Negative Regulation of Yeast Eps15-like Arp2/3 Complex Activator, Pan1p, by the Hip1R-related Protein, Sla2p, during Endocytosis▣ⓥ

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Control of actin assembly nucleated by the Arp2/3 complex plays a crucial role during budding yeast endocytosis. The yeast Eps15-related Arp2/3 complex activator, Pan1p, is essential for endocytic internalization and proper actin organization. Pan1p activity is negatively regulated by Prk1 kinase phosphorylation after endocytic internalization. Phosphorylated Pan1p is probably then dephosphorylated in the cytosol. Pan1p is recruited to endocytic sites ~25 s before initiation of actin polymerization, suggesting that its Arp2/3 complex activation activity is kept inactive during early stages of endocytosis by a yet-to-be-identified mechanism. However, how Pan1p is maintained in an inactive state is not clear. Using tandem affinity purification–tagged Pan1p, we identified End3p as a stoichiometric component of the Pan1p complex, and Sla2p, a yeast Hip1R-related protein, as a novel binding partner of Pan1p. Interestingly, Sla2p specifically inhibited Pan1p Arp2/3 complex activation activity in vitro. The coiled-coil region of Sla2p was important for Pan1p inhibition, and a *pan1* partial loss-of-function mutant suppressed the temperature sensitivity, endocytic phenotypes, and actin phenotypes observed in *sla2ΔCC* mutant cells that lack the coiled-coil region. Overall, our results establish that Sla2p's regulation of Pan1p plays an important role in controlling Pan1p-stimulated actin polymerization during endocytosis.

INTRODUCTION

The actin cytoskeleton plays an important role in cellular processes including cell polarity development, cell migration, cytokinesis, and endocytosis. Endocytosis is critical for controlling nutrient uptake, plasma membrane homeostasis, synaptic machinery recycling, and signaling pathway regulation. In budding yeast, endocytosis requires dynamic interactions between the plasma membrane and the actin cytoskeleton (Engqvist-Goldstein and Drubin, 2003). In both yeast and mammals, a transient burst of actin polymerization at endocytic sites is essential for endocytic internalization (Merrifield *et al.,* 2002; Kaksonen *et al.,* 2003). Mutations in actin cytoskeletal genes, including the Arp2/3 complex, or treatment of yeast with the actin polymerization inhibitor, latrunculin A, block endocytic vesicle internalization (Ayscough *et al.,* 1997; Lappalainen and Drubin, 1997; Belmont

and Drubin, 1998; Martin *et al.,* 2005). Therefore, actin polymerization is thought to provide force for plasma membrane invagination, vesicle scission, and/or movement into the cytoplasm (Munn, 2001; Engqvist-Goldstein and Drubin, 2003; Kaksonen *et al.,* 2006).

Recent analyses using yeast genetics and real-time, live-cell fluorescence microscopy demonstrated that proteins are recruited to endocytic sites, or cortical patches, in a highly predictable order. In yeast, clathrin is recruited to cortical patches ~1–2 min before vesicle internalization (Kaksonen *et al.,* 2005; Newpher *et al.,* 2005). Early endocytic components, including Pan1p (Eps15-related Arp2/3 complex activator), Las17p (yeast WASP), Sla1p (an adaptor for NPFx(1,2)D-mediated endocytosis) and Sla2p (yeast Hip1R), then appear at cortical patches and are later joined by actin, Abp1p and the Arp2/3 complex (Kaksonen *et al.,* 2003). Abp1p, a marker for actin polymerization in cortical patches, has a patch lifetime of 10–15 s, whereas early endocytic components, including the Arp2/3 activators Pan1p and Las17p, have lifetimes ranging from 30 to 40 s (Kaksonen *et al.,* 2003). Thus, the Arp2/3 activators Pan1p and Las17p arrive at endocytic sites ~25 s before actin assembly is initiated. How activities of Pan1p and Las17p are regulated and how the Arp2/3 complex is recruited to endocytic sites are not well understood. One possibility is that the Arp2/3 complex binding regions of these activators are masked by interactions with other proteins, which dissociate in response to yet-to-be-identified stimuli, triggering activation of these activators. Las17p's activity is inhibited by Sla1p and Bbc1p

Table 1. Yeast strains used in this study

Strain	Genotype	Source
DDY1810	*Mata leu2 ura3-52 lys2-801 trp1 prb1-1122 pep4-3 pre1-451*	Drubin lab
DDY2887	*Mata leu2 ura3-52 lys2-801 trp1 prb1-1122 pep4-3 pre1-451 PAN1-S TAP-TEV-ZZ::TRP1*	Drubin lab
DDY3326	*Mata his3-Δ200 leu2-3,112 ura3-52 SLA1-GFP::KanMX6 ABP1-RFP::HIS3*	This study
DDY3327	*Matα his3-Δ200 leu2-3, 112 ura3-52 pan1Δ::pan1Δ855-1480::LEU2 SLA1-GFP::KanMX6 ABP1-RFP::HIS3*	This study
DDY3328	*Matα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1Δ855-1480::LEU2 las17Δ547-633-13myc::HIS3 SLA1-GFP::KanMX6 ABP1-RFP::HIS3*	This study
DDY3329	*Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1ΔWA::LEU2*	This study
DDY3330	*Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 sla2Δ::LEU2*	This study
DDY3331	*Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 sla2Δ:LEU2 pan1Δ::pan1ΔWA::LEU*	This study
DDY3332	*Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 sla2Δ::sla2ΔCC::URA3 pan1Δ::PAN1::LEU*	This study
DDY3333	*Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 sla2Δ::sla2ΔCC::URA3 pan1Δ::pan1ΔWA::LEU*	This study
DDY3334	*Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 sla2Δ::sla2ΔCC::URA3 PAN1-GFP:KanMX6 ABP1-RFP::HIS3*	This study
DDY3335	*Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 sla2Δ::sla2ΔCC::URA3 pan1Δ::pan1ΔWA-GFP:KanMX6 ABP1-RFP::HIS3*	This study

All strains are derived from strain S288C.

(Rodal *et al.*, 2003), and the combination of *sla1Δ* and *bbc1Δ* mutations leads to formation of dramatic actin protrusions that associate with endocytic sites (Kaksonen *et al.*, 2005). Importantly, how Pan1p activity is inhibited at early stages of endocytosis is not known.

Pan1p is an essential protein that is involved in both the internalization step of endocytosis and the organization of the actin cytoskeleton (Tang and Cai, 1996; Wendland *et al.*, 1996). Pan1p interacts with many endocytic proteins, suggesting that Pan1p forms the core of an endocytic complex. The two Eps15 homology (EH) domains located at the N-terminal region of Pan1p bind to the NPF motifs of Ent1/2p and Yap180A/Bp, which also interact with clathrin (Wendland and Emr, 1998; Wendland *et al.*, 1999). The central coiled-coil region of Pan1p contains a WH2-like region, which binds to filamentous actin (Toshima *et al.*, 2005), and a C-terminal acidic region, which binds to the Arp2/3 complex (Duncan *et al.*, 2001). Pan1p also has two N-terminal LR (long repeat) regions, which contain 18 Prk1p consensus phosphorylation sequences ([L/I/V/M]xx[Q/N/T/S]xTG), and also interact with Sla1p and the EH domain protein End3p (Tang *et al.*, 1997; Zeng and Cai, 1999; Tang *et al.*, 2000; Huang *et al.*, 2003). The Ark1p/Prk1p kinases have been suggested to negatively regulate the Pan1p-Sla1p-End3p interaction and Pan1p's Arp2/3 complex activation activity (Zeng *et al.*, 2001; Toshima *et al.*, 2005). Inhibition of both Ark1p and Prk1p activities caused a severe endocytic defect and abnormal cytoplasmic actin aggregates, and a mutant in which the Prk1p-targeted threonines in Pan1p were mutated to alanines exhibited a phenotype similar to the *ark1Δ prk1Δ* mutant, demonstrating that Pan1p is a key Prk1p target in vivo (Cope *et al.*, 1999; Sekiya-Kawasaki *et al.*, 2003; Toshima *et al.*, 2005). Moreover, phosphorylation by Prk1p inhibits Pan1p's abilities to bind to F-actin and to activate the Arp2/3 complex (Toshima *et al.*, 2005). Therefore, these observations suggest that phosphoregulation of Pan1p by Prk1p is an important mechanism to shut off Arp2/3-mediated actin polymerization on endocytic vesicles. Importantly, when Pan1p is dephosphorylated and whether dephosphorylation of Pan1p causes initiation of actin assembly during endocytosis are not known.

Among early endocytic components, Sla2p arrives at cortical patches slightly earlier than Sla1p and is important for clathrin organization and for progression of early endocytic patches to the late stages of endocytosis (Newpher and Lemmon, 2006). Sla2p is a modular protein that is composed of an N-terminal AP180 N-terminal homology (ANTH) domain that interacts with phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2; Sun *et al.*, 2005], a coiled-coil region that interacts with clathrin (Henry *et al.*, 2002; Newpher and Lemmon, 2006), and a C-terminal talin-like actin-binding domain (Yang *et al.*, 1999). Sla2p appears to regulate actin polymerization because *sla2Δ* cells exhibit continuous actin assembly at nonmotile endocytic sites (Kaksonen *et al.*, 2003). An identical phenotype was also observed when Hip1R (mammalian Sla2p homologue) expression was lowered by RNA interference (Engqvist-Goldstein *et al.*, 2004), suggesting that Sla2p and Hip1R share a conserved function and that these proteins negatively regulate actin assembly associated with endocytic vesicle formation.

In this study, we identified Sla2p as a novel binding partner of Pan1p. Using biochemical and genetic analyses, we showed that Sla2p specifically inhibits Pan1p Arp2/3 complex activation activity and regulates actin assembly during endocytic vesicle internalization.

MATERIALS AND METHODS

Yeast Strains, Growth Conditions, and Plasmids

The yeast strains used in this study are listed in Table 1. Yeast strains were grown in standard rich media (YPD) or synthetic media (SM) supplemented with the appropriate amino acids. The carbon sources used were 2% glucose, 2% galactose, or 2% raffinose, depending on the experiment. C-terminal tandem affinity purification (TAP) or GFP tags were integrated at the endogenous loci of the gene using the method described in Longtine *et al.* (1998). The *sla2ΔCC* mutant was generated as follows: First, to create a *sla2* integration plasmid, the EcoRI fragment of the *SLA2* gene was cloned into pBluescript II SK, and the SmaI fragment of the *URA3* gene was inserted into the NruI site 150 base pairs downstream of the *SLA2* ORF (PBS-SLA2-URA3). *sla2ΔCC* (Δ1081-2187) was generated by ligating the NarI-digested PCR product amplified by primers 5′-CCGGCGCCGGCGCCCAGAAGTCCGGCTG-CATTTGTGCC-3′ and 5′-CCGGCGCCGGCGCCGAACCGTTATTGAACATAC-AATCTG-3′ using PBS-*SLA2*-URA3 as a template. To integrate the *sla2ΔCC* mutant at the endogenous locus, the plasmid was digested with EcoRI and transformed into *sla2Δ::LEU2/SLA2* diploid strains. Integrated *sla2ΔCC* mutants were selected on SC plates lacking uracil and sporulated to obtain *sla2ΔCC* mutants. The Yap180Ap and Ent2p expression plasmids were constructed by inserting their ORFs into BamHI- and NotI-digested pRS426 containing the GAL1-10 promoter, a TEV protease recognition site, and encoding the myc epitope (Rodal *et al.*, 2003). The Sla1p and End3p expression plasmids were constructed by inserting their ORFs into the BamHI site of pBS1479 (Rigaut *et al.*, 1999). The Prk1p and Pan1-TAP expression plasmids were constructed as described previously (Toshima *et al.*, 2005). Sla2p truncation constructs were generated as described previously (Yang *et al.*, 1999).
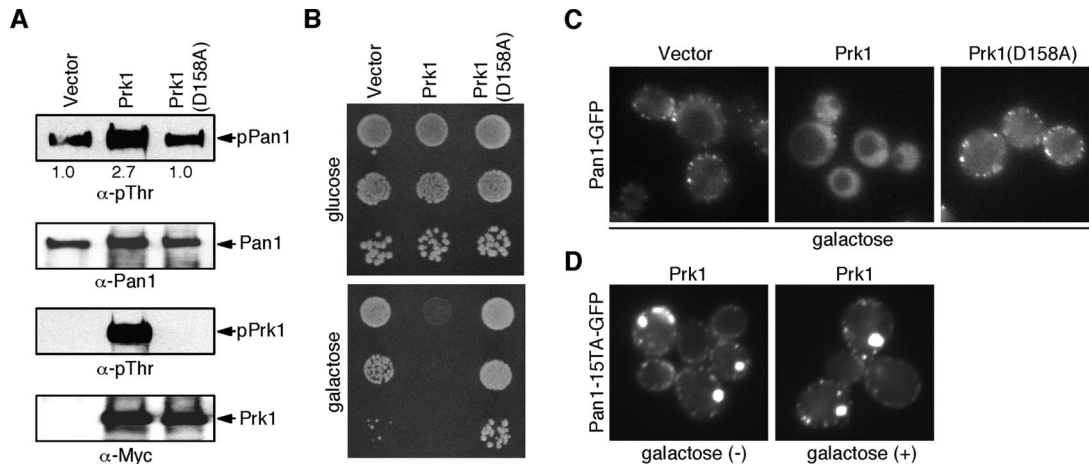
Figure 1. Localization of phosphorylated Pan1p in vivo. (A) In vivo phosphorylation of Pan1p by Prk1p. Pan1p were precipitated with TAP-tag from cells overexpressing vector alone, Prk1 or Prk1(D158A) and immunoblotted with anti-pThr or anti-Pan1p antibody. Relative phosphorylation levels are indicated under the top panel. Prk1 and Prk1(D158A) were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-pThr or anti-Myc antibody. (B) Viability of cells overexpressing Prk1 or Prk1(D158A). Cells were grown in YPD media overnight, and serial dilutions were spotted on glucose and galactose plates. (C) Localization of Pan1-GFP in cells overexpressing Prk1 or Prk1(D158A). The same cells as in B were grown to an OD600 of ~0.3 in selective synthetic medium with 2% raffinose, and then Prk1 or Prk1(D158A) was induced with 2% galactose for 120 min. (D) Localization of Pan1-15TA-GFP in cells overexpressing Prk1. Pan1-15TA cells were grown to an OD600 of ~0.3 in selective synthetic medium with 2% raffinose (left), and then Prk1 was induced with 2% galactose for 120 min (right).

Protein Expression and Purification

DDY1810 strains expressing the relevant plasmid were grown to saturation in 20 ml SD supplemented with all amino acids except uracil. This inoculum was added to 1.5 l of synthetic media with 2% wt/vol raffinose and dropout uracil and was grown to OD600 of 1.0. Protein expression was induced upon addition of 30 g bacto-peptone, 15 g yeast extract, and a final concentration of 2% wt/vol galactose for 8–12 h, as described (Toshima *et al.*, 2005). Cells were harvested by centrifugation, washed with 500 ml water, resuspended at a 1/5 wt/vol ratio of yeast to water, and drop-frozen in liquid N_2. Cells were lysed five times in a Waring blender with liquid N_2, as described (Goode *et al.*, 1999). TAP-tagged proteins (Pan1p, Sla1p, and End3p) were purified according to the TAP method as described elsewhere (Rigaut *et al.*, 1999). The Pan1p-End3p complex was purified from the DDY1810 strain overexpressing both End3p and TAP-tagged Pan1p, using the same method (Rigaut *et al.*, 1999). TEV-myc–tagged proteins (Yap180Ap and Ent2p) and GST proteins were purified as described (Duncan *et al.*, 2001 and Rodal *et al.*, 2003, respectively). Arp2/3 complex (Martin *et al.*, 2005) and yeast actin (Goode *et al.*, 1999) were purified as described previously. Pyrene-labeled rabbit muscle actin was prepared as described elsewhere (Cooper *et al.*, 1983).

Actin Filament Cosedimentation and Actin Assembly Assay

Pan1p (0.5 μM) and 0, 1, or 2 μM GST-fused Sla2-CC were added to 2 μM of assembled yeast actin, incubated for 30 min at room temperature, and centrifuged for 30 min in a TLA100 rotor (Beckman, Fullerton, CA) at 90,000 rpm. Pellets and supernatants were separated on 9% SDS-PAGE gels that were subsequently stained with Coomassie blue. To measure actin assembly kinetics, pyrene fluorescence was monitored as described previously (Goode *et al.*, 1999). Actin (2 μM yeast monomeric actin and 0.1 μM pyrene-labeled rabbit muscle actin) was centrifuged at 90,000 rpm in a TLA100 rotor (Beckman) for 45 min to remove nuclei before use. Reaction conditions were as follows: 53 μl actin in G-buffer (50 mM Tris-Cl, pH 7.5, 0.2 mM CaCl$_2$, 0.2 mM ATP, and 0.2 mM DTT) was mixed rapidly with 7 μl initiation buffer (250 mM KCl, 20 mM MgCl$_2$, and 5 mM ATP) and 10 μl HEKG5 (20 mM HEPES, pH 7.5, 1 mM EDTA, 50 mM KCl, and 5% glycerol) containing 10 nM Arp2/3, and/or Pan1p and then immediately transferred to a cuvette in the fluorometer. Fluorescence data were collected by using a Fluoromax 3 fluorometer (Jobin-Yvon Horiba, Edison, NJ).

Immunoblotting and In Vitro Pulldown Assay

Immunoblot analysis was performed as described previously (Toshima *et al.*, 2005). Anti-Pan1p (Duncan *et al.*, 2001), anti-Sla2p (Yang *et al.*, 1999) and anti-Act1p antibodies (Goode *et al.*, 1999) were used at a dilution of 1:1000. Immunoreactive protein bands were visualized, using a SuperSignal West Pico Chemiluminescent Substrate System (Pierce, Rockford, IL). For in vitro pulldown assays, GST-Sla2 was overexpressed in DDY1810 and purified as described above. GST-Sla2 pelleted with glutathione-Sepharose beads (GE Healthcare, Waukesha, WI) was washed three times with wash buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5% NP40) and incubated with purified Pan1p

in 100 μl binding buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EGTA, 1 mM EDTA, 7.5% glycerol, 0.5% NP40, 10 mM β-mercaptoethanol, 1 mM PMSF, and 0.5 μg/ml leupeptin, antipain, pepstatin A, and aprotinin). After incubation overnight at 4°C, the precipitates were washed three times with wash buffer and used for immunoblotting. In vitro pulldown assays using other protein combinations were also performed in the same way.

Fluorescence Microscopy

Fluorescence microscopy was performed using an Olympus IX81 microscope equipped with a 100×/NA 1.40 (Olympus, Melville, NY) objective and Orca-ER cooled CCD camera (Hamamatsu, Bridgewater, NJ). Simultaneous imaging of red and green fluorescence was performed using an Olympus IX81 microscope, described above, and an image splitter (Dual-View; Optical Insights, Tucson, AZ) that divided the red and green components of the images with a 565-nm dichroic mirror and passed the red component through a 630/50-nm filter and the green component through a 530/30-nm filter.

RESULTS

Localization of Phosphorylated Pan1p In Vivo

In yeast and mammals, several Arp2/3 complex activators associate with endocytic sites where they appear to promote actin assembly (Schafer, 2002; Kaksonen *et al.*, 2006; Sun *et al.*, 2006). Our group previously showed that Eps15-related Arp2/3 complex activator, Pan1p, functions redundantly with WASP-related protein, Las17p, and that these proteins are important for initiating actin assembly during yeast endocytosis (Duncan *et al.*, 2001; Toshima *et al.*, 2005; Sun *et al.*, 2006). Pan1p is recruited to endocytic sites ~25 s earlier than Abp1p (Kaksonen *et al.*, 2003), a marker for actin assembly, suggesting that Pan1p's Arp2/3 complex activation activity is kept inactive during early stages of endocytosis. However, how Pan1p is maintained inactive before initiation of actin assembly has not been explored. One possibility is that phosphorylated/inactive Pan1p is recruited to cortical patches and that Pan1p dephosphorylation occurs immediately before the onset of actin polymerization (Toshima *et al.*, 2005). To test this possibility, we examined the effects of Prk1p kinase overexpression in cells. Prk1p overexpression resulted in about a 2.7-fold increase in the level of Pan1p phosphorylation, and a severe growth defect that depended on Prk1p kinase activity (Figure 1, A and B). Interestingly,

Figure 2. Pan1p binds to End3p and Sla2p. (A) Coomassie-stained gel of TAP-tagged Pan1p and its binding proteins purified in two steps using immunoglobulin G (IgG)-coupled beads and S protein beads (left panel). The TAP-tag was expressed at the Pan1p C-terminus in DDY1810 cells via a chromosomal integration (*PAN1-TAP*). Immunoblots showing that Sla2p is coisolated with TAP-tagged Pan1p (center and right panels). The center blot was probed with an anti-Pan1p antibody, and the right blot was probed with anti-Sla2p antibody. DDY1810 (*PAN1*) was used as a control. (B) Coomassie-stained gel of the purified Pan1p or Pan1p-End3p complex. TAP-tagged Pan1p alone (left lane) or TAP-tagged Pan1p and End3p (right lane) were expressed in DDY1810 and purified in two steps using IgG-coupled beads and calmodulin-coupled agarose. (C) Effect of Prk1p phosphorylation on Pan1p-End3p complex. Purified Pan1p-End3p complex was incubated with 0–0.3 μM Prk1p at 30°C, separated by SDS-PAGE, and analyzed using Coomassie blue staining. (D) Interaction between Pan1p and Sla2p in the two-hybrid analysis. 1, pDB (bait vector) + pPC86 (prey vector); 2, pDB-*PAN1* and pPC86; 3, pDB and pPC86-*SLA2*; 4, pDB-*PAN1* and pPC86-*SLA2*. DO LW, drop-out Leu and Trp; DO LWH, drop-out Leu, Trp, and His; 3-AT, 3-amino-1,2,4-triazole. (E) In vitro binding of Pan1p to Sla2p. Purified Pan1p was pulled down with GST-Sla2. The pelleted proteins were separated by SDS-PAGE and analyzed using Coomassie blue staining. (F) Coomassie-stained gel for a cosedimentation assay using Pan1p truncation mutants. Purified GST-Sla2p was pulled down with indicated TAP-tagged Pan1 truncation mutants and analyzed as in E.
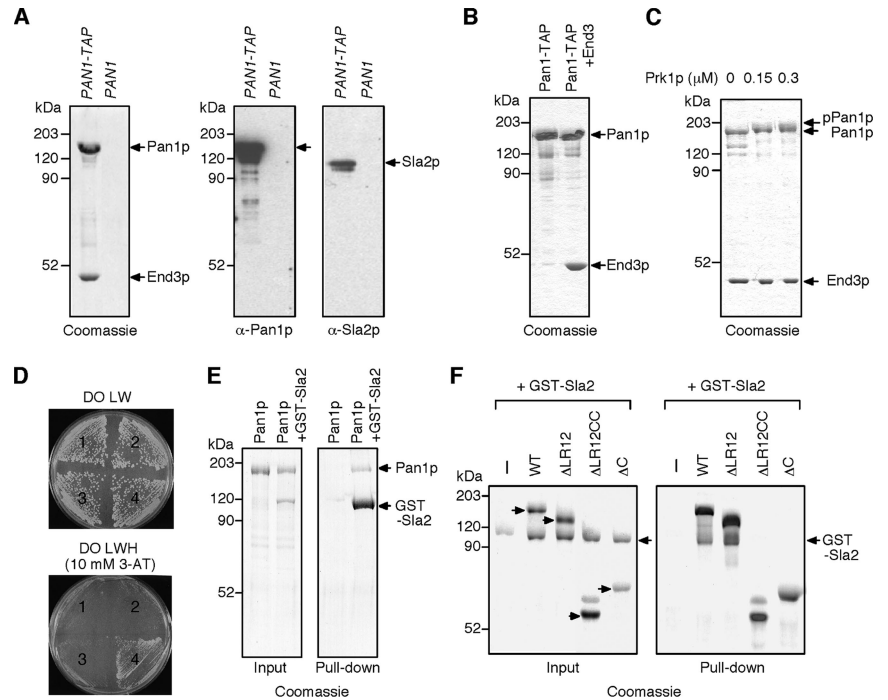
Prk1p overexpression caused Pan1p to be mislocalized in the cytosol, although Pan1p cortical localization was not significantly altered by overexpressing a kinase-dead mutant of Prk1p, Prk1(D158A) (Figure 1C). In contrast, the localization of Pan1-15TAp, in which 15 potential Prk1p phosphorylation sites in Pan1p were mutated to alanine (Toshima *et al.*, 2005), at cortical patches was not affected by Prk1 overexpression (Figure 1D). These observations suggest that Prk1p-phosphorylated Pan1p localizes in the cytosol and that Pan1p dephosphorylation in the cytosol may be required for recruitment to endocytic sites. Therefore, Pan1p phosphorylation is not likely to maintain Pan1p in an inactive state during early stages of endocytosis.

Purification of Pan1p-binding Proteins

To determine how Pan1p might be inactivated at cortical patches, we next investigated Pan1p protein–protein interactions. Pan1p is known to interact with several endocytic proteins, such as Sla1p, End3p, Ent1/2p, and Yap180A/Bp (Wendland and Emr, 1998, 1999; Tang *et al.*, 2000; Krogan *et al.*, 2006). To purify the endogenous Pan1p complex, a TAP tag was fused to the C terminus of Pan1p and expressed from the endogenous *PAN1* locus in a protease-deficient yeast strain. Interacting proteins were identified by mass spectrometry and immunoblotting, revealing that native End3p and Pan1p appear to form a stable complex (Figure 2A). To further examine the interaction between these proteins, we purified the Pan1p complex from yeast overexpressing TAP-tagged full-length Pan1p in strains overexpressing or not overexpressing End3p (Figure 2B). Similar to the native complex, Pan1p and End3p were purified as a stable complex in about a 1:1 binding stoichiometry (Figure 2B, right lane). It was previously reported that the interaction between Pan1p and End3p is negatively regulated by

Prk1p phosphorylation (Zeng *et al.*, 2001). However, in our experiments, phosphorylation of the Pan1p-End3p complex by Prk1p kinase did not affect the stability of the complex (Figure 2C). We previously showed that endogenous Pan1p purified from *ark1Δ prk1Δ* cells also makes a complex with End3p (Toshima *et al.*, 2005). These observations, taken together, suggest that End3p can stably bind to Prk1p-phosphorylated Pan1p, as well as to the unphosphorylated form of Pan1p.

Because Sla2p appears at cortical patches at almost the same timing as Pan1p (Kaksonen *et al.*, 2003), we also examined whether Sla2p copurifies with TAP-tagged Pan1p. Interestingly, we identified Sla2p as a novel Pan1p-interacting protein (Figure 2A). The interaction between Sla2p and Pan1p was much weaker, suggesting that this interaction might be more transient when compared with the Pan1p-End3p complex (Figure 2A). Both Pan1p and Sla2p are reported to bind to F-actin, but we did not find actin copurifying with TAP-tagged Pan1p, suggesting that this interaction is not mediated by F-actin (data not shown). We further confirmed the Pan1p-Sla2p interaction using the yeast two-hybrid system (Figure 2D). To examine the binding in vitro, we performed pulldown assays with purified Pan1p and GST-Sla2p and demonstrated that Pan1p binds to Sla2p in vitro as well as in vivo (Figure 2E). We next purified TAP-tagged Pan1p containing deletions of the LR regions (ΔLR12), the LR regions and the coiled-coil region (ΔLR12CC), or the C-terminal region including the coiled-coil region (ΔCC) (Toshima *et al.*, 2005; Figure 2F) and attempted to pull down GST-Sla2 with these proteins. Pan1-ΔLR12 could bind to Sla2p, but neither ΔLR12CC nor ΔCC bound to Sla2p (Figure 2F). Therefore, Sla2p appears to bind to the central coiled-coil region of Pan1p, which contains the WH2-like region that binds to F-actin (Toshima *et al.*, 2005).
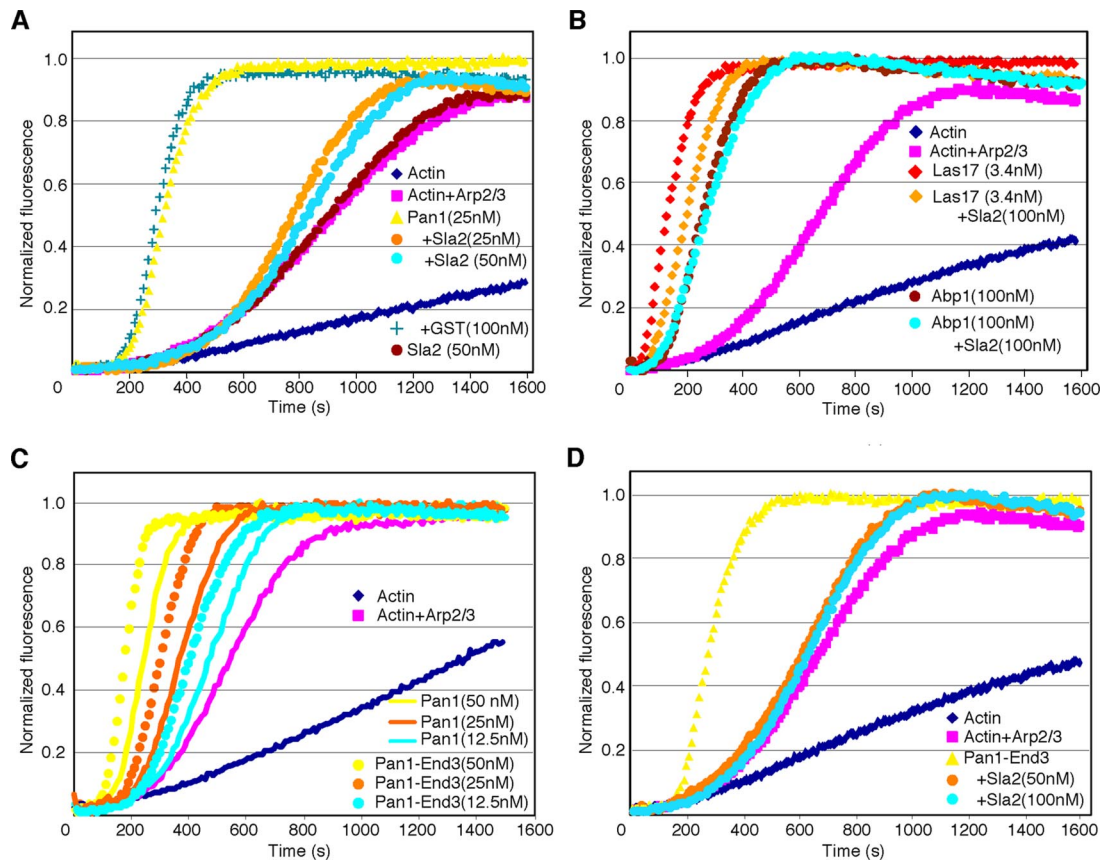
Figure 3. Effects of Sla2p on Pan1p-induced actin nucleation. (A) Pan1p-mediated Arp2/3 activation is inhibited by Sla2p. Graph shows assembly of 2 μM yeast actin and 0.1 μM pyrene-labeled rabbit muscle actin, with 10 nM yeast Arp2/3 complex and 25 nM Pan1p with or without indicated amounts of GST-Sla2p. GST or Sla2p alone was also added to the reaction as a control. (B) Effect of Sla2p on Las17p- or Abp1p-induced actin nucleation. Las17p with or without Sla2p was added to identical reactions of actin and Arp2/3 complex. (C) The Pan1p-End3p complex exhibits similar level of Arp2/3 activation activity to Pan1p. Indicated amounts of Pan1p or Pan1p-End3p complex was added to identical reactions of actin and Arp2/3 complex. (D) Pan1p-End3p complex mediated Arp2/3 activation is inhibited by Sla2p. Indicated amounts of GST-Sla2p were added to identical reactions of actin, Arp2/3 complex, and 50 nM Pan1p-End3p complex.

Inhibition of Pan1p's Activity by Sla2p

To examine the effect of Pan1p-interacting proteins on Pan1p's ability to activate the Arp2/3 complex, pyrene-actin assembly assays were performed. As reported previously (Toshima *et al.,* 2005), 25 nM of full-length Pan1p enhances Arp2/3-mediated actin polymerization (Figure 3A). However, preincubation of Pan1p with 25 nM GST-Sla2p dramatically reduced its Arp2/3 activation activity, whereas GST alone, or Sla2p in the absence of Pan1p, did not affect actin assembly (Figure 3A). The effect of Sla2p was specific because neither Las17p, nor Abp1p activation of Arp2/3 was significantly inhibited by Sla2p, even when using 100 nM Sla2p with 3.4 nM Las17p (Figure 3B). The Pan1p-End3p complex exhibited slightly higher Arp2/3 activation activity than Pan1p alone (Figure 3C). The activity of the Pan1p-End3p complex was also inhibited by Sla2p (Figure 3D), suggesting that Sla2p regulates the native form of the Pan1p complex. To examine the effects of other previously identified Pan1p binding partners on Pan1p Arp2/3 complex activation activity, we purified Sla1p, Yap180Ap, End3p, and Ent2p (Figure 4A) and tested their effects on Pan1p using the pyrene-actin assembly assay. Sla1p and Yap180Ap slightly reduced Arp2/3 complex activation by Pan1p, but these effects were modest compared with those of Sla2p (Figure 4, B and C). Although the Pan1p-End3p complex exhibited a slightly higher activity compared with Pan1p alone (Fig-

ure 3C), End3p, when purified separately from Pan1p and added into the reaction, had no effect on Pan1p's activity (Figure 4D). This result might indicate that the N-terminal End3p-binding region of Pan1p was not correctly folded when purified separately from End3p and that it therefore could not form a stable complex with End3p. Ent2p had no effect on Pan1p's activity (Figure 4E). These results further support the conclusion that Sla2p specifically inhibits Pan1p's Arp2/3 complex activation activity.

The Coiled-Coil Region of Sla2p Binds to Pan1p and Inhibits Pan1p Activity

To identify the region of Sla2p that is important for the inhibition of Pan1p's activity, we purified GST-fused Sla2p containing deletions of the ANTH domain and the proline-rich region (Δ33-360), the C-terminal talin-like domain (Δ768-968), a part of the coiled-coil and talin-like domains (Δ501-968), the whole coiled-coil and talin-like domains (Δ360-968), or the N-terminal ANTH and the coiled-coil domains (33-750) (Figure 5, A and B). As shown in Figure 5C, truncation mutants that retained the coiled-coil region were able to fully or partially inhibit Pan1p's activity (also see summarized result in Figure 5A, right). In addition, deletions that lacked the coiled-coil region, Sla2Δ360-968 and Sla2Δ33-750, failed to inhibit Pan1p activity. We next purified the
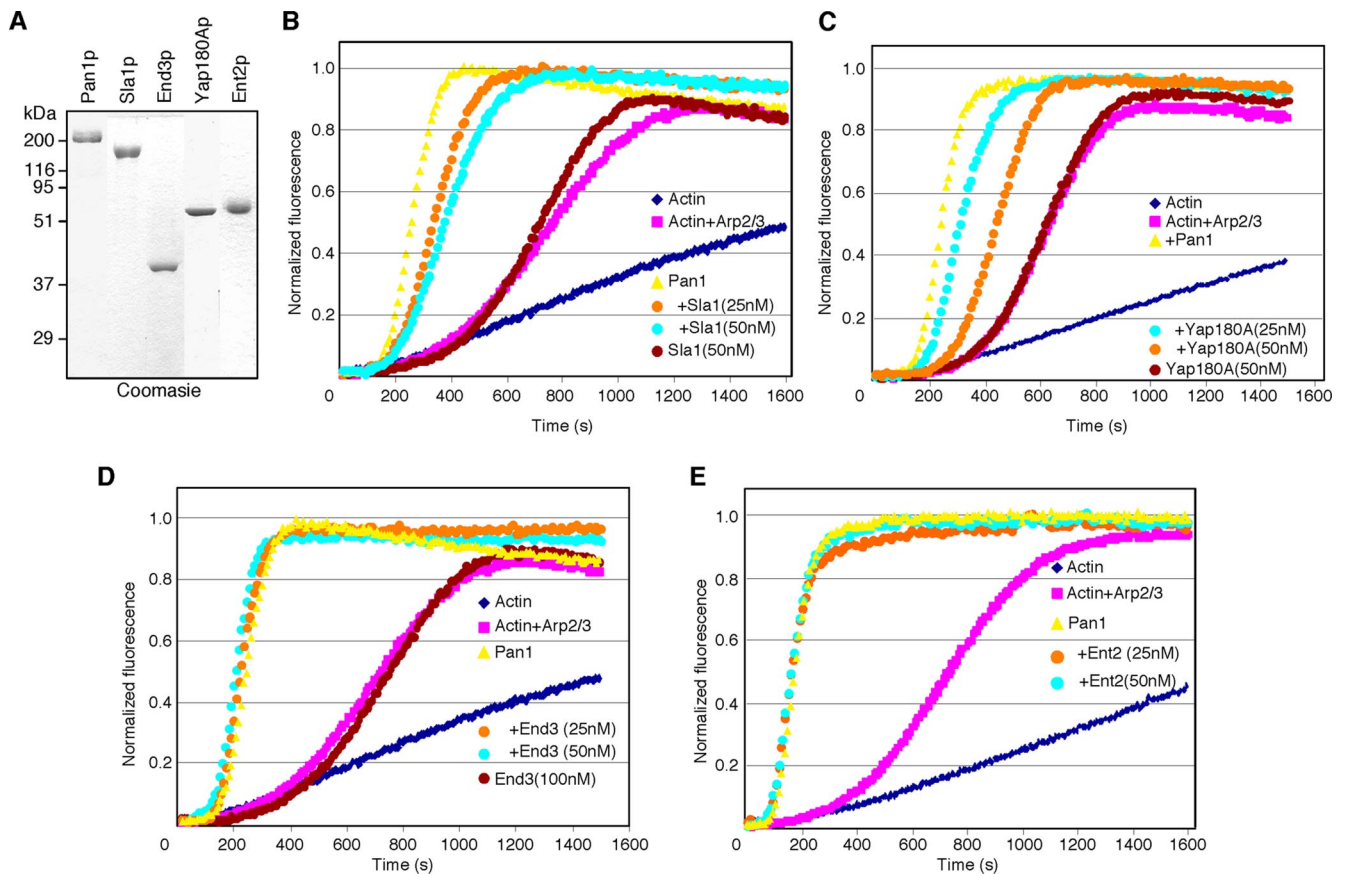
Figure 4. Effects of Pan1p binding proteins on Pan1p's activity. (A) Coomassie-stained gels of purified proteins. (B–E) Indicated amounts of Sla1p (B), Yap180Ap (C), End3p (D), and Ent2p (E) were added to assembly of 2 μM yeast actin and 0.1 μM pyrene-labeled rabbit muscle actin, with 10 nM yeast Arp2/3 complex and 25 nM Pan1p.

coiled-coil region (355-733aa) of Sla2p (GST-CC; Figure 5, A and D) and performed in vitro binding and actin assembly assays. GST-CC directly bound to Pan1p (Figure 5D) and inhibited Pan1p's Arp2/3 activation activity (Figure 5E), although a higher concentration of GST-CC was necessary to fully inhibit Pan1p's activity. This result suggests that either the 355-733aa region of Sla2p is not sufficient for forming correct 3D structure of the CC region or that a slightly wider region is required for complete inhibition.

A Pan1 Partial Loss-of-Function Mutation Partially Suppresses the Temperature Sensitivity of the sla2Δ Mutant

Cells lacking Sla2p are temperature sensitive for growth at 34°C and exhibit abnormal actin comet tail-like structures associated with endocytic complexes at the cell cortex (Yang *et al.,* 1999; Kaksonen *et al.,* 2003). We have shown that Sla2p directly binds to Pan1p and inhibits its Arp2/3 complex activation activity in vitro. Therefore, Pan1p may be constitutively active in *sla2Δ* cells, and a Pan1p loss-of-function mutant might suppress *sla2Δ* mutant phenotypes. To test this possibility, we crossed two Pan1 inactive mutants, the *pan1-101* or *pan1-KE*, which impair Arp2/3 complex binding or F-actin binding, respectively (Duncan *et al.,* 2001; Toshima *et al.,* 2005), to the *sla2Δ* mutant. Interestingly, the *pan1-KE* mutation partially suppresses the growth defect of the *sla2Δ* mutant at 34°C, but the *pan1-101* mutant does not (Figure 6A). This result suggests that Sla2p might inhibit Pan1p's activity by competing with F-actin. In support of this hy-

pothesis, the addition of GST-CC markedly reduced the interaction between Pan1p and F-actin (Figure 6B), consistent with the observation that Sla2p binds to the Pan1p coiled-coil region, including the WH2-like region (Figure 2F). Because Pan1-KEp might retain some activity, and the *pan1-KE* mutation might therefore only suppress the phenotype of the *sla2Δ* mutant partially, we next used a *pan1* mutant that lacks both F-actin binding and Arp2/3 binding regions (*pan1-ΔWA*; Figure 6C). Immunoblotting showed that Pan1-ΔWAp is expressed at levels similar to wild-type Pan1p (Figure 6D). As expected, the *pan1-ΔWA* mutant exhibited a more severe synthetic growth phenotype when combined with the *arp2-1* mutant than either the *pan1-101* or *pan1-KE* mutants (Figure 6E). However, although the *pan1-ΔWA* mutant suppressed the temperature sensitivity of the *sla2Δ* mutant a little more efficiently than the *pan1-KE* mutant, the suppression was still partial (Figure 6F). This result suggests that the *sla2Δ* mutant phenotype is caused in part by inappropriate activation of Pan1p and additionally by loss of other Sla2p functions, such as PIP2 binding via the ANTH domain (Sun *et al.,* 2005) and/or actin binding via the talin-like domain (McCann and Craig, 1997).

The pan1-ΔWA Mutant Suppresses the Temperature Sensitivity, Endocytic Defect, and Actin Phenotypes of sla2ΔCC Mutant

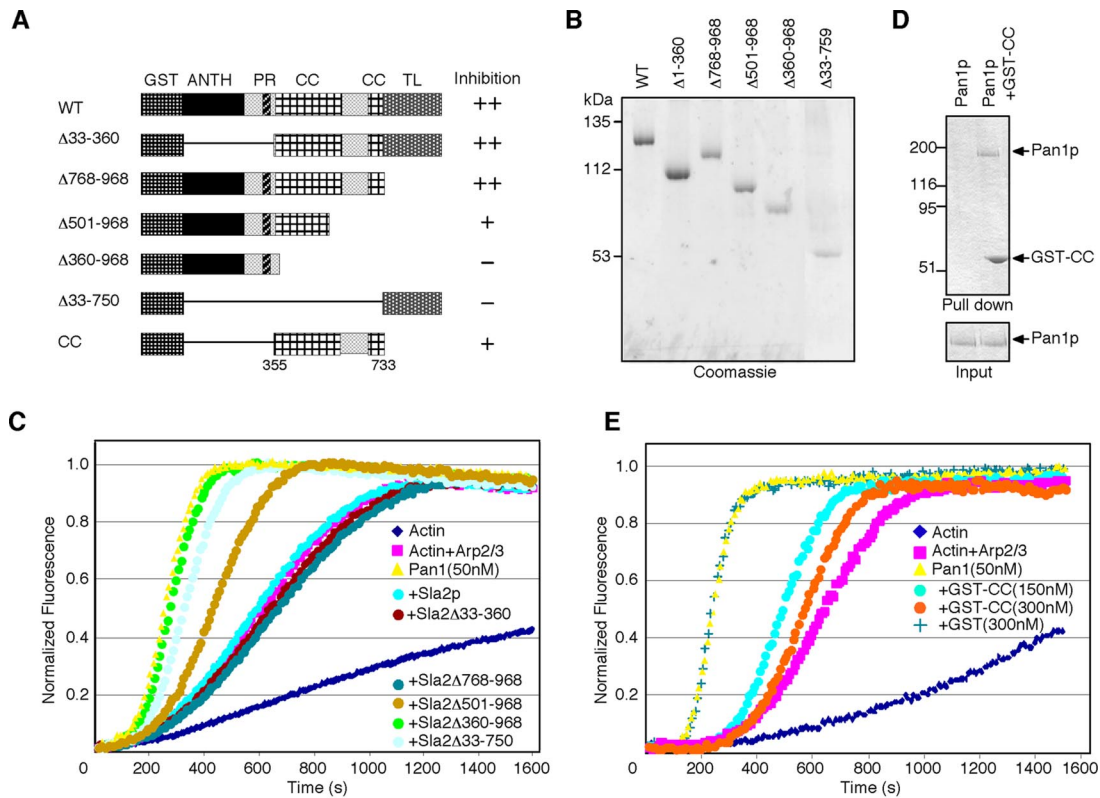We showed that the coiled-coil region of Sla2p inhibits Pan1p's Arp2/3 complex activation activity (Figure 5). There-

Figure 5. The coiled-coil region of Sla2p binds to Pan1p and inhibits Pan1p activity. (A) Diagram of GST-fused Sla2p constructs. The ANTH (AP180 N-terminal homology) domain, proline-rich (PR), predicted coiled-coil (CC), and talin-like (TL) regions are indicated. The results shown in C are summarized to the right of each diagram under "inhibition." (B) Coomassie-stained gels of purified GST-fused Sla2 mutants. (C) Effects of Sla2 truncation mutants on Pan1p's activity. Full-length Sla2p, Δ33-360, Δ768-968, Δ501-968, Δ360-968, or Δ33-750, 100 nM, was added to assembly reactions containing 2 μM yeast actin and 0.1 μM pyrene-labeled rabbit muscle actin, with 10 nM yeast Arp2/3 complex and 50 nM Pan1p. (D) In vitro binding of Pan1p to GST-CC. Purified Pan1p was pulled down with (right) or without (left) GST-CC. The precipitates were separated by SDS-PAGE and analyzed using Coomassie blue staining. (E) Effect of GST-CC on Pan1p's activity. Indicated amounts of GST-CC were added to identical reactions of actin and Arp2/3 complex.

fore, a *sla2* mutant lacking the coiled-coil region might result in a phenotype caused by uncontrolled Pan1p Arp2/3 complex activation. To test this possibility, we first made a *sla2ΔCC* mutant that lacks amino acids 360–729 of Sla2p and integrated this construct into the endogenous *SLA2* locus (Figure 7A). Sla2ΔCCp was expressed at levels similar to the wild-type Sla2p protein (Figure 7B). Although the growth rate of the *sla2ΔCC* mutant was similar to that of the wild-type strain at 25°C, *sla2ΔCC* cells exhibited a severe defect in growth at 39°C (Figure 7C). Importantly, the *pan1-ΔWA* mutant suppressed the temperature sensitivity of the *sla2ΔCC* mutant at 39°C (Figure 7C), and it suppressed the endocytosis defect that was observed for the *sla2ΔCC* mutant at 39°C (Figure 7D). We also examined Pan1p and actin dynamics in the *sla2ΔCC* mutant using cells coexpressing Pan1-GFP and Abp1-RFP. In wild-type cells, Pan1p and Abp1p have lifetimes of ~36 and 13.6 s, respectively (Figure 7E, top panels; Supplementary Movie 1). In contrast, the *sla2ΔCC* mutant exhibited abnormal actin tail structures (~74.6%, n = 126 patches) and impaired Pan1p internalization into the cytosol at 37°C (Figure 7E, center panels; Supplementary Movie 1), much like the *sla2Δ* mutant. Similar to the growth phenotype and endocytosis defect, actin tail formation (~5.7%, n = 132 patches) and the defect in Pan1p internalization were mostly suppressed by the *pan1-ΔWA* mutation (Figure 7E, bottom panels; Supplementary Movie 1),

suggesting that the Sla2p coiled-coil region regulates Pan1p both in vitro and in vivo.

DISCUSSION

Conserved Role for Hip1R/Sla2 Family Proteins in Actin Cytoskeleton Regulation

In both yeast and mammals, a transient burst of actin assembly occurs coincident with endocytic vesicle internalization (Merrifield *et al.*, 2002; Kaksonen *et al.*, 2003). Arp2/3 complex activators play a key role in actin assembly, but how activities of these proteins are regulated is poorly understood. Our group previously showed that cells lacking the *SLA2* gene engage in continuous actin nucleation from nonmotile endocytic sites (Kaksonen *et al.*, 2003). A similar phenotype is also observed in human cells depleted of a Sla2p-related protein, Hip1R, by RNA interference (Engqvist-Goldstein *et al.*, 2004). These observations suggested that proteins of the Hip1R/Sla2 family might negatively regulate actin assembly during endocytosis. We showed here that Sla2p interacts with Pan1p and inhibits its Arp2/3 complex activation activity, possibly by preventing Pan1p's binding to F-actin. We also showed that a Pan1p loss-of-function mutant suppressed the continuous actin assembly phenotype observed in the *sla2ΔCC* mutant. Taken together, these findings clearly demonstrate that Sla2p negatively regulates
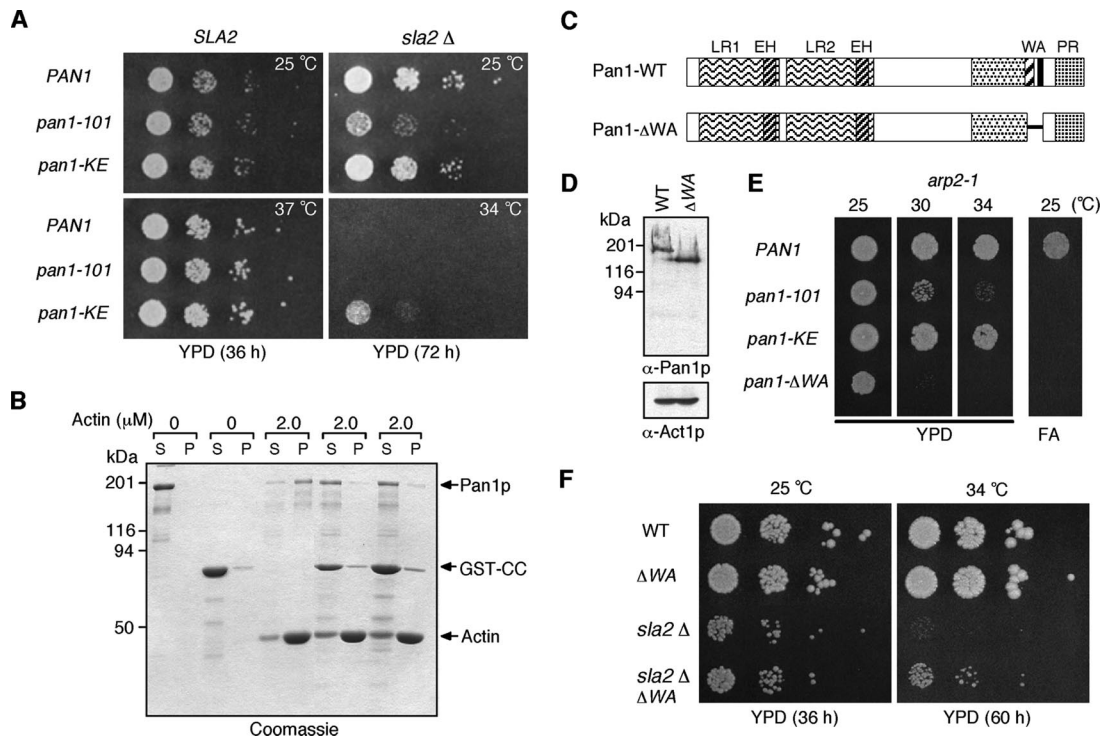
Figure 6. A Pan1 partial loss-of-function mutation partially suppresses the temperature sensitivity of the *sla2Δ* mutant. (A) The *pan1-KE* mutation partially suppresses the temperature sensitivity of the *sla2Δ* mutant at 34°C. A dilution series of cells was plated on YPD medium and incubated for the indicated times at 25, 34, or 37°C. (B) SDS-PAGE of a cosedimentation assay using full-length Pan1p, GST-CC, and F-actin. The gel was stained with Coomassie blue. P, pellets; S, supernatants. Indicated amounts of yeast actin were used with 100 nM Pan1p and 0, 250, or 500 nM GST-CC. (C) Diagram of wild-type Pan1p and Pan1ΔWA mutant. (D) Immunoblots showing that Pan1ΔWA is expressed at levels similar to wild-type Pan1p. Top panel was probed with anti-Pan1p antibody and bottom panel was probed with anti-Act1p antibody as a loading control. (E) Plates showing growth phenotypes of double-mutant combinations. Only *pan1ΔWA* results in a synthetic temperature sensitivity at 30°C when combined with *arp2-1*. All *pan1* mutants result in synthetic lethality with *arp2-1* on 3% formamide (FA) plates at 25°C. (F) The *pan1ΔWA* mutation partially suppresses the temperature sensitivity of the *sla2Δ* mutant at 34°C. A dilution series of cells was plated on YPD medium and incubated for indicated time at 25 or 34°C, respectively.

Pan1p's activity during endocytosis. Interestingly, the formation of abnormal actin structures observed in Hip1R-depleted human cells was shown to be suppressed by knockdown of another Arp2/3 complex activator, cortactin, which localizes at clathrin-coated pits and functions in clathrin-mediated endocytosis (Cao *et al.,* 2003; Engqvist-Goldstein *et al.,* 2004). Thus, negative regulation of actin assembly by the Hip1R/Sla2 family of proteins at endocytic site is likely to be a conserved mechanism for regulating endocytic actin assembly in yeast and mammals.

Possible Regulatory Mechanism for Pan1p Activation

One possible factor that might regulate the interaction between Pan1p and Sla2p is the lipid PtdIns(4,5)P2. Our group previously showed that Sla2p directly binds to PtdIns(4,5)P2 through the ANTH domain (Sun *et al.,* 2005). The ANTH domain of Sla2p is shown to be important for proper actin organization and endocytic internalization (Sun *et al.,* 2005), but a physiological role of the interaction of Sla2p with PtdIns(4,5)P2 was not identified. Yeast expresses three synaptojanin-like proteins (Sjl1, Sjl2, and Sjl3), which are phosphoinositide phosphatases, and an *sjl1Δ sjl2Δ* double mutant exhibited severe defects in both actin organization and endocytosis (Singer-Kruger *et al.,* 1998; Simonsen *et al.,* 2001; Czech, 2003). Recently, it has been reported that Sjl2p recruitment to endocytic sites is dependent on F-actin. Therefore PtdIns(4,5)P2 may be converted to PI subsequent to actin as-

sembly (Stefan *et al.,* 2005). Interestingly, a strain lacking synaptojanin-like proteins, which probably overproduces PtdIns(4,5)P2, induces abnormal, enlarged plasma membrane structures in an actin-dependent manner, and the formation of the abnormal membrane structures is suppressed by Sla2p overexpression (Stefan *et al.,* 2005). These observations suggest that the accumulation of PtdIns(4,5)P2 at endocytic sites could induce actin polymerization. Binding of PtdIns(4,5)P2 to Sla2p also suggests that accumulation of PtdIns(4,5)P2 might lead to dissociation of Sla2p from Pan1p and activation of Pan1p. Mutants of Pan1p show genetic interactions with mutants of Sjl1p (Wendland and Emr, 1998), further suggesting the importance of PtdIns(4,5)P2 regulation for Pan1p function.

The coiled-coil region of Sla2p was also reported to be important for interaction with Clc1p, the light chain subunit of yeast clathrin (Newpher and Lemmon, 2006). Although a *sla2* mutant lacking the coiled-coil region (289-583aa) completely disrupts Clc1p binding in vitro, the physiological role of the clathrin-Sla2p interaction has yet to be established. Our group previously showed that Sla1p patch lifetime is reduced by 30% in *clc1Δ* or *chc1Δ* cells (Kaksonen *et al.,* 2005), suggesting that the absence of mature clathrin structures could advance the timing of initiation of actin polymerization at endocytic sites. Therefore, clathrin may act as a negative regulator of the Pan1p-Sla2p interaction to control the timing of endocytic vesicle internalization.
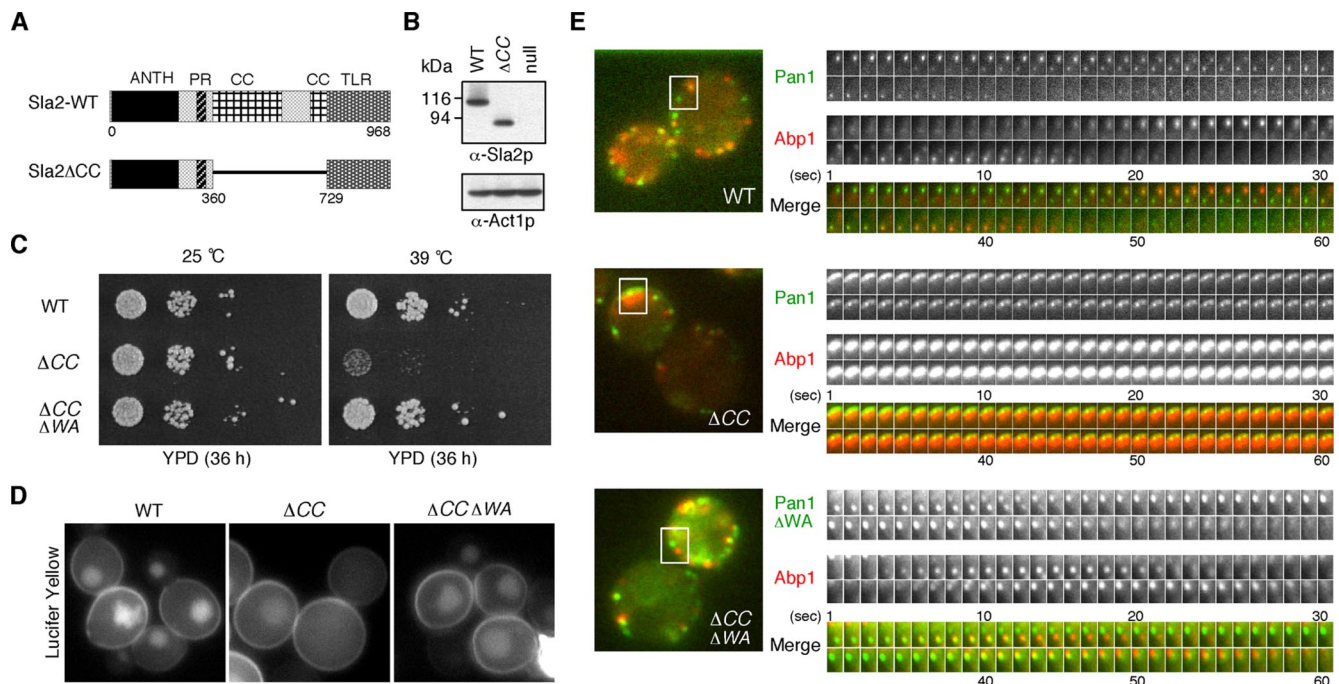
Figure 7. The *pan1ΔWA* mutation suppresses the temperature sensitivity, endocytic defect, and actin phenotype of *sla2ΔCC* mutants. (A) Diagram of wild-type Sla2p and the Sla2ΔCC mutant. (B) Immunoblots showing that Sla2ΔCC is expressed at similar levels to wild-type Sla2p. Top panel was probed with an anti-Sla2p antibody and bottom panel was probed with anti-Act1p antibody as a loading control. (C) Plates showing growth phenotypes of *sla2ΔCC* mutant or *sla2ΔCC pan1ΔWA* double mutant. Dilution series of these mutants were plated on YPD medium and incubated for 36 h at 25 or 39°C, respectively. (D) The *pan1ΔWA* mutation suppresses the endocytosis defect observed in the *sla2ΔCC* mutant. Lucifer yellow uptake was performed for 60 min at 39°C and was assessed by fluorescence microscopy. (E) Left panels are single frames from a two-color movie showing Abp1-RFP (red) and Pan1-GFP (green) in wild-type (top), *sla2ΔCC* (middle), or *sla2ΔCC pan1ΔWA* (bottom) cells. Right panels are time series of boxed area in left panels. The time to acquire one image pair was 1 s.

End3p Stably Binds to Pan1p and Functions Together with Pan1p throughout Endocytosis

The association of Pan1p-Sla1p-End3p in a complex has been suggested to be negatively regulated by Prk1p (Zeng and Cai, 1999; Zeng *et al.*, 2001). However, our results showed that Pan1p stably binds to End3p and that this interaction is not affected by Prk1p phosphorylation. In a previous study, Zeng *et al.* (2001) showed that the phosphorylated Pan1p LR2 (long repeat 2) fragment tagged with HA does not associate with GST-End3p. However, whether the pre-formed Pan1p-End3p complex can be dissociated by phosphorylation was not determined. In addition, Pan1p fragments containing both LR1 and LR2 regions bind to End3p about twofold more strongly than the LR2 fragment alone (Tang *et al.*, 1997), suggesting that the LR1 region also contributes to the strength of this interaction. Prk1p appears to phosphorylate the Pan1p complex after endocytic internalization (Sekiya-Kawasaki *et al.*, 2003). Therefore, it follows that our experimental conditions may better reflect the in vivo state of Pan1p-End3p. Earlier observations that Pan1p cortical patch localization decreases in *end3Δ* cells add further support to the importance of a stable interaction between Pan1p and End3p (Tang *et al.*, 1997; Kaksonen *et al.*, 2005). Besides Pan1p, the Pan1p binding proteins, Sla1p and Ent1/2p, are also Prk1p substrates (Watson *et al.*, 2001; Zeng *et al.*, 2001). Compared with the interaction between Pan1p and End3p, the interactions of these proteins with Pan1p appear much weaker because we did not find these proteins associated with TAP-tagged Pan1p. This finding suggests that these interactions might occur transiently, and potentially could be negatively regulated by Prk1p phosphorylation.

Phosphoregulation of Pan1p Localization during Endocytosis

Our results demonstrate that the phosphorylated form of Pan1p probably localizes in the cytosol and is dephosphorylated by a yet-to-be-identified phosphatase. One of the candidates to regulate the dephosphorylation of Prk1p-phosphorylated endocytic proteins is the Ser/Thr protein phosphatase-1 (PP1/Glc7p). A *glc7* mutant has been shown to affect cortical actin organization (Andrews and Stark, 2000). Scd5p, which is identified as a multicopy suppressor of a clathrin-deficient mutant (Nelson and Lemmon, 1993; Nelson *et al.*, 1996), plays a crucial role in endocytosis and cortical actin organization (Henry *et al.*, 2002), and Scd5p binding to the PP1/Glc7p is important for function (Chang *et al.*, 2002; Henry *et al.*, 2002). Furthermore, deletion of *PRK1* suppresses the phenotypes of an *scd5* mutant that has a mutation in the PP1-binding site (Henry *et al.*, 2003; Huang *et al.*, 2003), suggesting that Scd5p-PP1 could antagonize the function of Prk1p-dependent phosphorylation of endocytic proteins. A recent study showed that Scd5p recruitment to the cell surface is not essential for its function, and that Scd5p/PP1 can act on its targets in the cytosol (Chang *et al.*, 2006). These results support our idea that phosphorylated Pan1p could be dephosphorylated in the cytosol.

Pan1p Activity Is Important for Initiating Actin Assembly during Yeast Endocytosis

Pan1p functions redundantly with Las17p, and these proteins are known to be important for endocytic internalization (Duncan *et al.*, 2001; Toshima *et al.*, 2005; Sun *et al.*, 2006). We previously showed that a double mutant of *pan1Δ855-1480*, which lacks the C-terminal region containing the

F-actin binding and Arp2/3 activating sites, and the *las17ΔWCA* mutant, has a severe endocytic defect (Toshima *et al.*, 2005). However, we had not determined which step of endocytosis is impaired in the double mutant. In wild-type cells, Sla1p has a lifetime of ~35.8 s, appearing at endocytic sites ~24.2 s earlier than Abp1p, which has a shorter lifetime (~13.6 s) and is internalized into the cytosol together with Abp1p (Supplementary Figure S1A; Kaksonen *et al.*, 2003). The *pan1Δ855-1480* or *las17ΔWCA* single mutant extended the Sla1-GFP lifetime (~60.0 and ~58.0 s, respectively) and delayed actin polymerization (~48.0 s) at endocytic sites (Supplementary Figures S1, B and C). In addition, the double mutant of *pan1Δ855-1480* and *las17ΔWCA* exhibited a significant delay in the initiation of actin assembly (~156.1 s; Supplementary Figure S1D). Interestingly, the double mutant also exhibited a severe defect in Sla1p movement, which represents invagination of the plasma membrane into the cytosol (Supplementary Figure S1E; Kaksonen *et al.*, 2003). These observations indicate that Pan1p and Las17p are important for initiating actin assembly and endocytic vesicle internalization. Our group previously showed that a double mutant of *pan1ΔWA* and *las17ΔWCA* exhibits a delay in the initiation of actin assembly (~89.6 s), although the single mutant of *pan1-ΔWA* barely affected the timing of actin assembly (Sun *et al.*, 2006). The *pan1Δ855-1480* mutant likely affects a Pan1 activity in addition to the ability to activate the Arp2/3 complex. Compared with Las17p, Pan1p has much lower Arp2/3 activation activity in vitro (Sun *et al.*, 2006). It remains possible that a yet-to-be-identified activator of Pan1p exists and strongly activates Pan1p in vivo, just as verprolin stimulates the Arp2/3-activating activity of the budding yeast type I myosin (Geli *et al.*, 2000; Lechler *et al.*, 2001; Sirotkin *et al.*, 2005; Sun *et al.*, 2006).

In summary, our data suggest that Pan1p's Arp2/3 activation activity is inhibited by binding to Sla2p during early stages of endocytosis. The coiled-coil region of Sla2p binds to the F-actin–binding region of Pan1p and inhibits Pan1p's activity. The *sla2ΔCC* mutant exhibited constitutive actin polymerization at endocytic sites, and the actin phenotype was suppressed by a *pan1* loss-of-function mutation. Thus, these results suggest that Sla2p functions as a key regulator for initiating actin assembly during endocytic internalization. We propose that Pan1p is negatively regulated on the plasma membrane by Sla2p and on endocytic vesicles by Prk1p. It is now important to identify the positive regulators that counter the actions of Sla2p and Prk1p on Pan1p.

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