A Novel Function of Arp2p in Mediating Prk1p-specific Regulation of Actin and Endocytosis in Yeast

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The yeast protein Pan1p plays essential roles in actin cytoskeleton organization and endocytosis. It couples endocytosis with actin polymerization through its dual function in endocytic complex assembly and activation of the actin polymerization initiation complex Arp2/3p. Phosphorylation of Pan1p and other components of the endocytic complex by the kinase Prk1p leads to disassembly of the coat complex and the termination of vesicle-associated actin polymerization. A homologous kinase, Ark1p, has also been implicated in this regulatory process. In this study, we investigated the distinct roles of Prk1p and Ark1p. We found that the nonkinase domains determined the functional specificity of the two kinases. A short region located adjacent to the kinase domain unique to Prk1p was found to be required for the kinase to interact with Arp2p. Further studies demonstrated that the Prk1p-Arp2p interaction is critical for down-regulation of Pan1p. These findings reveal that, in addition to its role in the nucleation of actin polymerization, Arp2p also mediates what appears to be an auto-regulatory mechanism possibly adapted for efficient coordination of actin assembly and disassembly during endocytosis.

INTRODUCTION

Endocytosis is a plasma membrane-originated vesicular trafficking process that has been known to rely on actin dynamics for vesicle formation and movement (Engqvist-Goldstein and Drubin, 2003). In budding yeast, endocytosis occurs at the sites that coincide with the cortical actin patches, which comprise an array of proteins involved in various aspects of endocytosis and actin dynamics (Engqvist-Goldstein and Drubin, 2003; Kaksonen *et al.*, 2006; Moseley and Goode, 2006). These proteins, many of which have homologous counterparts in higher eukaryotic cells, have been categorized into several functional modules proposed to be responsible for specific tasks during endocytosis (Kaksonen *et al.*, 2003; Kaksonen *et al.*, 2005). The molecular mechanism by which these proteins cooperate to achieve the sequential events of endocytosis has yet remained largely unknown.

Actin assembly during endocytosis is initiated by the Arp2/3 complex, whose activity is stimulated by a number of nucleation promoting factors (NPFs) including Las17p (WASP), Pan1p, Abp1p, and Myo5p (Sun *et al.*, 2006). The need for involving multiple NPFs in endocytosis apparently stems from the requirement for actin assembly at different stages of endocytosis. While Las17p is responsible for the actin assembly at endocytic sites for membrane invagination, Myo5p is most important in the actin polymerization event during the inward movement of the vesicles (Sun *et al.*, 2006). Pan1p, on the other hand, functions redundantly with Las17p in promoting actin assembly at endocytic sites (Duncan *et al.*, 2001; Toshima *et al.*, 2005, 2007; Sun *et al.*, 2006).

Pan1p is a special endocytic protein in that it is involved in a diverse range of events in endocytosis and actin dynamics. In addition to its part in promoting actin assembly as a nucleation promoting factor, Pan1p also acts as the root component of the endocytic complex by virtue of its ability to interact with many endocytic proteins including End3p, Sla1p (Cin85), Sla2p (Hip1), Ent1/2p (epsin), Yap1801/2p (AP180), and Scd5p (Wendland *et al.*, 1996; Tang *et al.*, 1997; Wendland and Emr, 1998; Tang *et al.*, 2000; Huang *et al.*, 2003; Toshima *et al.*, 2007). Furthermore, Pan1p is able to bind directly to actin filaments (Toshima *et al.*, 2005), thus providing an anchor point for actin meshwork to associate with the endocytic complex. The fact that these functions are required at different phases of vesicle formation and internalization begs a regulatory mechanism to modulate and coordinate the activities of Pan1p during these stages. Pan1p appears on the cortical patches shortly before actin assembly can be detected (Kaksonen *et al.*, 2003). A recent study showed that, during this period of time, the ability of Pan1p to recruit and activate the Arp2/3 complex is inhibited by its binding protein Sla2p (Toshima *et al.*, 2007). This would conceivably enable Pan1p to assemble an early endocytic complex in place before membrane invagination is allowed to occur. Consistently, Las17p is similarly inhibited by Sla1p during this time (Rodal *et al.*, 2003; Sun *et al.*, 2006).

The overall function of Pan1p is under a negative regulation by Prk1p, a member of the Prk1 family of kinases, which is composed of Prk1p, Ark1p, and Akl1p (Smythe and Ayscough, 2003). Although none is essential for life, deletion of *PRK1* and *ARK1* genes together renders the cell temperature sensitive and severely defective in actin organization and endocytosis, indicating that some crucial functions are shared between these two kinases (Cope *et al.*, 1999). Prk1p phosphorylates a number of endocytic proteins *in vivo*, including Pan1p, Sla1p, Scd5p, Yap1801/2p, and Ent1/2p (Zeng *et al.*, 2001; Watson *et al.*, 2001; Huang *et al.*, 2003; Henry *et al.*, 2003), with Pan1p and Sla1p containing most abundant consensus phosphorylation sites of (L/I/V/

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M)xx(Q/N/T/S)xTG (Huang *et al.*, 2003). Phosphorylation by Prk1p leads to dissociation between Pan1p and Sla1p (Zeng *et al.*, 2001) and inhibition of Pan1p's ability to activate the Arp2/3p complex and to bind to actin filaments (Toshima *et al.*, 2005). This suggests that phosphorylation of Pan1p by Prk1p is a regulatory step in endocytosis to allow the vesicles to be detached from actin and uncoated. Compared with Prk1p, Ark1p and Akl1p have been barely studied and their functional characteristics are essentially unknown. While they probably recognize the similar phosphorylation motifs as Prk1p (Zeng *et al.*, 2001; Henry *et al.*, 2003), the functions of the three kinases in vivo are not entirely identical, as they have distinct genetic interactions with other endocytic proteins (Cope *et al.*, 1999). Previous reports showed that the cortical patch localization of both Prk1p and Ark1p is mainly dependent on Abp1p, through the binding between their C-terminal polyproline motifs and the SH3 domain of Abp1p (Cope *et al.*, 1999; Fazi *et al.*, 2002). However, deletion of *ABP1* does not result in a similar phenotype as seen in the *ark1 prk1* mutant, suggesting that Abp1p is not the only anchor for the kinases.

In this study, we set out to analyze the distinct functions between Prk1p and Ark1p. Through genetic and biochemical analysis, we discovered that the divergent nonkinase regions differentiate the functions of the two kinases. A unique sequence located next to the kinase domain of Prk1p is required for the kinase to interact with Arp2p and this interaction is principally responsible for phosphorylation of Pan1p in vivo.

MATERIALS AND METHODS

Strains, Growth Conditions, and General Methods

The yeast strains used in this study are listed in Table 1. Yeast cells were grown in standard yeast extract-peptone-dextrose (YEPD) or dropout medium supplemented with appropriate amino acids. In experiments requiring the expression of genes under the *GAL1* promoter, raffinose instead of dextrose was used as the carbon source and galactose was added later for *GAL1* induction. Bacterial strains were grown in LB medium containing 100 mg of ampicillin (Sigma, St. Louis, MO) to maintain plasmids. Staining of actin filaments with rhodamine-phalloidin was performed as described (Zeng and Cai, 1999), using the Leica DMAXA microscope (Deerfield, IL). Preparation of

yeast extracts, immunoprecipitation, immunoblotting, and the in vitro kinase assays followed previous procedures (Zeng *et al.*, 2001).

Plasmid and Strain Constructions

The plasmid constructs used in this study are listed in Table 2. Mutations in pGEX-SRmut-ARK1299HA, Ark1D159Y, Prk1ARHA-316 were generated by PCR with mutagenic primers and confirmed by sequencing analysis. To make pARK1n-PRK1c-HA and pPRK1n-ARK1c-HA constructs, the kinase domains of *ARK1* and *PRK1* were fused with the C-termini of *PRK1* and *ARK1*, respectively, and confirmed by sequencing analysis.

To obtain YMC511, YMC512, YMC503, and YMC509, plasmids pPAN1-Myc-304 were linearized within the *PAN1* gene by BamHI and integrated into W303-1A, YMC410, YMC411, and YMC414, respectively. To obtain YMC505, YMC506, and YMC507, plasmids pMyc-SLA1-304 were linearized within the *SLA1* gene by BamHI and integrated into W303-1A, YMC410, YMC411, respectively. To obtain YMC510, plasmids Arp2-HA-305 were linearized within the *ARP2* gene promoter by BspEI and integrated into W303-1A. The integrations were confirmed by PCR and sequencing analysis.

Two-Hybrid Assay, Protein Binding, and Coimmunoprecipitation

For the yeast two-hybrid assay, DNA fragments of *ARP2* and *PRK1D158Y* were fused to the hemagglutinin (HA)-tagged *GAL4* activation domain of pGADT7 or the Myc-tagged DNA-binding domain of pGBKT7 as described in Table 2. Plasmids were cotransformed into the strain SFY526 and the expression of each fusion protein was confirmed by Western blotting with anti-HA or anti-Myc antibodies. The β -galactosidase activities were measured as instructed by the manufacturer (CLONTECH, Palo Alto, CA). For glutathione *S*-transferase (GST) fusion protein binding, 400 μ l of $\sim$ 3 mg of purified His-tag proteins was incubated with GST fusion protein coupled beads for 1 h at 4°C, washed five times with RIPA, and eluted into SDS-PAGE sample buffer. Coimmunoprecipitation of Prk1p and Arp2p followed the procedure described in Lechler *et al.* (2000).

Escherichia coli Coexpression Kinase Assay

The bicistronic expression plasmids for *E. coli* coexpression assay were generated as follows: The coding regions of the substrates (SR, SRmut, or LR1, LR2) followed by three stop codons, a translational enhancer, and a Shine-Dalgarno sequence (5'-CGTGTCTCGTCTAATAATTTTGTCTTAAG AAGGAGATATA3'; Tan, 2001) were fused to the kinase domains of either Ark1p or Prk1p (containing an HA tag at the C-termini) by PCR and then cloned in frame into the BamHI and XhoI sites of pGex-4T-1 (Amersham Biosciences, Piscataway, NJ). The expression plasmids were transformed into *E. coli* BL21. The expression of GST fusion proteins and HA-tagged kinases were induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside at 30°C for 1 h. The purified GST-fusion substrates were subjected to SDS-PAGE and Western blot to show the phosphothreonine status. The nitrocellulose membranes with blotted GST fusion proteins were stained with Coomassie blue to visualize the GST fusion proteins.

Table 1. Yeast strains used in this study

Strains	Genotype
W303-1A	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1</i>
SFY526	MAT a <i>ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 canr gal4-542 gal80-538 URA3:: GAL1-lacZ</i>
YMC410	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3</i>
YMC411	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 akl1Δ::URA3</i>
YMC412	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 akl1Δ::URA3 ark1Δ:: LEU2</i>
YMC413	MAT a <i>ade2 trp1 can1 leu2 his3 ura3 pan1-4 prk1Δ:: HIS3</i>
YMC414	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 ark1Δ:: LEU2</i>
YMC511	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 pan1::PAN1-Myc-TRP1</i>
YMC512	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 pan1:: PAN1-Myc-TRP1</i>
YMC503	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 akl1Δ::URA3 pan1:: PAN1-Myc-TRP1</i>
YMC504	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 akl1Δ::URA3 ark1Δ:: LEU2 pRS314-PAN1-Myc-TRP1</i>
YMC505	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 sla1:: Myc-SLA1-TRP1</i>
YMC506	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 sla1:: Myc-SLA1-TRP1</i>
YMC507	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 akl1Δ::URA3 sla1:: Myc-SLA1-TRP1</i>
YMC508	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 akl1Δ::URA3 ark1Δ::LEU2 pRS314-Myc-SLA1-TRP1</i>
YMC509	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 ark1Δ::LEU2 pan1:: PAN1-Myc-TRP1</i>
YMC510	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 arp2::Arp2-HA-LEU2</i>
YMC513	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 pan1:: PAN1-4-Myc-TRP1</i>
YMC514	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 prk1Δ::HIS3 pan1:: PAN1-4-Myc-TRP1</i>
YMC515	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 ark1Δ::LEU2 sla1:: Myc-SLA1-TRP1</i>
YMC516	MAT a <i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 ark1Δ::LEU2 prk1Δ::HIS3 sla1:: Myc-SLA1-TRP1</i>

Table 2. Plasmid constructs used in this study

Constructs	Description
pARK1-HA	<i>ARK1</i> coding region was generated by PCR, cloned in frame with a C-terminal HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>PRK1</i> promoter control in pRS316.
pPRK1-HA	<i>PRK1</i> coding region was generated by PCR, cloned in frame with a C-terminal HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>PRK1</i> promoter control in pRS316.
pARK1n-PRK1c-HA	The DNA coding region for Ark1p (1-299 aa) was fused with the DNA coding region for Prk1p (299-810 aa) by PCR, cloned in frame with a C-terminal HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>PRK1</i> promoter control in pRS316.
pPRK1n-ARK1c-HA	The DNA coding region for Prk1p (1-298 aa) was fused with the DNA coding region for Ark1p (300-638 aa) by PCR, cloned in frame with a C-terminal HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>PRK1</i> promoter control in pRS316.
pPRK1 ₂₉₈ -HA	The DNA coding region for Prk1p kinase domain (1-298 aa) was generated by PCR and cloned in frame with a C-terminal HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>PRK1</i> promoter control in pRS316.
pPRK1Δpp-HA	The DNA coding region for Prk1p (1-747 aa) was generated by PCR and cloned in frame with a C-terminal HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>PRK1</i> promoter control in pRS316.
pARK1Δpp-HA	The DNA coding region for Ark1p (1-606 aa) was generated by PCR and cloned in frame with a HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>ARK1</i> promoter control in pRS316.
pPRK1 ₃₁₉ -HA	The DNA coding region for Prk1p (1-319 aa) was generated by PCR and cloned in frame with a HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>PRK1</i> promoter control in pRS316.
pPRK1 _{AR} Δpp-HA	The DNA coding region for Prk1p (1-747 aa) with Prk1p (299-319 aa) replaced with Ark1p according region (300-320 aa) was generated by PCR and cloned in frame with a C-terminal HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>PRK1</i> promoter control in pRS316.
pPRK1 _{AR} -HA	The DNA coding region for Prk1p with Prk1p (299-319 aa) replaced with Ark1p according region (300-320 aa) was generated by PCR, cloned in frame with a C-terminal HA epitope followed by the <i>ADH1</i> terminator, and placed under <i>PRK1</i> promoter control in pRS316.
pPAN1-Myc-304	The DNA coding region for Pan1p (1252-1480 aa) was generated by PCR and cloned in frame with a C-terminal Myc epitope followed by the <i>ADH1</i> terminator in pRS304.
pPAN1-Myc	<i>PAN1</i> open reading frame was generated by PCR and cloned in frame with a C-terminal Myc epitope followed by the <i>ADH1</i> terminator, and placed under <i>PAN1</i> promoter control in pRS314.
pMyc-SLA1-304	Myc-Sla1p. <i>SLA1</i> open reading frame was generated by PCR and cloned in frame after three copies of the Myc epitope, under <i>SLA1</i> promoter control in pRS304.
pMyc-SLA1	Myc-Sla1p. <i>SLA1</i> open reading frame was generated by PCR and cloned in frame after three copies of the Myc epitope, under <i>SLA1</i> promoter control in pRS314.
pGADT7-ARP2	<i>ARP2</i> coding region was generated by PCR and cloned in frame into pGADT7.
pGBKT7-ARP2	<i>ARP2</i> coding region was generated by PCR and cloned in frame into pGBKT7.
pGBKT7-PRK1 ^{D158Y}	The coding region of Prk1 ^{D158Y} p was generated by PCR and cloned in frame into pGBKT7 (Zeng <i>et al.</i> , 2001).
pGBKT7-PRK1 ^{D158Y} (1-747)	The DNA coding region of Prk1 ^{D158Y} p (1-747 aa) was generated by PCR and cloned in frame in pGBKT7.
pGBKT7-PRK1 ^{D158Y} (1-319)	The DNA coding region of Prk1 ^{D158Y} p (1-319 aa) was generated by PCR and cloned in frame in pGBKT7.
pGADT7-PRK1 ^{D158Y} (1-298)	The DNA coding region for Prk1p (1-298 aa) was generated by PCR and cloned in frame into pGADT7.
pGEX-SR	GST-SR; The DNA coding region for Sla1p (1068-1244 aa) was generated by PCR and cloned in frame into pGEX-4T-1.
pGEX-SR-ARK1 ₂₉₉ -HA	For GST-SR and ARK1 ₂₉₉ -HA coexpression in <i>E. coli</i> . The DNA coding region for Sla1p (1068-1244 aa) followed by three stop codons, was fused with a translational enhancer, a Shine-Dalgarno sequence and Ark1p kinase domain coding region (1-299 aa) followed with a HA tag at C-terminal by PCR fusion method, and cloned into the BamHI and XhoI sites of pGEX-4T-1.
pGEX-SRmut-ARK1 ₂₉₉ -HA	For GST-SRmut and ARK1 ₂₉₉ -HA coexpression in <i>E. coli</i> . GST-SRmut is GST-SR variant with all TG to AG mutations.
pGEX-SR-ARK1 ^{D159Y} ₂₉₉ -HA	For GST-SR and ARK1 ^{D159Y} ₂₉₉ -HA coexpression in <i>E. coli</i> . ARK1 ^{D159Y} ₂₉₉ -HA is ARK1 ₂₉₉ -HA variant with D159Y mutation.
pGEX-SR-PRK1 ₂₉₈ -HA	For GST-SR and PRK1 ₂₉₈ -HA coexpression in <i>E. coli</i> . The DNA coding region for Sla1p (1068-1244 aa) followed by three stop codons, was fused with a translational enhancer, a Shine-Dalgarno sequence and Prk1p kinase domain coding region (1-298 aa) followed with a HA tag at C-terminal by PCR fusion method, and cloned in frame into the BamHI and XhoI sites of pGEX-4T-1.
pGEX-LR1-ARK1 ₂₉₉ -HA	For GST-LR1 and ARK1 ₂₉₉ -HA coexpression in <i>E. coli</i> . The DNA coding region for Pan1p (1-245 aa) followed by three stop codons, was fused with a translational enhancer, a Shine-Dalgarno sequence and Ark1p kinase domain coding region (1-299 aa) followed with a HA tag at C-terminal by PCR fusion method, and cloned in frame into pGEX-4T-1.
pGEX-LR1-ARK1 ^{D159Y} ₂₉₉ -HA	For GST-LR1 and ARK1 ^{D159Y} ₂₉₉ -HA coexpression in <i>E. coli</i> . ARK1 ^{D159Y} ₂₉₉ -HA is ARK1 ₂₉₉ -HA variant with D159Y mutation.
pGEX-LR1-PRK1 ₂₉₈ -HA	For GST-LR1 and PRK1 ₂₉₈ -HA coexpression in <i>E. coli</i> . The DNA coding region for Pan1p (1-245 aa) followed by three stop codons, was fused with a translational enhancer, a Shine-Dalgarno sequence and Prk1p kinase domain coding region (1-298 aa) followed with a HA tag at C-terminal by PCR fusion method, and cloned into the BamHI and XhoI sites of pGEX-4T-1.
pGEX-LR2-ARK1 ₂₉₉ -HA	For GST-LR2 and ARK1 ₂₉₉ -HA coexpression in <i>E. coli</i> . The DNA coding region for Pan1p (384-584 aa) followed by three stop codons, was fused with a translational enhancer, a Shine-Dalgarno sequence and Ark1p kinase domain coding region (1-299 aa) followed with a HA tag at C-terminal by PCR fusion method, and cloned into the BamHI and XhoI sites of pGEX-4T-1.

Continued

Table 2. *Continued*

Constructs	Description
pGEX-LR2-ARK1 ^{D159Y} ₂₉₉ -HA	For GST-LR2 and ARK1 ^{D159Y} ₂₉₉ -HA coexpression in <i>E. coli</i> . ARK1 ^{D159Y} ₂₉₉ -HA is ARK1 ₂₉₉ -HA variant with D159Y mutation.
pGEX-LR2-PRK1 ₂₉₈ -HA	For GST-SR and PRK1 ₂₉₈ -HA coexpression in <i>E. coli</i> . The DNA coding region for Pan1p (384-584 aa) followed by three stop codons, was fused with a translational enhancer, a Shine-Dalgarno sequence and Prk1p kinase domain coding region (1-298 aa) followed with an HA tag at C-terminal by PCR fusion method, and cloned in frame into the BamHI and XhoI sites of pGEX-4T-1.
pGAL-HA-PRK1	The PRK1 coding region was generated by PCR, cloned in frame with the HA epitope, and placed under GAL1 promoter control in pRS316 (Zeng and Cai, 1999).
pGAL-HA-ARK1	The ARK1 coding region was generated by PCR, cloned in frame with the HA epitope, and placed under GAL1 promoter control in pRS316.
pGAL-HA-ARK1 ^{D159Y}	The ARK1 ^{D159Y} coding region was generated by PCR, cloned in frame with the HA epitope, and placed under GAL1 promoter control in pRS316.
pGAL- PRK1 ^{D158Y} -EGFP	PRK1 ^{D158Y} coding region was generated by PCR, cloned in frame with a C-terminal EGFP epitope followed by the ADH1 terminator, and placed under GAL1 promoter control in pRS316.
pGAL- PRK1 ^{D158Y} ₃₁₉ -EGFP	The DNA coding region for Prk1 ^{D158Y} p (1-319 aa) was generated by PCR, cloned in frame with a C-terminal EGFP epitope followed by the ADH1 terminator, and placed under GAL1 promoter control in pRS316.
pGAL- PRK1 ^{D158Y} ₂₉₈ -EGFP	The DNA coding region for Prk1 ^{D158Y} p (1-298 aa) was generated by PCR, cloned in frame with a C-terminal EGFP epitope followed by the ADH1 terminator, and placed under GAL1 promoter control in pRS316.
pGAL- PRK1 ^{D158Y} _{AR} -EGFP	The DNA coding region for Prk1 ^{D158Y} p with Prk1p (299-319 aa) replaced with Ark1p according region (300-320 aa) was generated by PCR, cloned in frame with a C-terminal EGFP epitope followed by the ADH1 terminator, and placed under GAL1 promoter control in pRS316.
pGAL-PRK1 ^{D158Y} _{AR} Δpp-EGFP	The DNA coding region for Prk1 ^{D158Y} p (1-747 aa) with Prk1p (299-319 aa) replaced with Ark1p according region (300-320 aa) was generated by PCR, cloned in frame with a C-terminal EGFP epitope followed by the ADH1 terminator, and placed under GAL1 promoter control in pRS316.
pGAL-PRK1 ^{D158Y} _{AR} Δpp-Myc	The DNA coding region for Prk1 ^{D158Y} p (1-747 aa) with Prk1p(299-319 aa) replaced with Ark1p according region (300-320 aa) was generated by PCR, cloned in frame with a C-terminal Myc epitope followed by the ADH1 terminator, and placed under GAL1 promoter control in pRS316.
pGAL-PRK1 ^{D158Y} Δpp-Myc	The DNA coding region for Prk1 ^{D158Y} p (1-747 aa) was generated by PCR, cloned in frame with a C-terminal Myc epitope followed by the ADH1 terminator, and placed under GAL1 promoter control in pRS316.
pGEX-PRK1 ₃₁₉	GST-PRK1319; The DNA coding region for Prk1p (1-319 aa) was generated by PCR and cloned in frame into pGEX-6p-1.
pGEX-PRK1 ₂₉₈	GST-PRK1298; The DNA coding region for Prk1p (1-298 aa) was generated by PCR and cloned in frame into pGEX-6p-1.
pET-ARP2	His-ARP2; ARP2 coding region without intron was generated by PCR and cloned in frame into pET-Duet-1.
ARP2-HA-305	The DNA coding region for Arp2p with Arp2p promoter was generated by PCR and cloned in frame with a C-terminal HA epitope followed by the ADH1 terminator in pRS305.
pPRK1Δpp-GFP	The DNA coding region for Prk1p (1-747 aa) was generated by PCR and cloned in frame with a C-terminal GFP epitope followed by the ADH1 terminator, and placed under PRK1 promoter control in pRS316.
pARK1Δpp-GFP	The DNA coding region for Ark1p (1-606 aa) was generated by PCR and cloned in frame with a C-terminal GFP epitope followed by the ADH1 terminator, and placed under ARK1 promoter control in pRS316.
pARK1-GFP	ARK1 coding region was generated by PCR, cloned in frame with a C-terminal GFP epitope followed by the ADH1 terminator, and placed under PRK1 promoter control in pRS316.
pPRK1-GFP	PRK1 coding region was generated by PCR, cloned in frame with a C-terminal GFP epitope followed by the ADH1 terminator, and placed under PRK1 promoter control in pRS316.
pARK1 _{PR} -HA	The DNA coding region for Ark1p with Ark1p (300-320 aa) replaced with Prk1p according region (299-319 aa) was generated by PCR, cloned in frame with a C-terminal HA epitope followed by the ADH1 terminator, and placed under ARK1 promoter control in pRS316.
pPAN1-4-Myc-304	The DNA coding region for Pan1p (1-849 aa) was generated by PCR and cloned in frame with a C-terminal Myc epitope followed by the ADH1 terminator in pRS304.
pGBKT7-ARK1 ^{D159Y} _{PR}	The DNA coding region of Ark1 ^{D159Y} p, with Ark1p (300-320 aa) replaced with Prk1p according region (299-319 aa) was generated by PCR and cloned in frame in pGBKT7.

RESULTS

Kinase Activity of Ark1p

Ark1p is essential for endocytosis and the normal actin patch structures in the absence of Prk1p (Cope *et al.*, 1999). Unlike Prk1p, however, the targets of Ark1p have not been clearly demonstrated. As the two kinases are functionally redundant, at least some of the substrates of Prk1p are expected to be shared by Ark1p. We first examined the kinase activity of Ark1p using Sla1p as a substrate. As

shown in Figure 1A, the C-terminal region of Sla1p (GST-SR) containing multiple Prk1p recognition motifs was readily phosphorylated by immunoprecipitated HA-Prk1p, as has been reported previously (Zeng *et al.*, 2001). HA-Ark1p prepared in the same way could also phosphorylate GST-SR, albeit much less effectively (Figure 1A). A mutation at the putative catalytic site of Ark1p (Ark1^{D159Y}) largely abolished the kinase activity (Figure 1A). This experiment was repeated using only the N-terminal kinase domains of Prk1p and Ark1p, and the Ark1p kinase domain again exhibited a

Figure 1. Phosphorylation of Sla1p and Pan1p by Ark1p. (A) In vitro phosphorylation of Sla1p by Ark1p. Wild-type Ark1p, the kinase-inactivated Ark1^{D158Y}, and wild-type Prk1p were expressed as HA-tagged proteins and quantified by immunoprecipitation and Western analysis. Equal amounts of the immunoprecipitated proteins were used in the kinase reactions (right panel). Phosphorylation results were shown as autoradiography and the input substrates were visualized by the Coomassie Blue staining (left panel). (B) Phosphorylation of Sla1p-SR, Pan1p-LR1, and Pan1p-LR2 by Ark1p and Prk1p in *E. coli*. GST fusion substrates, indicated below the panels, coexpressed with respective kinases, indicated above the panels, were purified after 1-h induction and analyzed by SDS-PAGE and Western analysis. Lane 4 (CIP) in each panel is same as lane 1 (Ark1) but treated with the phosphatase before loading. The band of added phosphatase is indicated by asterisk. (C) In vivo phosphorylation status of Sla1p and Pan1p in different kinase deletion mutants. Top, Myc-Sla1p was immunoprecipitated from cell lysate prepared from wild-type (YMC505) and five different kinase deletion strains: *prk1Δ* (YMC506), *prk1Δ akl1Δ* (YMC507), *ark1Δ* (YMC515), *prk1Δ ark1Δ* (YMC516), and *prk1Δ ark1Δ akl1Δ* (YMC504/508), at 30°C. The immunoprecipitates from wild type (YMC505) were incubated with 1 μ l of CIP for 30 min at 37°C before loading. Bottom, Myc-tagged Pan1p was immunoprecipitated from cell lysate prepared from wild-type (YMC511) and three different kinase deletion strains: *prk1Δ* (YMC512), *prk1Δ akl1Δ* (YMC503), and *prk1pΔ ark1Δ akl1Δ* (YMC504), separated on SDS gels, and probed by the mouse anti-Myc antibody. The membrane was stripped and probed again with the rabbit anti-phosphothreonine antibody. The immunoprecipitates from wild type (YMC511) were incubated with 1 μ l of CIP for 30 min at 37°C before loading.

much weaker activity than that of Prk1p (data not shown). To investigate whether this was due to an intrinsic low activity of Ark1p or to a suboptimal assay condition, we tested the kinase activity in a bacterial coexpression system. This system has been used successfully to establish the kinase activity of SRPK1 over its substrate ASF/SF2 (Yue *et al.*, 2000) and to test the effect of phosphorylation on protein interactions (Shaywitz *et al.*, 2002). GST-SR was coexpressed with the HA-tagged kinase domain of either Ark1p or Prk1p in a bicistronic expression vector. The expression of the kinases was confirmed by immunoblotting to be at similar levels (data not shown). As shown in Figure 1B, GST-SR coexpressed with either kinase domain became equally extensively phosphorylated, whereas no phosphorylation could be observed when coexpressed with Ark1^{D158Y} (Figure 1B, top panel). The extensive phosphorylation of GST-SR caused the protein band to be up-shifted from 40 up to ~60 kDa. The phosphorylation was confined to the predicted Prk1 recognition sites, as converting all the threonine residues in these consensus sites to alanine (GST-SRmut) by site-directed mutagenesis abolished phosphorylation by either Ark1p or Prk1p, even though numerous other threonine residues were still present. Similar experiments were also carried out with two Pan1p N-terminal long repeats (LR): LR1 and LR2 (Zeng and Cai, 1999). Ark1p phosphorylated LR1 and LR2 as efficiently as Prk1p (Figure 1B, bottom panels). These results showed that Ark1p is able to phosphorylate at least two of the Prk1p's native targets, Pan1p and Sla1p, in vitro.

We next determined whether Ark1p contributes to the phosphorylation of Sla1p and Pan1p in vivo. Proteins immunoprecipitated from wild-type, *prk1Δ*, *ark1Δ*, *prk1Δ akl1Δ*, and *prk1Δ ark1Δ akl1Δ* cells were examined by immunoblotting with an anti-phospho-threonine antibody. The level of Sla1p phosphorylation from *prk1Δ*, *ark1Δ*, *prk1Δ ark1Δ*, or *prk1Δ akl1Δ* cells was similar to that of wild-type cells, whereas no signal was detected from the triple deletion *prk1Δ ark1Δ akl1Δ* cells or after phosphatase treatment (CIP), indicating that Ark1p could indeed phosphorylate Sla1p in vivo (Figure 1C). In fact, the level of Sla1p phosphorylation remained essentially unchanged as long as one of the three kinases was functional (data not shown). In contrast, deletion of the *PRK1* gene alone resulted in a drastic reduction in the Pan1p phosphorylation (Figure 1C), suggesting that Pan1p, unlike Sla1p, is more restricted to the regulation by Prk1p. These results revealed that, despite the presence of similar phosphorylation motifs in both proteins, Pan1p and Sla1p are subjected to different modes of regulation by different Prk1p-like kinases in vivo.

Nonkinase Domains Mediate Distinct Genetic Interactions

Previous genetic studies have suggested some functional differences between Ark1p and Prk1p (Cope *et al.*, 1999). Consistent with the Pan1p phosphorylation in vivo being primarily dependent on Prk1p, we also found that a temperature-sensitive mutant of *PAN1*, *pan1-4*, could be suppressed by deletion of *PRK1* but not *ARK1* (Zeng and Cai, unpublished data). *pan1-4* is a nonsense mutation in the C-terminal part of LR2 near the End3p binding region (a C-to-T mutation at nucleotide 2545). The truncated protein of 848 aa is unable to bind to End3p at the nonpermissive temperature and therefore is hyperphosphorylated at 37°C (Tang and Cai, unpublished data). Consequently, the temperature sensitivity of *pan1-4* can be suppressed by either deletion of *PRK1* or overproduction of End3p (Tang *et al.*, 1997; Zeng and Cai, 1999), both of which abrogate the hyperphosphorylation of the mutant protein. That deletion of

ARK1 could not suppress *pan1-4* implies that this kinase is not critical for the phosphorylation of Pan1p in vivo. As the two kinases share extensive sequence similarity in their N-terminal kinase domain, we suspected that any in vivo functional distinctions between them should be resulted from their divergent C-terminal regions. To evaluate the functional importance of the nonkinase domains, we swapped their kinase domains (1-298 aa of Prk1p and 1-299 aa of Ark1p) to create two chimeric kinases named Prk1n-Ark1c (with the Prk1p kinase domain) and Ark1n-Prk1c (with the Ark1p kinase domain), as shown in Figure 2A. Both fusion genes were expressed from the *PRK1* promoter, and the expression levels of Prk1n-Ark1c and Ark1n-Prk1c were confirmed by Western blotting to be same as their wild-type counterparts (Supplementary Figure A). After introduction into *prk1Δ ark1Δ* cells, both fusion genes were able to rescue the mutant's temperature sensitivity at 37°C and its actin and endocytosis defects (Figure 2B, left panel and data not shown), indicating that the fusion kinases were functional. Interestingly, after they were introduced into *pan1-4 prk1Δ* cells, it was Ark1n-Prk1c that imitated the activity of Prk1p to reconstitute the temperature-sensitivity in the mutant (Figure 2B, right panel). Consistently, the level of Pan1p phosphorylation at 30°C was similarly high in the cells expressing Prk1p and Ark1n-Prk1c and equally low in Ark1p- and Prk1n-Ark1c-containing cells (Figure 2C). These results together confirmed our prediction that it is the non-kinase domain that is responsible for the distinct functions of Prk1p and Ark1p kinases.

Ark1p, But Not Prk1p, Relies on the poly-P Motif to Function

One important function of the nonkinase domains is to direct proper localization of the protein in the cell. The localization of Ark1p and Prk1p to the cortical actin patches has been shown to be achieved via an interaction between the C-terminal poly-P motif of the kinases and an SH3 domain of Abp1p (Cope *et al.*, 1999; Fazi *et al.*, 2002). However, deletion of *ABP1* does not produce a similar phenotype as observed in *prk1Δ ark1Δ*. In addition, unlike Ark1p, whose cortical association was completely eliminated in the *abp1* mutant, the cortical localization of some Prk1p proteins has been noted to be Abp1p-independent (Cope *et al.*, 1999; Fazi *et al.*, 2002). Prk1p, therefore, may have an additional way to be localized cortically. To reassess the functions of the polyproline motif, we created *prk1* and *ark1* mutants lacking only the polyproline motifs and transformed them into *prk1Δ ark1Δ* cells. The expression levels of Prk1pΔPP and Ark1pΔPP were confirmed by Western blotting to be same as their wild-type counterparts (Supplementary Figure B). As shown in Figure 3A, Prk1pΔPP could complement the temperature sensitivity of *prk1Δ ark1Δ* at 37°C, while Ark1pΔPP could not. Consistent with this, Prk1pΔPP was still able to localize to the cortical patches, while Ark1pΔPP was diffused in the cell (Figure 3B). Moreover, Prk1pΔPP also rescued the actin defects in the *prk1Δ ark1Δ* cells to the extent indistinguishable from the wild type, whereas considerable actin aggregates were still visible in the cells expressing Ark1pΔPP, although in smaller sizes than those in *prk1Δ ark1Δ* cells (Figure 3C). These results suggest that Prk1p, but not Ark1p, could function independently of the polyproline motif. This also explains an earlier observation that the *prk1Δ abp1Δ* mutant acquired a similar actin defect as in the *prk1Δ ark1Δ* mutant whereas the *ark1Δ abp1Δ* mutant did not (Cope *et al.*, 1999).

Figure 2. Domain swap between Prk1p and Ark1p. (A) Schematic illustration of Prk1p, Ark1p and the chimeric kinases. Prk1n-Ark1c is made of the kinase domain of Prk1 (1-298 aa) and the nonkinase domain of Ark1 (300-638 aa), and Ark1n-Prk1c is made of the kinase domain of Ark1 (1-299 aa) and the nonkinase domain of Prk1 (299-810 aa). (B) Left, the *prk1Δ ark1Δ* mutant (YMC414) was transformed with the plasmids containing different kinase genes as indicated on the left side of the panel. The resultant strains were patched on selective medium, let grow at 30°C, and then replica-plated on a fresh plate and let grow at 37°C. Right, the *pan1-4 prk1Δ* mutant (YMC413) was transformed with the plasmids as indicated and the cells were grown to log phase and spotted onto a selective plate and incubated at 30 (left) or 37°C (right). Photographs were taken after each plate was incubated for 2 d. (C) The *prk1Δ ark1Δ* mutant containing Myc-Pan1p (YMC509) was transformed with the different kinase constructs. Myc-Pan1p was immunoprecipitated from cell lysates prepared from strains indicated above the panel, gel separated and probed sequentially by mouse anti-Myc and rabbit anti-phosphothreonine antibodies. The phosphorylation level of Pan1-Myc in each sample was measured by densitometer and normalized against its protein amount. The relative phosphorylation intensities were calculated and presented as bar graphs shown below.

Identification of Arp2p as a New Anchor Protein for Prk1p

The above results prompted us to start searching for new Prk1p anchor proteins that may interact with the C-terminal region of Prk1p in a polyproline-independent manner. We used the two-hybrid system to test interactions of Prk1pΔPP with a number of known actin patch-associated proteins including Pan1p, Sla1p, End3p, Scd5p, Sac6p, Cap1p, Cap2p, Arp2p, and Arp3p. Among them, Arp2p, the core component of Arp2/3 complex, was found capable of binding to Prk1pΔPP but not to Ark1pΔPP (Figure 4A). Using

Figure 3. Ark1p, but not Prk1p, depends on C-terminal poly-P for function. (A) The *prk1Δ ark1Δ* mutant (YMC414) was transformed with single-copy plasmid carrying *PRK1*, *ARK1*, *PRK1ΔPP*, and *ARK1ΔPP* (with deletions in the polyproline stretch). The resultant strains were patched on selective medium at 30°C (left), replica-plated on a fresh plate, and incubated at 37°C (right). Photographs were taken after each plate was incubated for 2 d. (B) Prk1p, but not Ark1p, can localize to cortical patches without the C-terminal poly-P motif. Plasmids carrying GFP tagged Prk1p, Ark1p, Prk1ΔPP, and Ark1ΔPP, each under their native promoters, were transformed into *prk1Δ* (YMC410) or *ark1Δ* (YMC409), and the transformants were examined under a fluorescent microscope. (C). Rhodamine-phalloidin staining of actin filaments in the cells described above. Scale bars, 5 μm.

various deletion constructs of Prk1p, the region necessary for interacting with Arp2p was localized to a 21-amino acid region (299-319 aa) next to the kinase domain. To determine whether the interaction between Prk1p and Arp2p is direct, the binding between His-tagged Arp2p and GST-Prk1₁₋₃₁₉ was investigated. Equal amounts of bead-immobilized GST-Prk1₁₋₃₁₉ and GST-Prk1₁₋₂₉₈ (kinase domain only) were mixed with equal amount of purified His-Arp2, respectively. After washing, the bound proteins were analyzed by Western blot with anti-GST and anti-His antibodies. As shown in Figure 4B, His-Arp2 could be precipitated by GST-Prk1₁₋₃₁₉ but not by GST-Prk1₁₋₂₉₈, indicating that the 21-amino acid motif is required for the direct binding of Prk1p to Arp2p. The interaction between Prk1p and Arp2p was further confirmed by coimmunoprecipitation. The *PRK1* gene without the C-terminal poly-P region, *PRK1ΔPP*, was tagged with the Myc-epitope and placed under the *GAL1* promoter to enhance its expression. After galactose induction for 2 h, cell lysates were prepared and Myc-Prk1p was precipitated. As shown in Figure 4C, HA-Arp2 could be coimmunoprecipitated by the anti-Myc antibody. The coimmunoprecipitation was abolished if the 21 aa region of Prk1p was replaced by the corresponding region from Ark1p (Prk1_{AR}ΔPP).

To test whether the 21-aa region was sufficient for Prk1p to achieve the cortical localization, GFP fusion proteins of wild-type Prk1p, Prk1₁₋₃₁₉, Prk1₁₋₂₉₈, Prk1_{AR}, and Prk1_{AR}ΔPP were placed under the control of *GAL1* promoter and transformed into wild-type cells. The cortical GFP signals were observed only in the cells expressing Prk1-GFP, Prk1₁₋₃₁₉-GFP, and Prk1_{AR}-GFP. On the other hand, the GFP signals of Prk1₁₋₂₉₈-GFP and

Figure 4. Identification of Arp2p as a new adapter protein for Prk1p. (A) Two-hybrid interaction between Prk1p and Arp2p. (B) In vitro binding of Arp2p with Prk1p. GST fusion proteins of Prk1₁₋₃₁₉ and Prk1₁₋₂₉₈ were immobilized on glutathione-agarose beads and incubated with His-Arp2. Bound proteins were analyzed by Western blotting with the anti-His antibody and the GST fusion proteins were detected by the anti-GST antibody. (C) Coimmunoprecipitation of Prk1p and Arp2p. Yeast extracts in equal amounts prepared from YMC510 (*arp2::Arp2-HA*) and YMC510 containing pGAL-PRK1^{D158Y}ΔPP-Myc or pGAL-Prk1^{D158Y}ARΔPP-Myc were subjected to anti-Myc immunoprecipitation. The bound proteins were analyzed by immunoblotting with anti-HA antibody. Extracts used are shown below the gel. The extract lane is the extracts from the pGAL-PRK1^{D158Y}ARΔPP-Myc containing strain.

Prk1_{AR}ΔPP-GFP were diffused in the cytoplasm (Figure 5). This result indicates that either the Arp2p-interacting region or the Abp1p-interacting region is sufficient for Prk1p to be localized to cortical patches. Moreover, we also noted that more than 90% of the Prk1-GFP and Prk1₁₋₃₁₉-GFP patches colocalized with the actin patches.

The region from Arp2p involved in the interaction with Prk1p has remained undefined, however. None of the constructs containing truncated Arp2p was able to confer positive interactions with Prk1p when tested in our two-hybrid assay (data not shown).

Pan1p Phosphorylation by Prk1p Depends on the Prk1p-Arp2p Interaction

To evaluate the functional significance of the Arp2p-Prk1p interaction, wild-type Prk1p, Prk1₁₋₃₁₉ and Prk1₁₋₂₉₈ and Prk1_{AR} (with the polyproline region) were transformed into *prk1Δ ark1Δ* and *pan1-4 prk1Δ* cells, respectively, and their expressions were confirmed by Western analysis (Supplementary Figure C). The resultant strains were tested for growth at 37°C. Wild-type Prk1p, Prk1₁₋₃₁₉, and Prk1_{AR} could rescue the temperature sensitivity of *prk1Δ ark1Δ* at 37°C, whereas the kinase domain alone (Prk1₁₋₂₉₈) failed to do the same (Figure 6A, left panel). Similarly, wild-type Prk1p, Prk1₁₋₃₁₉, and Prk1_{AR} could rescue the actin defect and the defect in Lucifer Yellow uptake of the *prk1Δ ark1Δ* cells, whereas Prk1₁₋₂₉₈ and Prk1_{AR}ΔPP could not (Figure 6B). This result suggests that so long as the localization to the cortical patches is achieved, either by the interaction with Arp2p or with Abp1p, Prk1p will be able to perform

Figure 5. The Arp2p binding region of Prk1p is required for its cortical localization. Wild-type cells (W303-1A) were transformed with plasmids carrying pGAL-PRK1^{D158Y}-GFP, pGAL-PRK1^{D158Y}₁₋₃₁₉-GFP, pGAL-PRK1^{D158Y}₁₋₂₉₈-GFP, pGAL-PRK1^{D158Y}_{AR}-GFP, and pGAL-PRK1^{D158Y}_{ARΔPP}-GFP. After 1-h galactose induction, cells were fixed and stained with rhodamine (Rd)-phalloidin. Because overexpression of Prk1p could disturb actin cytoskeleton, an inactive kinase (D158Y) was used in this experiment.

the essential functions shared between itself and Ark1p. However, only the interaction with Arp2p enables Prk1p to perform Prk1p-specific functions, as only wild-type Prk1p and Prk1₁₋₃₁₉ could restore the temperature sensitivity to *pan1-4 prk1Δ* cells at 37°C, whereas Prk1_{AR}, despite its ability to localize to the cortical patches and to rescue the double kinase deletion mutant, failed to do so (Figure 6A, right panel). Consistently, replacement of the same region in Ark1p with the 21 aa Arp2p binding region of Prk1p allowed the new kinase (Ark1_{PR}) to carry out Prk1p-specific functions, as it was now able to restore the temperature sensitivity in *pan1-4 prk1Δ* cells at 37°C (Figure 6C).

As explained earlier, an ability to reinstate temperature sensitivity to *pan1-4 prk1Δ* cells is an indicator of the ability to phosphorylate Pan1p in vivo. To verify the phosphorylation of Pan1p in vivo as the Prk1p-specific function mediated by Arp2p, we first analyzed the phosphorylation status of Pan1p in the *prk1Δ ark1Δ* mutant carrying Prk1₁₋₃₁₉, Prk1₁₋₂₉₈, or Prk1_{AR}. Indeed, Prk1₁₋₃₁₉ restored Pan1p phosphorylation close to the wild-type level, whereas Prk1₁₋₂₉₈ had no effect (Figure 7A). Prk1_{AR}, which is a functional equivalent of Ark1p, was able to increase the Pan1p phosphorylation level only slightly, approximately to the residual Pan1p phosphorylation level remained in the *prk1Δ* mutant (~20% of the wild-type level). The *pan1-4* mutant protein (Pan1-4p), as noted previously, exhibited a high steady-state level of phosphorylation at 37°C in the presence of wild-type

Prk1p (Figure 7B). The similar high level of phosphorylation was maintained by the kinase variants that possessed the Arp2p-binding capacity, i.e., Ark1n-Prk1c and Ark1_{PR}, but not by those that did not (Ark1, Prk1n-Ark1c, and Prk1_{AR}; Figure 7C). This result confirms that the interaction with Arp2p is responsible for the Prk1p-specific phosphorylation of Pan1p and suggests that Arp2p and Abp1p recruit the Prk1p kinase for different regulatory purposes.

DISCUSSION

Overlapping versus Distinct Functions of Prk1p and Ark1p

Compared with Prk1p, Ark1p has been much less studied and its phosphorylation targets are poorly understood. It is, however, deemed functionally redundant to Prk1p by the fact that the mutants of either *prk1* or *ark1* have no obvious phenotypes but simultaneous loss of both proteins is disastrous for cell growth, due to the severe defects in actin organization and endocytosis. A typical case was reported by Sekiya-Kawasaki *et al.* (2003), where inhibition of Prk1p in the *ark1* mutant led to rapid and Arp2/3-dependent accumulation of aggregated endocytic intermediates composed of vesicles wrapped by actin filaments. The functional redundancy of Prk1p and Ark1p must reside in the ability of the pair to phosphorylate an overlapping spectrum of sub-

Figure 6. Role of the interaction with Arp2p in the function of Prk1p. (A) The *prk1Δ ark1Δ* (YMC414) and *pan1-4 prk1Δ* (YMC413) strains were transformed with the constructs as indicated. The resultant cells were grown to log phase and spotted on selective medium and incubated at 30 (left) and 37°C (right). Photographs were taken after cells were grown for 2 d. (B) Endocytosis and actin structures of different *prk1* mutants. The YMC414 (*prk1Δ ark1Δ*) cells carrying different constructs as indicated were subjected to staining for actin filaments and the Lucifer yellow uptake assay. Scale bars, 5 μ m. (C) Left, YMC414 was transformed with plasmids containing different kinase genes as indicated. The resultant strains were grown to log phase and spotted onto a selective plate and incubated at 30 (left) or 37°C (right). Center, YMC413 was transformed with plasmids containing different kinase genes as indicated. The resultant strains were grown to log phase and spotted onto a selective plate and incubated at 30 (left) or 37°C (right). Photographs were taken after each plate was incubated for 2 d. Right, expression of Ark1p and Ark1_{PRP} in *prk1Δ ark1Δ* mutant. TCA extracts from indicated cells were analyzed by immunoblotting with anti-HA and anti-G6PDH.

strates. In this report, we demonstrate by a bacterial coexpression system that the kinase domains of Ark1p and Prk1p were equally efficient in phosphorylating Sla1p and Pan1p. In addition, the in vivo phosphorylation of Pan1p and, in particular, Sla1p, became dependent on Ark1p in the absence of Prk1p and Ark1p. Our finding that Ark1p could phosphorylate Pan1p in vitro and in vivo is consistent with the report that mutations of all potential Ark/Prk1 phosphorylation sites in Pan1p resulted in actin abnormalities similarly present in *prk1Δark1Δ*, but absent from *prk1Δakl1Δ* cells (Toshima *et al.*, 2005).

It remains to be resolved whether Ark1p and Prk1p recognize the identical set of motifs in Pan1p and Sla1p. Henry *et al.* (2003) previously showed by the in vitro kinase assay that the preferred recognition motif for Ark1p was LxxAxTG instead of LxxQ/TxTG, which had been determined for Prk1p (Zeng and Cai, 1999; Huang *et al.*, 2003). The significance of this minor deviation, however, is not known. Inter-

estingly, we found that the in vivo phosphorylation level of Pan1p was primarily dependent on Prk1p, as it decreased nearly 80% in the *prk1* mutant, whereas the Sla1p phosphorylation level remained unchanged with any one of the three kinases present. This result could not be explained simply by the suggested motif preference mentioned above, as the vast majority (97%) of the 19 and 10 potential Prk1/Ark1 sites in Pan1p and Sla1p, respectively, are non-LxxAxTG sites (Huang *et al.*, 2003). Clearly, distinct regulatory mechanisms must be at work to allow different kinases to differentiate their targets.

The distinct functions of Prk1p and Ark1p were first uncovered by genetic analyses. Loss of Prk1p, but not Ark1p, was found to be lethal to the *sla2Δ* mutant and caused severe actin defects in *abp1Δ* cells (Cope *et al.*, 1999). We also knew that a temperature-sensitive mutant of Pan1p, *pan1-4*, could be suppressed by *prk1Δ*, but not by *ark1Δ*. Until the present study, the molecular basis underlining the distinct genetic interactions has remained unknown.

Nonkinase Regions Account for Distinct Functions of Ark1p and Prk1p

Prk1p and Ark1p share little sequence similarity beyond their highly homologous kinase domains. It is reasonable to speculate that the distinct functions of Ark1p and Prk1p may be mediated by the divergent C-terminal regions. This was proved to be the case by the domain swap experiments. It is rather striking that the Pan1p phosphorylation was recovered to the near wild-type level by the kinase domain of Ark1p fused to the nonkinase domain of Prk1p, whereas the Prk1p kinase domain became incompetent to perform what used to be its native task after it acquired the nonkinase domain of Ark1p. Clearly, the nonkinase domains are critical for the differential activities of Prk1p and Ark1p, at least as manifested in the phosphorylation of Pan1p.

The only element from the nonkinase region that has been known to be important for the function of the kinases is a short proline rich (poly-P) motif, which is thought to be responsible for the patch localization of Ark1p and Prk1p via interaction with Abp1p (Fazi *et al.*, 2002). The poly-P motif, however, ought not to be the sole determinant for patch localization of the kinases, as deletion of *ABP1* elicited no obvious actin and endocytic defects (Holtzman *et al.*, 1993; Cope *et al.*, 1999), and no noticeable changes in the in vivo phosphorylation level of Pan1p (Supplementary Figure). In addition, while it is true that Ark1p depends exclusively on its poly-P motif for patch localization, Prk1p is able to achieve the same destination without it. By exploring this discrepancy between Prk1p and Ark1p, we identified another sequence element in the C-terminal region of Prk1p, a 21-aa motif adjacent to the kinase domain, as the second patch-anchoring point. This 21-aa motif, which is required for Prk1p to interact with Arp2p, accounts for ~80% of the Pan1p phosphorylation level in vivo, whereas the remaining is contributed by the poly-P motif, which is also shared with Ark1p. Although the interaction with Abp1p via poly-P by itself is sufficient to allow Prk1p to support cell survival and apparently normal actin organization and endocytosis by maintaining an essential level of Pan1p phosphorylation, there may be some subtle events that will require more extensive phosphorylation of Pan1p achievable only through the interaction with Arp2p. While the functional difference between Abp1p- and Arp2p-anchored Prk1p kinase remains to be comprehended, it is clear that the interaction with Arp2p serves as the primary actin patch-anchoring point.

Figure 7. Comparison of phosphorylation levels of Pan1-4p in various kinase mutants. (A) Phosphorylation status of Pan1p in different *prk1* mutants. The *prk1Δ ark1Δ* mutant containing Myc-Pan1p (YMC509) was transformed with the different kinase constructs as indicated. Myc-Pan1p was immunoprecipitated, and SDS gel separated and probed sequentially by anti-Myc and anti-phosphothreonine antibodies (left). The phosphorylation level of Pan1-Myc in each sample was normalized against its protein amount. The relative phosphorylation intensities were calculated and presented as bar graphs (right). (B) Endogenously expressed Pan1-4p-Myc was immunoprecipitated from YMC514 (*prk1Δ*) and YMC513 (PRK1) cells at either 25°C (lanes 1 and 3) or 37°C (lanes 2 and 4) for 3 h, and SDS gel was separated and sequentially immunoblotted with anti-PThr and anti-Myc antibodies. The phosphorylation level of Pan1-4p-Myc in each sample was measured by densitometer and normalized against its protein amount. The relative phosphorylation intensities were calculated and presented as bar graphs. (C) Phosphorylation status of Pan1-4p in different *prk1* mutants. Endogenously expressed Pan1-4p-Myc was immunoprecipitated at 37°C from YMC514(*prk1Δ*) cells containing pPrk1-HA-316, pArk1-HA-316, pArk1n-Prk1c-HA-316, pPrk1n-Ark1c-HA-316, pRS316, pPrk1AR-HA-316, and pArk1PR-HA-316. The relative phosphorylation intensities were calculated and presented as bar graphs.

choring point for Prk1p to efficiently phosphorylate Pan1p in vivo.

The Implication of Arp2p as an Anchor Protein for Prk1p

Pan1p is a key component of the cellular machinery responsible for actin organization and endocytosis. It not only acts as a scaffold for assembly of the endocytic complex by interacting with End3p, Sla1p, Sla2p, Ent1/2p, Yap1801/2p, and Scd5p, among others, but also provides a link between the vesicle and its propulsion, i.e., the Arp2/3-containing actin polymerization initiation complex and, as an NPF, even helps fire it up. It is well established that phosphorylation of Pan1p by Prk1p is an important mode of regulation during endocytosis (Toshima *et al.*, 2005). It marks a turning point for actin assembly at the endocytic sites. The discovery of the novel function of Arp2p as a new Prk1p anchor protein raises an intriguing possibility that an auto-regulatory mechanism may at work in this process. As Arp2p and Prk1p come to the cortical patches at about same time, possibly recruited

together by NPFs, it appears that the actin assembly machinery is equipped with a brake as it starts working. It is conceivable that the Pan1p-promoted actin assembly at the endocytic sites may only need to be very transient, and such brief burst of actin polymerization may be sufficient for that particular step of event, to induce membrane invagination, for instance. This hypothesis is in line with the recent finding that the NPF activity of Pan1p is kept inhibited by its binding protein Sla2p (Toshima *et al.*, 2007). Although it remains unknown how Pan1p is activated from Sla2p, the finding from this study has provided some insight into how it may be quickly inactivated again by the Arp2p-mediated phosphorylation.

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REFERENCES

- Cope, M. J., Yang, S., Shang, C., and Drubin, D. G. (1999). Novel protein kinases Ark1p and Prk1p associate with and regulate the cortical actin cytoskeleton in budding yeast. *J. Cell Biol.* 144, 1203–1218.
- Duncan, M. C., Cope, M. J., Goode, B. L., Wendland, B., and Drubin, D. G. (2001). Yeast Eps15-like endocytic protein, Pan1p, activates the Arp2/3 complex. *Nat. Cell Biol.* 3, 687–690.
- Engqvist-Goldstein, A. E., and Drubin, D. G. (2003). Actin assembly and endocytosis: from yeast to mammals. *Annu. Rev. Cell Dev. Biol.* 19, 287–332.
- Fazi, B., Cope, M. J., Douangamath, A., Ferracuti, S., Schirwitz, K., Zucconi, A., Drubin, D. G., Wilmanns, M., Cesareni, G., and Castagnoli, L. (2002). Unusual binding properties of the SH3 domain of the yeast actin-binding protein Abp1, structural and functional analysis. *J. Biol. Chem.* 277, 5290–5298.
- Henry, K. R., D'Hondt, K., Chang, J. S., Nix, D. A., Cope, M. J., Chan, C. S., Drubin, D. G., and Lemmon, S. K. (2003). The actin-regulating kinase Prk1p negatively regulates Scd5p, a suppressor of clathrin deficiency, in actin organization and endocytosis. *Curr. Biol.* 13, 1564–1569.
- Holtzman, D. A., Yang, S., and Drubin, D. G. (1993). Synthetic-lethal interactions identify two novel genes, SLA1 and SLA2, that control membrane cytoskeleton assembly in *Saccharomyces cerevisiae*. *J. Cell Biol.* 122, 635–644.
- Huang, B., Zeng, G., Ng, A. Y., and Cai, M. (2003). Identification of novel recognition motifs and regulatory targets for the yeast actin-regulating kinase Prk1p. *Mol. Biol. Cell* 14, 4871–4884.
- Kaksonen, M., Sun, Y., and Drubin, D. G. (2003). A pathway for association of receptors, adaptors, and actin during endocytic internalization. *Cell* 115, 475–487.
- Kaksonen, M., Toret, C. P., and Drubin, D. G. (2005). A modular design for the clathrin- and actin-mediated endocytosis machinery. *Cell* 123, 305–320.
- Kaksonen, M., Toret, C. P., and Drubin, D. G. (2006). Harnessing actin dynamics for clathrin-mediated endocytosis. *Nat. Rev. Mol. Cell Biol.* 7, 404–414.
- Lechler, T., Shevchenko, A., and Li, R. (2000). Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. *J. Cell Biol.* 148, 363–373.
- Moseley, J. B., and Goode, B. L. (2006). The yeast actin cytoskeleton: from cellular function to biochemical mechanism. *Microbiol. Mol. Biol. Rev.* 70, 605–645.
- Rodal, A. A., Manning, A. L., Goode, B. L., and Drubin, D. G. (2003). Negative regulation of yeast WASp by two SH3 domain-containing proteins. *Curr. Biol.* 13, 1000–1008.
- Sekiya-Kawasaki, M. *et al.* (2003). Dynamic phosphoregulation of the cortical actin cytoskeleton and endocytic machinery revealed by real-time chemical genetic analysis. *J. Cell Biol.* 162, 765–772.
- Shaywitz, A. J., Dove, S. L., Greenberg, M. E., and Hochschild, A. (2002). Analysis of phosphorylation-dependent protein-protein interactions using a bacterial two-hybrid system. *Sci. STKE* 2002, L11.
- Smythe, E., and Ayscough, K. R. (2003). The Ark1/Prk1 family of protein kinases. Regulators of endocytosis and the actin skeleton. *EMBO Rep.* 4, 246–251.
- Sun, Y., Martin, A. C., and Drubin, D. G. (2006). Endocytic internalization in budding yeast requires coordinated actin nucleation and myosin motor activity. *Dev. Cell* 11, 33–46.
- Tan, S. (2001). A modular polycistronic expression system for overexpressing protein complexes in *Escherichia coli*. *Protein Expr. Purif.* 21, 224–234.
- Tang, H. Y., Munn, A., and Cai, M. (1997). EH domain proteins Pan1p and End3p are components of a complex that plays a dual role in organization of the cortical actin cytoskeleton and endocytosis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 17, 4294–4304.
- Tang, H. Y., Xu, J., and Cai, M. (2000). Pan1p, End3p, and Sla1p, three yeast proteins required for normal cortical actin cytoskeleton organization, associate with each other and play essential roles in cell wall morphogenesis. *Mol. Cell. Biol.* 20, 12–25.
- Toshima, J., Toshima, J. Y., Duncan, M. C., Cope, J. T., Sun, Y., Martin, A. C., Anderson, S., Yates, J. R., III, Mizuno, K., and Drubin, D. G. (2007). Negative regulation of yeast Eps15-like Arp2/3 complex activator, Pan1p, by the Hip1R-related protein, Sla2p, during endocytosis. *Mol. Biol. Cell* 18, 658–668.
- Toshima, J., Toshima, J. Y., Martin, A. C., and Drubin, D. G. (2005). Phosphoregulation of Arp2/3-dependent actin assembly during receptor-mediated endocytosis. *Nat. Cell Biol.* 7, 246–254.
- Watson, H. A., Cope, M. J., Groen, A. C., Drubin, D. G., and Wendland, B. (2001). In vivo role for actin-regulating kinases in endocytosis and yeast epsin phosphorylation. *Mol. Biol. Cell* 12, 3668–3679.
- Wendland, B., and Emr, S. D. (1998). Pan1p, yeast eps15, functions as a multivalent adaptor that coordinates protein-protein interactions essential for endocytosis. *J. Cell Biol.* 141, 71–84.
- Wendland, B., McCaffery, J. M., Xiao, Q., and Emr, S. D. (1996). A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. *J. Cell Biol.* 135, 1485–1500.
- Yue, B. G., Ajuh, P., Akusjarvi, G., Lamond, A. I., and Kreivi, J. P. (2000). Functional coexpression of serine protein kinase SRPK1 and its substrate ASF/SF2 in *Escherichia coli*. *Nucleic Acids Res.* 28, E14.
- Zeng, G., and Cai, M. (1999). Regulation of the actin cytoskeleton organization in yeast by a novel serine/threonine kinase Prk1p. *J. Cell Biol.* 144, 71–82.
- Zeng, G., Yu, X., and Cai, M. (2001). Regulation of yeast actin cytoskeleton-regulatory complex Pan1p/Sla1p/End3p by serine/threonine kinase Prk1p. *Mol. Biol. Cell* 12, 3759–3772.