previous two-hybrid interaction of Glc7p with Scd5p, as well as a recent large scale proteomic complex analysis showing Scd5p copurification with yeast PP1 (26). In addition, our results show that the Glc7p interaction in vivo is dependent upon the first 534 amino acids of Scd5p.

A Putative PP1-binding Motif is Required for Interaction of Scd5p with Glc7p—To further define the region of Scd5p mediating interaction with PP1, a series of truncation constructs comprising C-terminal deletions of Scd5p were fused to the DNA binding domain of Gal4p (GBD) (see Fig. 2A) and tested by two hybrid analysis for interaction with Glc7p fused to the Gal4p activation domain (GAD). Scd5p-∆338 (residues 1-534) and Scd5p-∆523 (residues 1-349) strongly interacted with Glc7p, whereas Scd5p-∆645 (residues 1-227) could no longer bind yeast PP1 (Fig. 2B). A control prey, GAD-Gac1p showed no detectable interaction with any of Scd5p-truncation constructs. These two-hybrid results map the PP1 binding site to a region between amino acids 228 and 349 of Scd5p.
A number of mammalian and yeast PP1-binding proteins contain a consensus PP1-binding motif (R/K-V/I-x-F or R/K-x-V/I-x-F) through which they interact with the PP1 catalytic subunit, although the latter motif is most common in yeast (18,19). Interestingly, Scd5p contains two potential PP1 binding sequences: KVDF (amino acids 240-243) and KKVRF (amino acids 272-276) which are located in the Glc7p-binding region defined by the two hybrid Scd5p truncation analysis.

To determine whether the putative PP1-binding motifs in Scd5p are important for binding Glc7p, we mutated these sites singly or in combination, changing KVDF(240-243) to KAAA (Δ1) and KKVRF (272-276) to AKAAA (Δ2). These mutations were introduced into the pGBD-scd5-Δ338 plasmid to test for two hybrid interactions with GAD-Glc7p (Fig. 3). We found that Scd5p-Δ338-PP1Δ1 containing KVDF(240-243)KAAA showed wild-type interaction, whereas interaction of Glc7p with Scd5p-Δ338-PP1Δ2 containing KKVRF(272-276)AKAAA was reduced approximately 10 fold. Mutation of both sites (Δ1Δ2) completely disrupted Glc7p binding (Fig. 3A and 3B). These results indicated that the second PP1 binding motif is most important for association of Scd5p with Glc7p, but the first site might contribute to the interaction.

The Second PP1 Binding Motif (KKVRF) is Crucial for Scd5p Function—To determine whether these potential PP1-binding motifs are important for Scd5p function, mutations in each or both of the sites were introduced into the full length SCD5 expressed from its own promoter on a CEN LEU2 plasmid and tested for complementation of the scd5 null allele. YCp plasmids carrying scd5-PP1 binding site mutations were transformed into SL4121, which carries a scd5-Δ::TRP1 disruption but is viable because of the presence of SCD5 on a URA3 2µ plasmid. Following plasmid shuffling on 5-FOA to force loss of the URA3 plasmid, the YCp, LEU2
plasmids became the sole source of Scd5 protein. While cells expressing only \textit{scd5-PP1Δ1} grew normally at 25°C and 37°C, \textit{scd5-PP1Δ2} cells were temperature sensitive for growth at 37°C (Fig. 4A). Moreover, the double mutant (Δ1Δ2) failed to complement a \textit{scd5} null mutation at any temperature (Fig. 4A).

Immunoblot analysis showed that Scd5p-PP1Δ1 was expressed at levels identical to the wild type protein and Scd5p-PP1Δ2 was even slightly more abundant than normal Scd5p (Fig. 4B). We were unable to examine expression of the double mutant protein directly, since the strain is inviable. However, preliminary studies from expression of the double mutant protein in the presence of a functional copy of \textit{SCD5} suggest that the double mutation causes protein instability\textsuperscript{2}. This would explain the lack of interaction in the two hybrid analysis and the lack of complementation of the null mutation at all temperatures. Thus, we conclude that the second PP1 binding site is most important for interaction of Glc7p with Scd5p and for Scd5p’s function.

\textit{The scd5-PP1Δ2 Mutation Causes Defects in Endocytosis and Actin Organization}—Recently our laboratory has shown that Scd5p plays a critical role in endocytosis and actin cytoskeleton organization (27). Scd5p also colocalizes with cortical actin patches and physically or genetically interacts with a number of cortical actin patch components, many of which are also important for actin organization and endocytosis (27). Therefore, we examined whether the Scd5p-PP1 binding site mutations affect these processes.

We first assayed endocytosis of lucifer yellow (LY), a fluid phase marker which accumulates in the vacuole upon internalization. Both \textit{scd5-PP1Δ1} and \textit{scd5-PP1Δ2} cells internalized the dye efficiently at 25°C (Fig. 5A). When cells were shifted to 37°C for 15 min, \textit{scd5-PP1Δ1} cells still showed normal uptake, but endocytosis of LY by \textit{scd5-PP1Δ2} cells was completely blocked (Fig. 5A). Receptor-mediated endocytosis of radiolabelled α-factor by its
receptor, Ste2p, was normal in cells with *scd5-PP1Δ1* at 24˚C and 37˚C. In contrast, α-factor uptake was impaired in *scd5-PP1Δ2* cells at 24˚C and 37˚C, although the defect at 24˚C was less severe (Fig. 5B). Thus, the *scd5-PP1Δ2* mutation causes defects in both fluid-phase and receptor-mediated endocytosis.

We next examined the effect of the Scd5p-PP1-binding motif mutations on the actin cytoskeleton by staining cells with Alexa–564 phalloidin to visualize assembled filamentous (F) actin. In yeast, actin cables, which are bundles of actin filaments, extend from the mother cell into the bud for polarized delivery of organelles and other materials into the growing daughter cell. Cables reorient towards the mother/daughter cell neck during cytokinesis. Cortical patches appear at the site of bud emergence and then localize primarily to the growing bud. Late in the cell cycle they concentrate at the bud neck for septum formation and cytokinesis.

Cells expressing *scd5-PP1-Δ1* exhibited normal actin structures throughout the cell cycle at 25˚C and 37˚C, similar to those observed in wild type cells (Fig. 6). The *scd5-PP1-Δ2* strain grown at 25˚C also displayed a relatively normal polarized distribution of cortical actin patches and cables. In contrast, actin structures were aberrant in *scd5-PP1-Δ2* cells shifted to 37˚C (Fig. 6). In small- and medium-budded cells many actin patches were polarized to the daughter cells, but significant depolarization to mother cells was also observed. In addition, actin cables were often misoriented and much thinner or hardly visible, as compared to those seen in the wild-type and *scd5-PP1-Δ1* cells. In large-budded cells increased numbers of actin patches were seen distributed throughout cells with *scd5-PP1-Δ2*. These patches were often much larger than normal and an actin ring at the bud neck was rarely observed. In addition, actin cables were barely visible.
Cells were also stained with anti-actin antibodies, which allows visualization of both F- and G-actin. The *scd5-PP1-Δ2* cells often (up to 11% of cells at 37°C) contained G-actin bars, which are thought to be aggregates of monomeric or disassembled actin (Fig. 6), whereas none of the wild-type and *scd5-PP1-Δ1* cells displayed the actin bar phenotype (data not shown). The overall size of *scd5-PP1-Δ2* cells throughout the cell cycle was also larger than normal at both 25°C and 37°C (see Figs. 6, 7), consistent with effects on the actin cytoskeleton and polarized growth.

*Overexpression of GLC7 Suppresses scd5-PP1Δ2*—Often when phenotypes in yeast are caused by a mutation that affects the productive interaction of two proteins, the defects can be suppressed by overexpression of the interacting partner. Thus we tested whether overexpression of PP1 can suppress the phenotypes caused by the *scd5-PP1Δ2* mutation. We found *scd5-PP1Δ2* cells carrying a vector control (YEp24) were inviable at the restrictive temperature of 37°C, whereas growth was rescued when cells were transformed with *GLC7* expressed from a multicopy plasmid (Fig. 7A). While LY uptake was still defective in the *scd5-PP1-Δ2* mutant overexpressing *GLC7* (not shown), the actin organization phenotype was also partially suppressed by YEp*GLC7* (Fig. 7B). Nearly 50% of mutant cells overexpressing *GLC7* displayed highly polarized actin patches and normally oriented actin cables, similar to wild-type cells with YEp*GLC7* (Fig. 7B). In addition, significant numbers of large budded cells had actin at the site of cytokinesis in the *scd5-PP1-Δ2* strain overexpressing *GLC7*. These overexpression studies provide further evidence that Scd5p interaction with PP1 is important for Scd5p function.
DISCUSSION

Scd5p interacts with the yeast PP1 homologue, Glc7p, through its PP1-binding motif—Mutational analysis of a number of PP1 regulatory proteins has shown that alteration of the highly conserved V/I and/or F residues in their PP1 binding motif disrupts or severely weakens interaction with PP1 and prevents specific functional targeting of the phosphatase (20-24). In this paper we provide evidence that Scd5p interacts with the GLC7-encoded PP1 in vivo and that, like other PP1 regulatory proteins, a V/I-x-F binding motif in Scd5p is important for PP1 association and the biological function of Scd5p.

The Glc7p-interacting region on Scd5p was mapped to a region containing two potential PP1-binding motifs, KVDF (240-243) and KKVRF (272-276). Another potential PP1 binding motif is found at residues 29-33 (PPVSF) (19), but an N-terminal fragment of Scd5p (amino acids 1-227) could not associate with Glc7p, indicating that this is not a Glc7p binding site. Mutational analysis further revealed that the KKVRF signal is crucial for interaction of Scd5p with Glc7p, while the KVDF motif is not likely to bind to the PP1 hydrophobic channel in the C-terminus of PP1. Although KVDF contains the highly conserved V/I and F residues, it contains an acidic residue within the core sequence. Peptide panning experiments to identify PP1 binding sequences found that the most frequent residues in the second position of the V/I-x-F motif were His or Arg (19). In addition, previous work has shown that phosphorylation of serine at this site prevents binding of G_M to mammalian PPlc (46). This is also predicted by the crystal structure (18), suggesting that Asp and possibly Glu are not favorable either. However, a KVEF motif found in mammalian aurora kinase appears to be important for PP1 binding, suggesting glutamic acid would be compatible in some contexts (47). Nevertheless our data suggest that the Asp residue in the KVDF sequence of Scd5p prevents interaction with residues comprising the
hydrophobic pocket of Glc7p, or, alternatively, the KVDF sequence may be inaccessible on the protein surface of Scd5p for binding to the Glc7p hydrophobic channel.

While alteration of the KKVRF motif in Scd5p severely impaired interaction with Glc7p, Scd5p appears to have additional contacts on the Glc7p protein surface, since changing KKVRF to AKAAA did not completely disrupt binding to Glc7p or Scd5p function at the permissive temperature and resulted in a temperature sensitive growth phenotype. However, the mutation did cause some phenotypic consequences at 24°C, as reduced α-factor uptake and the actin bar phenotype were already observed at this temperature, even though the protein was completely stable. We note that some PP1-binding proteins, such as M110 (48,49), AKAP220 (50), and PP1 inhibitors (51-53), bind PP1 through V/I-x-F motifs, but also make additional contacts on the protein surface of PP1, which increase stability of the association or regulate activity and specificity. In addition, in vivo other proteins might form a complex with Scd5p and PP1 to stabilize their interaction. Although we found that Scd5p mutated at both the KVDF and KKVRF motifs was not able to rescue the \textit{scd5Δ} mutation, the lack of physical interaction with Glc7p by two hybrid analysis or functional complementation by the double mutant protein most likely results from instability of the protein².

Role of Scd5p-PP1 interaction in endocytosis and actin cytoskeleton organization — In addition to causing temperature sensitive growth, \textit{scd5-PP1Δ2} dramatically blocked both receptor-mediated and fluid-phase endocytosis and had a significant effect on actin organization. Therefore, the critical role of Scd5p in endocytosis and actin organization most likely requires its interaction with and targeting of Glc7p. Supporting this, we found that overexpression of Glc7p could partially rescue Scd5p phenotypes resulting from mutation of the KKVRF PP1 binding motif. In addition, previous studies have shown that some \textit{glc7} mutations cause a cortical actin
defect (54). A role for PP1 in regulating actin organization has been demonstrated in animal cells as well. For example, neurabin I (Nrb I) binds F-actin and recruits PP1 to control cell morphology (24,55). When the PP1-binding motif in Nrb I was altered, the mutant protein failed to bind PP1 and to induce filopodia formation (24).

PP1 regulatory proteins target PP1 to distinct subcellular locations or promote association with specific substrates to reverse or counter the consequences of regulatory phosphorylation by kinases (1-3). One example in yeast is the regulation of a glycogen synthase by a Gac1p/PP1 holoenzyme in glycogen synthesis. Gac1p targets PP1 to a glycogen synthase (Gsy2p) and reverses phosphorylation and inactivation of Gsy2p by cyclin-dependent kinase Pho85p (7,56-59). By analogy, Scd5p may direct PP1 to dephosphorylate actin patch-associated components for regulation of actin organization and endocytosis. Our recent work (27) has shown that Scd5p partially colocalizes with actin patches and physically associates with actin patch proteins, such as Sla2p/End4p and Rvs167p (60,61), both of which are also important for endocytosis (62,63). In addition, Sla2p’s cortical localization is dependent upon Scd5p (27).

Recent studies indicate that yeast actin-regulating kinases (ARKs), including Prk1p and Ark1p, play a role in actin organization and endocytosis (64,65). Prk1p and Ark1p localize to cortical actin patches (64,65) and likely promote actin patch disassembly by phosphorylating target proteins, since many cortical actin-associated proteins collapse into large F-actin aggregates in arkl prkl double mutant cells (64). Prk1p negatively regulates the interaction of actin patch/endocytic factors Pan1p, End3p and Sla1p by phosphorylating a repeated motif (L/IxxQxTG) found in Pan1p and Sla1p (65,66). Prk1p also regulates Pan1p interacting proteins, Ent1/2p, which contain Pan1p-consensus sequences (67). As a PP1 targeting subunit, Scd5p/Glc7p could counter kinases, such as ARKs, that act on cortical actin components.
Interestingly, Scd5p also contains sites that could be targets of regulatory phosphorylation, including a central repeat that has motifs related to those found in other Prk1p substrates. Thus, Scd5p, itself, could be regulated by ARKs or other kinases. This raises the possibility that Scd5p is also a PP1 substrate, and PP1’s binding reverses regulatory phosphorylation on Scd5p. This would be similar to the role of Glc7p binding to Reg1p in association with Snf1 kinase for glucose repression. Glc7p dephosphorylates both Snf1p and Reg1p during a regulatory cascade that turns off Snf1p in high glucose conditions (68). Further studies are under way to determine whether PP1 regulates Scd5p directly and what are the targets of PP1 in association with Scd5p for regulation of actin organization and endocytosis in yeast.
ACKNOWLEDGMENTS

We thank Marian Carlson, Michael Stark, Michael Hall, Elizabeth Jones, David Botstein, and Clarence Chan who provided plasmids, strains, and antibodies. We also thank Kelly Tatchell for materials and helpful discussions. This work was supported by NIH grant R01 GM55796 (S.K.L.) and the Deutsche Forschungsgemeinschaft, project SFB 352 (M.G.). K.R.H. was supported by an individual National Research Service Award Minority Predoctoral Fellowship (F31 GM20082).
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FOOTNOTES

1 The abbreviations used are: PP1, type I Ser/Thr protein phosphatase; YEPD, yeast extract peptone dextrose; 5-FOA, 5-fluoroorotic acid; GST, glutathione S-transferase; HA, hemagglutinin; GBD, Gal4p binding domain; GAD, Gal4p activation domain; PMSF, phenylmethyl sulphonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; LEU, leucine; URA, uracil; ADE, adenine; LY, Lucifer yellow; ARK, actin regulating kinase.

2 J. Chang, unpublished observations.
FIGURE LEGENDS

FIG. 1. Scd5p associates with Glc7p in vivo. pJSC2 (GST-SCD5), pJSC12 (GST-scd5-Δ338), and a negative control, pDG101 (GST-bms1-828-1000) were transformed into a protease deficient strain, BJ2168, expressing HA-Glc7p. GST fusion proteins were induced for expression and affinity-purified from protein extracts using glutathione-Sepharose 4B beads. Samples were subjected to SDS-PAGE and immunoblot analysis, probing with anti-GST antibodies to detect GST-fusions (panel A) or anti-HA monoclonal antibodies to detect HA-Glc7p (panel B). Blots of equal amounts of whole cell extracts were probed with anti-HA antibodies to confirm equal expression of HA-Glc7p (panel C). The ~70-kDa bands detected by anti-GST antibodies (panel A; lanes 2 and 3) are likely degradation products of GST-Scd5 fusion proteins.

FIG. 2. Two putative PP1-binding motifs in Scd5p are located within the region that binds Glc7p. (A) Schematic diagram of Scd5p. Scd5p (872 amino acids) contains two putative PP1-binding motifs (KVDF and KKVRF), three repeats of 20 amino acids (gray boxes), and nine repeats of 12 amino acids (black boxes). Arrowheads indicate the positions of stop codons that generate Scd5p-C-terminal truncations of 645, 523, and 338 amino acids used in two hybrid analysis shown in panel B. (B) Two-hybrid interaction of Scd5p truncation proteins with Glc7p. Yeast strain YPJ96-4 (MATα) was transformed with Gal4 binding domain (GBD) bait plasmids pKRH20 (GBD-scd5-Δ645), pJSC6 (GBD-scd5-Δ523), or pNT1 (GBD-scd5-Δ338) and SL3004 (MATα) was transformed with Gal4 activation domain (GAD) prey plasmids pGAD-GLC7 or pGAD-GAC1. Baits and preys were combined by the mating method described in Experimental Procedures and then diploids were spot plated on medium lacking leucine and uracil (C-LEU-
URA) to monitor growth or medium lacking leucine, uracil, and adenine (C-LEU-URA-ADE) to assay for two hybrid activation of the \textit{GAL2:ADE2} reporter. Plates were grown for 3 days at 30˚C. Note that full length \textit{SCD5} fused to GBD self-activated, so it was not included in panel B.

**FIG. 3.** \textbf{Effect of Scd5p PP1-binding motif mutations on the interaction of Scd5p with Glc7p.} (A) YPJ96-4 (MATa) was transformed with pGBDU (empty vector), pNT1 (pGBD-scd5-Δ338), pJSC18 (pGBD-scd5-Δ338-PP1Δ1), pJSC19 (pGBD-scd5-Δ338-PP1Δ2), or pJSC20 (pGBD-scd5-Δ338-PP1Δ1Δ2). These were mated to SL3004 (MATα) transformed with pGAD (empty vector) or pGAD-GLC7 and diploids were spotted and grown on selective medium as described in Fig. 2B. (B) Two-hybrid interactions shown in panel A were quantified by measuring β-galactosidase activity. Results are the average of three independent transformants expressed in Miller units ± standard deviation.

**FIG. 4.** \textbf{Test for rescue of scd5-Δ::TRP1 inviability by Scd5p containing PP1-binding motif mutations.} (A) SL4121 (scd5-Δ::TRP1 + YEpSCD5 (URA3 plasmid)) was transformed with the \textit{CEN LEU2} plasmids pCC545 (SCD5), pJSC8 (scd5-PP1Δ1), pJSC9 (scd5-PP1Δ2), or pJSC10 (scd5-PP1Δ1Δ2) and grown on synthetic complete medium lacking leucine and uracil (C-LEU-URA) or medium lacking leucine and containing 5-fluoroorotic acid (C-LEU+ 5-FOA) to select against the YEpSCD5 plasmid with the \textit{URA3} marker. Plates were grown for three days at 25˚C or 37˚C as indicated. (B) Western blot analysis of PP1 binding motif mutants. Equal amounts of proteins from cell extracts of wild type (SL4415), \textit{scd5-PP1Δ1} (SL4416), and \textit{scd5-PP1Δ2} (SL4417) were analyzed by immunoblotting with anti-Scd5p antibodies.
FIG. 5. Fluid phase- and receptor-mediated endocytosis are defective in *scd5-PP1Δ2* cells at 37°C. (A) Lucifer yellow (LY) accumulation. Wild type (SL4415), *scd5-PP1Δ1* (SL4416), and *scd5-PP1Δ2* (SL4417) cells were preincubated at 25°C or 37°C for 15 min prior to addition of LY. Cells were further incubated at 25°C or 37°C for 1 h and immediately visualized using DIC (right panels) and fluorescence (left panels) microscopy. (B) Radiolabeled α-factor internalization. Wild type (SL4436; circles), *scd5-PP1Δ1* (SL4437; squares), and *scd5-PP1Δ2* (SL4438; triangles) cells were preincubated at 24°C or 37°C for 15 min. 35S-labeled-α-factor was added and samples were collected at indicated time points for determination of percent of cell-associated α-factor internalized. The results shown are the averages of three independent experiments.

FIG. 6. Actin organization is defective in *scd5-PP1Δ2* cells. Wild type (SL4418), *scd5-PP1Δ1* (SL4419), and *scd5-PP1Δ2* (SL4420) cells were grown to log-phase in YEPD at 25°C and preincubated at 25°C or 37°C for 3.5 h before fixation. Upper panels: Filamentous actin was stained with Alexa-568 phalloidin and visualized using a LSM 410 confocal microscope. Lower panels: Both F- and G-actins in *scd5-PP1Δ2* (SL4420) cells were visualized by indirect immunofluorescence, staining with anti-actin antibodies.

FIG. 7. Overexpression of *GLC7* suppresses the *scd5-PP1Δ2* growth and actin phenotypes. (A) Suppression of *scd5-PP1Δ2* temperature sensitive growth. SL4417 (*scd5-Δ::TRP1 + pJSC9 (scd5-PP1Δ2)) was transformed with YEp24 or YEpGLC7 and streaked for growth on YEPD for 3 days at 25°C or 37°C. (B) Partial rescue of the *scd5-PP1Δ2* actin organization phenotype. SL4420 transformed with YEp24 or YEpGLC7 or a wild type strain (SL4418) transformed with
YEpGLC7 were grown to log-phase in selective medium at 25°C and shifted to 37°C for 3.5h before fixation. Filamentous actin was stained with Alexa-568 phalloidin and visualized by fluorescence microscopy. Bar graph shows the percent of cells with normal actin organization in SL4420 cells (scd5-PP1Δ2) containing YEp24 or YEpGLC7.
| Strain    | Genotype                                                                 | Source/Reference |
|-----------|---------------------------------------------------------------------------|------------------|
| BJ2168    | MATα leu2 ura3-52 trp1 pep4-3 prb1-1122 prcl-407                           | E. Jones         |
| SL3004    | MATα ura3 his3 ade2-101 lys2-801 trp1-901 TYR1 gal4 gal80 leu2             | (27)             |
| SL4121    | MATα leu2 trp1 his3-Δ200 ura3-52 scd5-Δ::TRP1 scd1-v YEpSCD5               | This study       |
| SL4415    | MATα leu2 trp1 his3-Δ200 ura3-52 scd5-Δ::TRP1 scd1-v pCC545 [CEN, LEU2, SCD5] | This study       |
| SL4416    | MATα leu2 trp1 his3-Δ200 ura3-52 scd5-Δ::TRP1 scd1-v pJSC8 [CEN, LEU2, scd5-PP1Δ1] | This study       |
| SL4417    | MATα leu2 trp1 his3-Δ200 ura3-52 scd5-Δ::TRP1 scd1-v pJSC9 [CEN, LEU2, scd5-PP1Δ2] | This study       |
| SL4418    | MATα/ MATα leu2/ leu2 trp1/trp1 his3-Δ200/his3-Δ200 ura3-52/ura3-52 scd1-v/scd1-v scd5-Δ::TRP1/scd5-Δ::TRP1 pCC545 | This study       |
| SL4419    | MATα/ MATα leu2/ leu2 trp1/trp1 his3-Δ200/his3-Δ200 ura3-52/ura3-52 scd1-v/scd1-v scd5-Δ::TRP1/scd5-Δ::TRP1 pJSC8 | This study       |
| SL4420    | MATα/ MATα leu2/ leu2 trp1/trp1 his3-Δ200/his3-Δ200 ura3-52/ura3-52 scd1-v/scd1-v scd5-Δ::TRP1/scd5-Δ::TRP1 pJSC9 | This study       |
| SL4436    | MATα leu2 trp1 his3-Δ200 ura3-52 scd5-Δ::TRP1 scd1-v bar1-Δ::HIS-MX4 pCC545 | This study       |
| SL4437    | MATα leu2 trp1 his3-Δ200 ura3-52 scd5-Δ::TRP1 scd1-v bar1-Δ::HIS-MX4 pJSC8  | This study       |
| SL4438    | MATα leu2 trp1 his3-Δ200 ura3-52 scd5-Δ::TRP1 scd1-v bar1-Δ::HIS-MX4 pJSC9  | This study       |
| YPJ96-4   | MATα ade2 gal4-Δ gal80-Δ GAL2-ADE2 his3-Δ200 leu2-3,112 LYS::GAL1-HIS3 met2::GAL7-lacZ trp1-901 ura3-52 | (33)             |
Fig. 2

A

\[ \Delta 645 \ (1-227) \ \Delta 523 \ (1-349) \ \Delta 338 \ (1-534) \]

\[ \text{Scd5p} \]

\[ \uparrow \uparrow \]

KVDF KKVRF

B

| pGBD-scd5 truncations | \( \Delta 338 \) | \( \Delta 523 \) | \( \Delta 645 \) | \( \Delta 338 \) | \( \Delta 523 \) | \( \Delta 645 \) |
|-----------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| pGAD-\text{GAC1}     | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| pGAD-\text{GLC7}     | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |

C-LEU-URA  C-LEU-URA-ADE
Fig. 3

A

|        | pGBD | pGBD-scd5-Δ338 |
|--------|------|----------------|
|        | WT   | Δ1             |
|        | Δ2   | Δ1Δ2           |

**pGAD**

**pGAD-GLC7**

C-LEU-URA-ADE

**pGAD**

**pGAD-GLC7**

C-LEU-URA

B

**Beta-gal activity**

|        | vector | WT | Δ1 | Δ2 | Δ1Δ2 |
|--------|--------|----|----|----|------|
| **Beta-gal activity** | 0     | 20 | 15 | 15 | 0    |
Fig. 6

Phalloidin

WT

\( \Delta 1 \)

\( \Delta 2 \)

anti-actin

\( \Delta 2 \)

\( 25^\circ C \)  
\( 37^\circ C \)
Protein Phosphatase-1 binding to Scd5p is important for regulation of actin organization and endocytosis in Yeast
Ji Suk Chang, Kenneth Henry, Bianka L. Wolf, Maribel Geli and Sandra K. Lemmon

J. Biol. Chem. published online September 27, 2002

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