Novel Protein Kinases Ark1p and Prk1p Associate with and Regulate the Cortical Actin Cytoskeleton in Budding Yeast

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Abstract. Ark1p (actin regulating kinase 1) was identified as a yeast protein that binds to Sla2p, an evolutionarily conserved cortical actin cytoskeleton protein. A rk1p and a second yeast protein, Prk1p, contain NH2-terminal kinase domains that are 70% identical. Together with six other putative kinases from a number of organisms, these proteins define a new protein kinase family that we have named the Ark family.

Lack of both Ark1p and Prk1p resulted in the formation of large cytoplasmic actin clumps and severe defects in cell growth. These defects were rescued by wild-type, but not by kinase-dead versions of the proteins. Elevated levels of either Ark1p or Prk1p caused a number of actin and cell morphological defects that were not observed when the kinase-dead versions were overexpressed instead. A rk1p and Prk1p were shown to localize to actin cortical patches, making these two kinases the first signaling proteins demonstrated to be patch components. These results suggest that Ark1p and Prk1p may be downstream effectors of signaling pathways that control actin patch organization and function. Furthermore, results of double-mutant analyses suggest that Ark1p and Prk1p function in overlapping but distinct pathways that regulate the cortical actin cytoskeleton.

Key words: protein kinases • Saccharomyces cerevisiae • cyclin-G–associated kinases (GAKs) • endocytosis • actin

The actin cytoskeleton plays a pivotal role in governing cellular morphology and provides a structural framework for organelle transport and cytokinesis. The dynamic nature of actin filaments is essential for their ability to function; actin filaments turn over rapidly and therefore can rapidly be disassembled and reassembled in response to cues from the cell's environment. However, these dynamics imply instability, and instability necessitates tight regulation. Therefore, the actin cytoskeleton is controlled by a number of parallel and overlapping regulatory pathways. Small GTPases of the ras superfamily, such as Rho (Ridley and Hall, 1992), Rac (Ridley et al., 1992), and Cdc42 (Kozma et al., 1995; Nobes and Hall, 1995), are critical enzymes in these signaling pathways (for reviews see Hall, 1998; Tanaka and Takai, 1998). Kinases are often responsible for further amplifying and dispersing the signals necessary for actin modulation in response to the various needs of the cell. For example, the p21-activated kinases (PAKs) and the LIM kinases, directly or indirectly, are activated by Rho subfamily GTPases and have been shown to phosphorylate components of the actin-based cytoskeleton (Ramos et al., 1997; Sells and Chernoff, 1997; Arber et al., 1998; Yang et al., 1998). The final targets of these signaling pathways include actin-binding proteins such as actin depolymerizing factors (ADF), profilin, myosin heavy and light chains, and members of the ezrin, radixin, and moesin (ERM) family of proteins (for reviews see Sohn and Goldschmidt-Clermont, 1994; Tsukita and Yonemura, 1997; Lappalainen et al., 1998). Although many signaling proteins that are implicated in cytoskeleton regulation have been identified, the complex pathways regulating the actin cytoskeleton have not been elucidated fully. A more thorough dissection of known pathways and the identification of additional components of these pathways will be necessary before it will be possible to develop a complete understanding of how a cell changes its shape, membrane dynamics, organization, or motility in response to environmental and internal cues.

Several signal transduction proteins with homology to the above-mentioned mammalian GTPases and kinases have been implicated in the control of actin organization in the budding yeast Saccharomyces cerevisiae. These include the GTPases Cdc42p and Rho1, as well as downstream effectors of Cdc42 including the PAK kinases Cla4p and Ste20p (A dams et al., 1990; Johnson and Pringle, 1990;...
E by et al., 1998). Therefore, budding yeast provides a more simple, genetically tractable model organism in which to identify new components of these pathways.

In yeast, filamentous actin is found in two forms, cables and patches. A ctn cables are oriented along the mother-bud axis and are involved in processes such as organelle inheritance and vesicle targeting (Drubin et al., 1993; Simon et al., 1995). A ctn patches are motile structures found at regions of cortical expansion where they appear to be involved in endocytosis (Novick and Botstein, 1985; Kubler and Riezman, 1993; Doyle and Botstein, 1996; Waddle et al., 1996; Mulholland et al., 1997). Much of our understanding of actin dynamics in yeast has resulted from genetic studies that have allowed the identification of actin-binding proteins and have revealed redundant regulatory mechanisms (for review see Ayscough and Waddell, 1996; Botstein et al., 1997).

SLA2 (synthetic lethal with A bp1) was identified in a screen for mutations that are synthetic lethal with a null allele of the gene encoding A bp1p, a nonessential actin-binding protein (Holtzman et al., 1993). In yeast, Sla2p is required for the nucleation of cortical actin assembly in permeabilized yeast cells (Li et al., 1995). This protein contains an actin-binding tail-like domain. A null allele of SLA2 results in severe disruption of the actin cytoskeleton and defects in endocytosis (Holtzman et al., 1993; Waskell et al., 1997). There appear to be homologues of Sla2p in all eukaryotes. A human homologue, H1P1 (huntingtin-interacting protein 1), associates with huntingtin, the protein that is mutated in patients with Huntington’s disease (Kachman et al., 1997; Wanker et al., 1997).

The ultimate function of the signaling pathways described above is to modulate the assembly and disassembly of actin-based complexes that mediate a variety of cellular processes. Sla2p is thus a candidate target of signals intended to modulate the actin cytoskeleton. Here we report that a screen for proteins that interact with Sla2p and a separate screen for proteins that disrupt the actin cytoskeleton when overexpressed have identified two novel protein kinases that have a critical role in the regulation of the actin cytoskeleton in budding yeast.

**Materials and Methods**

A ll plasmids used in this work are listed in Table 1.

**Two-Hybrid Screen**

E residues 503-968 of Sla2p were fused to the Gal4p DNA-binding domain in pA S1-CYH2 generating pD D 373 (Yang, S., M J. T.V. Cope, and D.G. Drubin, manuscript submitted for publication). This construct expresses a product of the expected size (71 kD), which is detected by anti-Sla2p antibody (data not shown). A yeast Y 190 (Table II) strain containing pD D 373 was transformed with a library containing random cDNA fragments fused to the activating domain of Gal4p (Durfee et al., 1993) and was selected on synthetic medium lacking tryptophan, leucine, and histidine, and containing 50 μM 3-amino-1,2,4-triazole. Healthy colonies that also displayed β-galactosidase activity by filter lift assay were selected and the activation-domain fusion plasmids were isolated and sequenced from the primer “HA Internal” (GCT TAC CCCATACGATGTT).

**Sequence Alignment and Phylogenetic Analysis**

Sequence alignments were performed using the ClustalW software package (Thompson et al., 1994), implemented at the European Bioinformatics Institute web site at http://croma.ebi.ac.uk/clustalw/. The phylogenetic tree was determined from the alignment data, again using ClustalW. A n allowance was made for multiple substitutions (Kimura, 1983). Information from intervals in the alignment for which gaps are found in some sequences was not included, in order to avoid the inappropriate weighting of some sequences. The tree was tested (1,000 trials) for branching order confidence by bootstrapping (Felsenstein, 1985). Further information on this procedure (as applied to myosin motor domains) can be found on the World Wide Web at http://www.mrc-lmb.cam.ac.uk/myosin/myosin.html.

**Disruption of the ARK1 and the PRK1 Genes**

The ARK1 open reading frame (ORF) was precisely replaced by a DNA fragment containing the HIS3 gene. The primers SY 40 (GAGA AAGGAA ATTACCTCTGCA ATTAGTTTATTT T GACCA CACACTTACGCCTTACCCATACGATGTT) and SY 51 (CATTTACGCCGCTCTATCTCATTTACCCATACGATGTT) were amplified from pM S313 using PCR. D DY 426 was transformed with this fragment and the resulting transformants were screened for His⁺ colonies. Replacement of the ARK1 ORF with the HIS3-containing fragment was verified by PCR using the primers SY 51 (CGGAGTCGGCACA CCTCTATCGCTTATG) and SY 52 (CGTCTA GAGGAGAACATACCCATACGATGTT). The heterozygous (ARK1 ark1: HIS3) diploid was sporulated and ark1: HIS3 MATa (DDY 1407) and ark1:: HIS3 MATa (DDY 1408) haploid cells were isolated.

The PRK1 ORF was precisely replaced by a DNA fragment containing either the URA3 ORF or the LEU2 ORF. Primers SY 44 (GTGGTA CATGACCATATTGTGCA TTATCCCTTTGTTA GTGACGTTACCA AAAAACACCTGCGCTTTTCA CACCCG) and SY 45 (CATTGTTGTA CGCTTATATAATATCATGCTTATTTGTTA GTGACGTTACCA AAAAACACCTGCGCTTTTCA CACCCG) were used to PCR amplify URA3-containing fragments from pRS316 or LEU2-containing fragments from pRS315. Ura⁺ or Leu⁺ transformants of D DY 426 were isolated and verified for replacement of the PRK1 gene by PCR using the primers SY 61 (TGATGTA TATTGCGGTTCACCAG) and SY 62 (CGTGATCAGA GCGAAC GGTCTT), or the primers SY 45 and SY 62. Diploids heterozygous at the PRK1 locus were sporulated and prk1: URA3 MATa (DDY 1561), prk1:: LEU2 MATa (DDY 1560), prk1:: LEU2 MATa (DDY 1559), and prk1:: LEU2 MATa (DDY 1558) haploid were isolated. Finally, yeast in which the ARK1 ORF deletion mutation was marked with LEU2 was obtained by using the marker-swap method (Cross, 1997). The plasmid HL 3 (a kind gift from F. Cross) was digested with A paI and PstI to release the LEU2-disrupted HIS3 gene. The digested DNA was used to transform D DY 1407. Leu⁻/His⁺ yeast (DDY 1573) were identified and the new LEU2-marked disruption of the ARK1 gene was verified by PCR using the primers SY 63 and SY 51.

** Amplification of Genomic Fragments Containing the ARK1 and PRK1 Genes**

A genomic fragment containing the ARK1 ORF, plus 453 upstream base pair and 250 downstream base pair, was amplified from the S. cerevisiae cosmid 70944 by high-fidelity PCR using the primers SY 50 and SY 52. This fragment was inserted between the XboI sites of pRS316. The prk1 ORF-containing fragments from pRS316 or pRS315. Ura⁺ or Leu⁺ transformants of D DY 426 were isolated and verified for replacement of the PRK1 gene by PCR using the primers SY 61 (TGATGTA TATTGCGGTTCACCAG) and SY 62 (CGTGATCAGA GCGAAC GGTCTT), or the primers SY 45 and SY 62. Diploids heterozygous at the PRK1 locus were sporulated and prk1: URA3 MATa (DDY 1561), prk1:: LEU2 MATa (DDY 1560), prk1:: LEU2 MATa (DDY 1559), and prk1:: LEU2 MATa (DDY 1558) haploid were isolated. Finally, yeast in which the ARK1 ORF deletion mutation was marked with LEU2 was obtained by using the marker-swap method (Cross, 1997). The plasmid HL 3 (a kind gift from F. Cross) was digested with A paI and PstI to release the LEU2-disrupted HIS3 gene. The digested DNA was used to transform D DY 1407. Leu⁻/His⁺ yeast (DDY 1573) were identified and the new LEU2-marked disruption of the ARK1 gene was verified by PCR using the primers SY 63 and SY 51.

**Myc-Tagging of PrkIp**

A 6-my epitope-tag flanked by X mal sites (from pD D 557) was inserted into the X mal site present 11 codons into the prk1 ORF in pD D 556.
form DD558. This plasmid fully complemented the temperature-sensitive growth defects of ark1Δ prk1Δ double-null mutants.

**Indirect Immunofluorescence**

For rhodamine-phalloidin staining of filamentous actin when preservation of green fluorescent protein (GFP) fluorescence was not required, cells were grown to log-phase in YPD (Guthrie and Fink, 1991). To 1.5 ml of cells in YPD, 200 μl of a 37% formaldehyde solution was added and the culture was incubated at room temperature for 30 min to 1 h. The cells were washed two times in PBS containing 1 mg/ml BSA (PBS-BSA) and then resuspended in 50 μl PBS-BSA, to which 10 μl of rhodamine-phalloidin (Molecular Probes) solution was added (300 U in 1.5 ml methanol). After a 30-min incubation, the cells were washed three times in PBS-BSA and resuspended in mounting medium (Pringle et al., 1991) before visualization. When preservation of GFP fluorescence was required, cells were fixed in 2% formaldehyde for no longer than 30 min.

For actin immunofluorescence, a 1:2,000 dilution of guinea pig anti-actin serum was used (Mulholland et al., 1994). The myc epitope-tag was detected using a 1:50 dilution of rabbit polyclonal anti-myc antibodies (Santa Cruz Biotech.). Sla2p, Sac6p, and cofilin were detected using affinity-purified antibodies raised in rabbit, at dilutions of 1:50, 1:100, and 1:2,000 dilution of FITC-conjugated goat anti–guinea pig antibody (Cappel/Organon Technika Inc.), and 1:2,000 dilution of FITC-conjugated goat anti–rabbit antibody (Sigma Chemical Co.). Fixation and permeabilization of yeast cells was performed as described by Ay-Cy3-conjugated goat anti–rabbit antibody (Sigma Chemical Co.).

**GFP Fusion Constructs**

The ORFs for YNL020c (ARK1) and YIL095w (PRK1) were cloned into GFP expression vectors under control of the GAL1,10 promoter as follows. ARK1 was amplified by PCR using the primers JC_YNL020c_1 (GCTCTAGACTTATCCAAGGATAACTTTCG) and SY50 (CGTCACTAGTAAACCTCAATTTGGAAGAC) with pDD382 as template. The product was cloned into the XbaI site of pTS395, creating a plasmid (pDD555) encoding a chimeric protein with GFP at the COOH terminus of Ark1p. (The plasmids pTS395 and pTS408 are derivatives YCp50 [Ma et al., 1987], containing additionally the GAL1,10 promoter, wild-type GFP, and ACT1 terminator sequences, and are kind gifts from Tim Stearns, Stanford University). PRK1 was amplified by PCR using the primers JC_YIL095w_1 (CGGGGATCCATGAATACTCCACAGATATTCCACAGATTAG) and JC_YIL095w_2 (GCTCTAGATAAACCTCAATTTGGAAGAC) with pDD556 as the template. The product was inserted between the BamHI and XbaI sites of pTS408 to create a plasmid (pDD554) encoding a chimeric protein with GFP at the NH2 terminus of Prk1p. All PCR reactions were performed with low numbers of cycles and with accurate DNA polymerases (Pfu from Stratagene or Vent from New England Biolabs). The Ark1p-GFP (pDD555) construct complemented the growth defects of ark1Δ prk1Δ double-mutant cells at both 15 and 37°C. The GFP-Prk1p construct (pDD554) was itself deleterious to growth when overexpressed, but slow-growing colonies could form when this fusion protein was overexpressed in ark1Δ prk1Δ double-null cells at 37°C, whereas a control plasmid lacking an insert does not allow growth at 37°C.

<table>
<thead>
<tr>
<th>Table I. Plasmids Used in This Study</th>
<th>Name</th>
<th>Features*</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTS395‡</td>
<td>GFP(wt)</td>
<td>P_GAL1.10</td>
<td>Allows expression of COOH-terminal GFP fusion proteins under the inducible GAL1,10 promoter.</td>
</tr>
<tr>
<td>pTS408‡</td>
<td>GFP(wt)</td>
<td>P_GAL1.10</td>
<td>Allows expression of NH2-terminal GFP fusion proteins under the inducible GAL1,10 promoter.</td>
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<tr>
<td>pDD371</td>
<td>CEN, HIS3</td>
<td></td>
<td>An EcoRI fragment containing the SLA2 ORF, but lacking sequence encoding amino acids 360–575, in pRS313.</td>
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<tr>
<td>pDD373</td>
<td>CEN, TRP1</td>
<td>Sla2p residues 503–968 fused to the DNA-binding domain of Gal4p at the BamHI site of pASl-CYH2.</td>
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</tr>
<tr>
<td>pDD382</td>
<td>CEN, LEU2</td>
<td></td>
<td>A genomic fragment containing ARK1 plus 435 bp upstream and 250 bp downstream sequence cloned into the XbaI and SacI sites of pRS315.</td>
</tr>
<tr>
<td>pDD554</td>
<td>P_gal GFP-Prk1p</td>
<td>CEN, URA</td>
<td>The entire ORF of PRK1 fused to the COOH terminus of GFP (wild-type). Parent vector is pTS408.</td>
</tr>
<tr>
<td>pDD555</td>
<td>P_gal GFP-Ark1p</td>
<td>CEN, URA</td>
<td>The entire ORF of ARK1 fused to the NH2 terminus of GFP (wild-type). Parent vector is pTS395.</td>
</tr>
<tr>
<td>pDD556</td>
<td>CEN, URA</td>
<td>PKR1 ORF, plus 385 bp of 5’ UTR and 284 bp of 3’ UTR between SacI and SalI sites of pRS316.</td>
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</tr>
<tr>
<td>pDD557</td>
<td>6-myc source</td>
<td></td>
<td>6-myc tag flanked by Nos and XmaI sites inserted into the XmaI site of pBluescript II SK+*.</td>
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<tr>
<td>pDD558</td>
<td>CEN, URA</td>
<td>6-myc tagged PRK1 genomic fragment in pRS316.</td>
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<tr>
<td>pDD559</td>
<td>CEN, LEU</td>
<td>Same as pDD382, except with the K56A kinase-dead mutation within the ARK1 ORF and a silent mutation in the URA2 gene eliminating a unique AflII site.</td>
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<tr>
<td>pDD560</td>
<td>CEN, URA</td>
<td>Same as pDD556, except with the K56A kinase-dead mutation within the PRK1 ORF and a silent mutation in the URA3 gene eliminating a unique NolI site.</td>
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<tr>
<td>pDD561</td>
<td>P_gal GFP-Prk1p kinase-dead</td>
<td>CEN, URA</td>
<td>Same as pDD554, except with the kinase-dead mutation K56A.</td>
</tr>
<tr>
<td>pDD562</td>
<td>P_gal GFP-Ark1p kinase-dead</td>
<td>CEN, URA</td>
<td>Same as pDD555, except with the kinase-dead mutation K56A.</td>
</tr>
<tr>
<td>HL3</td>
<td>CEN, URA</td>
<td>CapR*</td>
<td>Marker-swapped plasmid containing the HIS3 gene disrupted by the LEU2 ORF (Cross, 1997). Parent vector is pBCKS* (Strategene).</td>
</tr>
</tbody>
</table>

* All plasmids listed are ampicillin-resistant, except HL3, which is chloramphenicol-resistant.

‡ A kind gift from T. Stearns.
**Induction of Galactose-regulated Expression**

Induction of GFP-tagged proteins from the GAL1,10 promoter was accomplished by first picking from solid media a single, isolated colony (containing the requisite vector) and inoculating it into liquid synthetic medium (SM) lacking uracil and containing 2% glucose. After growth overnight, a small sample was removed, washed in SM, and transferred to SM lacking uracil, but containing this time 2% raffinose and 2% galactose as carbon sources. After 8-10 h, the glucose repression is overcome and the GFP-tagged fusion constructs begin to be expressed. Cells were visualized 12-16 h after transfer to the raffinose-galactose-containing medium.

**Creation of Kinase-dead Mutations in Ark1p and Prk1p**

The codon encoding lysine 56 of Ark1p in pDD382 was converted to a codon encoding an alanine (K63A), using the primer JC_Ark1_mut (GGT-GCA-TGG-CCC-GAG-GTCATTGTC) with the "Transformer" site-directed mutagenesis kit (Clontech). In addition to making the K56A mutation, JC_Ark1_mut introduces a unique MscI site.

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**Table II. Yeast Strains Used in This Study**

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<th>Name</th>
<th>Genotype</th>
<th>Source</th>
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</thead>
<tbody>
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<td>DDY130</td>
<td>MATa his3::200 ura3-52 leu2-3,112 lys2-801</td>
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<td>DDY131</td>
<td>MATa his3-200 ura3-52 leu2-3,112 lys2-801 ade2-1</td>
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<tr>
<td>DDY318</td>
<td>MATa sac6Δ::LEU2 his3 ura3 leu2 lys2</td>
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<tr>
<td>(AAY1046)</td>
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<td></td>
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<td>MATa abp1Δ::LEU2 his3 Δ200 ura3-52 leu2-3,112</td>
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<tr>
<td>DDY426</td>
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<tr>
<td>DDY545</td>
<td>MATa slaΔ::URA3 his3-Δ200 ura3-52 leu2-3,112</td>
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<td>MATa rvs167Δ::TRP1 ura3 leu2 lys2 trp1</td>
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<td>MATa srvΔ::HIS3 ura3 leu2 his3 lys2</td>
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<td>P190</td>
<td>MATa gal4 gal180 ura3-52 leu2-3,112 his3 trpl-1::KanR + URA3::GAL::lacZ, LYS2::GAL::HIS3 cyhR</td>
<td>5</td>
</tr>
</tbody>
</table>

Sources: 1. Drubin lab; 2. Alison Adams; 3. this study; 4. David Amberg; 5. Steve Elledge.
to verify A rk1p and Prk1p expression and localization, by immuno-
fluorescence microscopy for actin distribution, and by differential
interference-contrast (DIC) microscopy for cell appearance and morphology.

Vital Staining Using FUN-1

Cells were tested for viability using the vital dye FUN-1 (Molecular
Probes). The cells were grown to log-phase in minimal medium and FUN-1
was added to a final concentration of 1 µM. After rotation at 25°C for 30
min, the cells were washed once and then resuspended in 250 µl of mini-
mal media for visualization. Living cells were able to internalize the stain
by the vacuole, where it could be metabolized to form aggregates that flu-
oresced brightly when observed using a rhodamine filter set. Dead cells,
contrast, displayed a uniform distribution of dye throughout the cell
and did not develop bright vacuolar aggregates.

Results

A Two-Hybrid Screen Reveals an Interaction between a
Novel Protein Kinase and a Component of the
Actin Cytoskeleton

Sla2p/Ep and/or Mop2p is a component of the yeast cortical
actin cytoskeleton implicated in the control of actin orga-
nization, endocytosis, and the maintenance of an ATPase
at the plasma membrane (Holzman et al., 1993; Raths et al.,
1993; Li et al., 1995; Na et al., 1995). To identify proteins
that interact with Sla2p and thus might be involved in the
assembly or function of cortical actin patches, a fusion of
residues 503-968 of Sla2p to the D NA binding domain of
Gal4p was used to screen a two-hybrid library of cDNA
fragments fused to the activation domain of Gal4p (Due-
fee et al., 1993). Of the six clones identified, two were de-
derived from the yeast gene Y NL020c. One clone encoded
residues 380-638, and the other encoded residues 218-552
of the protein. The remaining clones were single isolates
and have not yet been pursued further. Y N L020c encodes
a protein of 638 amino acids with a 300-residue region at
the NH2 terminus containing many elements conserved in
serine-threonine kinases (Fig. 1 A). Because of the find-
ings presented below, we have named this gene A RK1, for
actin regulating kinase 1.

Ark1p Homologues

Examination of the S. cerevisiae genome indicated that the
putative protein kinase domain of A rk1p is very similar in
amino acid sequence to the kinase domain of a protein en-
coded by the ORF Y IL095w. This protein was found in a
 genetic screen for modifiers of mammalian p53 activity in
yeast and has been named p53-regulating kinase 1, or
Prk1p (originally Pak1p; Thigalangam et al., 1995). The
kinase domains of the two proteins are 73% identical at
the amino acid level. Furthermore, a third S. cerevisiae
protein, encoded by the unstudied ORF Y BR059c, a
Schizosaccharomyces pombe protein, and proteins from
Caenorhabditis elegans, A rabidopsis thaliana, rat, and human
were also found to be similar to A rk1p and Prk1p
within the kinase domains, having between 30 and 48%
identity to the A rk1p kinase domain. This level of identity
is significantly higher than that observed with any other ki-
nases retrieved from the databases, according to the
BLAST algorithm (A Itschul et al., 1990). The rat and hu-
man proteins have been identified as cyclin-G–associated
kinases (G A K s) and the human G A K has been shown to be
a functional kinase in vitro (Kanaoka et al., 1997;
K imura et al., 1997). The kinase domains of these proteins
were reported previously to be most similar to those of
Nek1 and CDK2 (Kanaoka et al., 1997). However, our
analysis now places them in a separate family. We propose
to call this the A rk family, after A rk1p, the first member
of this family to be named for a biological process.

A sequence alignment shows the similarities between
the kinase domains of A rk1p, Prk1p, and six other A rk
family members (Fig. 1 B). The residues that are abso-
lutely conserved in all serine-threonine kinases are pre-
sent, but there are significant variations from the norm that
are distinguishing features of the eight kinase domains
shown here (Hanks et al., 1988; Hardie and Hanks, 1995).
Most notably, the largely invariant p-loop sequence (in-
volved in binding the nontransferrable phosphates of A T P
in other kinases conforms to the consensus G xG xG xV,
while the A rk family members have their own consensus
S/E G G F A/SxV Y (where x is any amino acid, S/E is serine
or glutamate, and A/S is alanine or serine). There is a
G xG xG motif very near the NH2 terminus of the G A K s
which has led to the suggestion that these residues might
constitute part of a p-loop (Kanaoka et al., 1997). How-
ever, based on the alignment shown in Fig. 1 B, we feel
that these residues are unlikely to comprise the true phos-
phate anchoring loop. This is because the first two glycines
are separated by a proline, an amino acid never seen in
this position in other kinases, and because other conserved
features are present surrounding the S/E G G F A/SxV Y
motif, but not surrounding the G xG xG motif in these ki-
nases. A crystal structure will be required to be certain
about this issue. Fig. 1 B also shows that several other resi-
dues are absolutely conserved in the kinase domains of the
A rk family members but are rarely found in kinases not in
this group.

To demonstrate objectively that these eight putative ki-
nases are more closely related to each other than to other
serine-threonine kinases, we performed a further multiple
alignment. This time, we also included representative ki-
nases from each of the classes defined in Hardie and
Hanks (1995) (alignment not shown). The resulting phylo-
genetic tree indicates that the eight kinase domains shown
in Fig. 1 B constitute a new family within the superfam-
ily of serine-threonine kinases (Fig. 1 C).

The Nonkinase Domains of Ark1p and Prk1p Are Not
Closely Related

Although the kinase domains of A rk1p and Prk1p are
highly similar, their nonkinase COOH-terminal domains
lack detectable similarity along most of their lengths.
A rk1p encodes a protein of 638 amino acids with a nonki-
nase COOH-terminal domain of ~340 amino acids, while
Prk1p encodes a protein of 810 amino acids with a nonki-
nase 510-amino acid COOH-terminal domain with no sig-
nificant extensive similarity to that of A rk1p. However, a
short conserved motif does exist close to the COOH ter-
minus of both proteins. A rk1p contains two copies, and

Cope et al. Ark1p and Prk1p Regulate Actin in Budding Yeast
Prk1p contains one copy, of the conserved motif PxPP-PKP. Proline-rich regions are known to mediate protein–protein interactions and can be bound by, for example, Src-homology 3 (SH3) domains. Several interactions between proline-rich motifs and SH3 domains are found among proteins found in yeast cortical actin patches, and it is possible that Ark1p and Prk1p also participate in such associations (Lila and Drubin, 1997).
Lack of Both Ark1p and Prk1p Leads to Severe Actin Abnormalities and Inviability at Extreme Temperatures: Kinase-active Ark1p or Prk1p Are Required to Rescue These Defects

To determine whether deletion of either the ARK1 or the PRK1 gene has a detectable effect on the actin cytoskeleton or on the growth characteristics of the cell, we replaced each of these genes individually with auxotrophic markers. Deletion of these genes singly caused little or no detectable defects in the actin cytoskeleton (Fig. 2, D–I). However, growth rates were slightly lower than in the parental wild-type strain (1.8 h doubling time at 30°C for Figure 2. Phenotypes of single deletions of ARK1 and PRK1, and of ark1Δ prk1Δ double-mutant yeast. Deletions in the ARK1 and PRK1 genes were created as described in Materials and Methods. All strains were grown to log-phase in rich liquid medium (YPD) at 30°C before fixation and visualization. (A–C) DDY130 (wild-type); (D–F) DDY1558 (prk1Δ::LEU2); (G–I) DDY1407 (ark1Δ::HIS3); (J–L) DDY1564 (ark1Δ::HIS3, prk1Δ::LEU2). (A, D, G, and J) Actin localization, visualized by rhodamine-phalloidin staining; (B, E, H, and K) nuclear and mitochondrial DNA localization visualized by DAPI staining; (C, F, I, and L) DIC images. Bar, 5 μm.
each single deletion mutant compared with 1.5 h for the wild-type (DDY 130).

Whereas the effects of genetically removing either Ark1p or Prk1p from the cell were negligible (Fig. 2, D–I), the effects of the removal of both proteins were profound (Fig. 2, J–L). Ark1Δ prk1Δ double mutants were inviable at 15 and 37°C, and grew optimally at 30°C. At 30°C, many of the double-mutant cells were substantially larger than the parental cells and had thicker bud necks (Fig. 2 L). Moreover, the double-mutant cells displayed a severely abnormal actin cytoskeleton. Most cells in the population (85%) contained one or more large clumps of actin (Fig. 2 J).

Similar clumps are not seen in wild-type cells. These clumps contain filamentous actin because they stain brightly with rhodamine-phalloidin. A ctin cortical patches and actin cables were still present, although the cortical patches no longer appeared uniform in size and they were not polarized. In addition, fewer cables could be observed in the double-mutant cells than in wild-type cells (50% of mutant cells contained visible cables versus 75% in the wild-type cells). Despite the severity of the actin defects, these cells did manage to form buds and divide. The doubling time of ark1Δ prk1Δ haploid cells at 30°C was ~3 h, however, compared with a doubling time of 90 min for the parental wild-type cells. An increase over wild-type in the number of multinucleate cells was not observed. Shifting cells from 30 to 37°C or to 15°C did not change actin distribution noticeably.

The phenotypes observed in the ark1Δ prk1Δ double-mutant cells were rescued when they were transformed with low-copy (CEN) plasmids bearing genomic fragments containing either ARK1 (pDD 382) or PRK1 (pDD 556) (data not shown). We then mutated in Ark1p and Prk1p a conserved lysine within the kinase domain that had been shown in a number of previous studies on other members of the protein kinase superfamily to result in a loss of protein kinase activity (Fig. 1 B; Hanks et al., 1988). For both Ark1p and Prk1p, a conserved lysine (K 56) was changed to an alanine. When ark1Δ prk1Δ double-mutant cells were transformed with low-copy plasmids bearing the kinase-dead mutants of ARK1 (pDD 559) and PRK1 (pDD 560), the phenotypes described above were not rescued (data not shown). These data suggest that Ark1p and Prk1p are active kinases, and that their kinase activities are necessary for their in vivo function(s).

The Actin Clumps Found in ark1Δ prk1Δ Cells Also Contain Cofilin, Sla2p, Sac6p, and Abp1p

To determine whether the actin clumps observed by rhodamine-phalloidin staining in ark1Δ prk1Δ cells were merely abnormal aggregates of filamentous actin derived from cables, or whether they also contained proteins normally found in cortical actin patches, we performed indirect immunofluorescence on these cells using antibodies directed against Sac6p (yeast fimbrin), Sla2p, and cofilin. All of these proteins are normally found in actin patches, Sac6p also associates with actin cables. All three of these proteins associated with the actin clumps in ark1Δ prk1Δ cells (Fig. 3). A bp1p-GFP also localizes to these clumps in vivo (data not shown). In addition, all four proteins are found to be present in the cortical actin patches that re-

Figure 3. A ctin clumps found in ark1Δ prk1Δ double mutants also contain Sac6p, cofilin, and Sla2p. ark1Δ prk1Δ double mutants were fixed and stained for actin (detected by FITC-conjugated secondary antibody) and for either Sac6p, cofilin, or Sla2p (detected by CY3-conjugated secondary antibody). (A–C) A ctin localization in ark1Δ prk1Δ double mutants. (D) Sac6p localization, (E) cofilin localization, and (F) Sla2p localization in the same cells shown in A–C. Bar, 5 μm.
main in the ark1Δ prk1Δ cells. As has been observed in wild-type cells, the localization of cortical Sla2p patches was not always coincident with that of cortical actin patches (Yang, S., M.J.T.V. Cope, and D.G. Drubin, manuscript submitted for publication).

The Actin Clumps Found in ark1Δ prk1Δ Cells Are Not Strictly Cortical

The presence of coflin, Sla2p, Sac6p, and App1p in the actin clumps of ark1Δ prk1Δ double-mutant cells is a characteristic shared with actin cortical patches. To determine whether these clumps were also associated with the cell cortex, we used confocal microscopy. The actin clumps were found associated with the cell cortex in only a minority of cases (Fig. 4). Confocal microscopy revealed that, while variable in size, the actin clumps could occupy a volume of the cytoplasm comparable to that of the nucleus. Thus, the clumps are not merely comprised of many actin patches aggregated at the cell surface. Rather, they are large masses of actin and other cortical patch proteins that accumulate in the absence of Ark1p and Prk1p.

Ark1p and Prk1p Are Associated with Cortical Actin Patches

Since Ark1p was found as a protein that interacts with Sla2p, and since Ark1p and Prk1p were shown to affect profoundly the organization of cortical actin cytoskeleton proteins, we determined the subcellular localization of Ark1p and Prk1p. First, we placed a 6-myc epitope-tag close to the NH2 terminus of each protein. In the case of Prk1p, this construct was capable of fully complementing the actin and growth phenotypes of the ark1Δ prk1Δ double-mutant cells when expressed from a low-copy plasmid under its own promoter. Indirect immunofluorescence of actin and of the myc epitope in cells expressing only the epitope-tagged version of Prk1p shows that this protein localizes to cortical actin patches (Fig. 5 A). These patches were appropriately organized according to the cell-cycle stage, indicating that expression of the myc-tagged protein was not causing the formation of abnormal structures or interfering with actin localization.

Epitope-tagged constructs of Ark1p appeared to be unstable. As an alternative approach to localizing Ark1p and to examine the in vivo localization of both Ark1p and Prk1p, we placed ARK1 and PRK1 in vectors that would express them as GFP fusion proteins under the control of the strong, inducible GAL1,10 promoter. When expressed in the presence of galactose, Ark1p-GFP was visible in cortical patch structures very similar in appearance and behavior (i.e., motility) to actin patches. When GFP and actin were both localized by indirect immunofluorescence, they were found to be coincident in patches at the cell cortex (Fig. 5 B). Kinase-dead Ark1p-GFP, which does not perturb the actin cytoskeleton (see below), also localized to cortical actin patches. Thus, we conclude that Ark1p localizes to cortical actin patches. GFP-Prk1p was also found to colocalize with cortical actin patches (Fig. 5 C).

Effects of Elevated Levels of Ark1p and Prk1p on Cell Morphology

Elevated levels of Ark1p and Prk1p lead to the formation of delocalized actin patches and actin bars (Fig. 5, B and C). Actin bars are intracellular aggregates of actin monomers and are therefore not labeled by rhodamine-phalloidin, a compound that binds to filamentous but not monomeric actin. A thin bars are in this respect different from the actin clumps observed in ark1Δ prk1Δ cells. Continued overexpression of either Ark1p or Prk1p leads to a variety of further and more severe effects (Fig. 6, A and B). In the case of Prk1p, this ultimately leads to inviability of the cell population. The initial consequences of overexpression of either Ark1p or Prk1p included cells with abnormally shaped buds, and apparent septation defects, multiple buds, and/or severely abnormal internal structures. Cells with abnormal internal structures were determined to be dead using the vital dye FUN-1 (Fig. 6 C). 4–6 h after induction of Prk1p-GFP overexpression, ~35% of budded cells had abnormal buds and/or multiple buds, and ~25% of cells were dead. Most dead cells contained actin bars. Continued growth of Prk1p-GFP-expressing cells (24 h) in galactose-containing media resulted in lethality for the majority of cells, although some did survive and micro-colonies were formed after 5–7 d growth at 30°C (data not shown). While overexpression of Ark1p caused similar

Figure 4. A stereo-pair showing a three-dimensional reconstruction of ark1Δ prk1Δ double-mutant yeast in which the actin has been labeled using rhodamine-phalloidin. The three-dimensional reconstruction from confocal serial sections was produced using Imageworks software running on a Silicon Graphics workstation.
bud morphology, septation, and multibudded phenotypes after 4–6 h after derepression (Fig. 6 B), fewer dead cells were seen (15–20%) and this percentage did not exceed 25% after continued overexpression (24 h).

The fact that Prk1p overexpression is lethal to most cells was independently demonstrated in this laboratory in a screen for proteins that cause death upon overexpression (DUO). The DUO screen was performed on yeast cells transformed with a galactose-inducible genomic library to select for transformants that grew on glucose plates (expression inhibited) and that died on galactose plates (expression induced). The PRK1 gene was identified in a subsequent visual screen as a gene that caused lethality and abnormal cell morphology when present at elevated levels. The overexpression phenotypes caused by this untagged Prk1p were identical to those described above.

As reported above, the kinase activities of Ark1p and of Prk1p are necessary for either protein to rescue the phenotypes of ark1Δ prk1Δ double-mutant yeast. To test whether the overexpression phenotypes were also dependent on kinase activity, we placed the kinase-dead variants of the proteins, also as GFP-containing chimeras, under the control of the GAL1,10 promoter. Overexpression of the kinase-dead variant of Prk1p did not cause inviability (Fig. 7 A; compare dilution series i to dilution series iv). However, the kinase-dead variant of Prk1p retained the ability to cause delocalization of actin patches (data not shown). As mentioned above, the kinase-dead variant of Ark1p localized to cortical patches, but when present at elevated levels no longer caused delocalization of actin patches (Fig. 7 B), nor did it cause abnormally budded or multibudded cells, death, or any of the phenotypes that were exhibited by cells containing elevated levels of wild-type Ark1p. Furthermore, using the kinase-dead mutant, the colocalization of Ark1p with cortical actin patches (and not actin cables) was confirmed under conditions where the actin cytoskeleton itself is unperturbed.

Ark1p and Prk1p Localization in Cells Lacking Specific Cortical Actin Cytoskeleton Proteins

The COOH-terminal, nonkinase domain of Ark1p interacts with Sla2 in the two-hybrid assay (Fig. 1) and Sla2p partially colocalizes with actin in cortical patches (Yang, S., M.J.T.V. Cope, and D.G. Drubin, manuscript submitted for publication). Thus, it was of interest to determine whether A rk1p and Prk1p were capable of being localized to cortical patches in the absence of Sla2p. Interestingly, both A rk1p and Prk1p were still found in patches at the cortex in sla2Δ cells (Fig. 8). However, localization of both proteins to cortical patches was reduced dramatically in abp1Δ cells. Ark1p and Prk1p localize to cortical patches in sac6Δ, srv2Δ, and in rvs167Δ cells. Yeast containing sac6Δ, srv2Δ, or rvs167Δ mutations have abnormal actin cortical patch morphologies, whereas abp1Δ yeast do not (Adams et al., 1989; Holtzman et al., 1993; Lila and Drubin, 1997). Overexpression of A rk1p and Prk1p results in further disruption of actin in each of these null mutant strains (even in abp1Δ cells); however, it is clear that A rk1p and Prk1p remain colocalized with actin in all the mutants tested, except abp1Δ cells.
Genetic Interactions Involving ARK1 and PRK1 Provide Evidence for Parallel Pathways in Actin Cytoskeleton Regulation

The synthetic lethality that results when ark1Δ and prk1Δ are combined suggests that these proteins function in parallel to regulate an essential process or processes. To gain deeper insight into how Ark1p and Prk1p might regulate the actin cytoskeleton, we constructed a number of double mutants containing a null allele of ARK1 or of PRK1, together with a null allele of a gene implicated in cortical actin cytoskeleton function. The genes chosen for testing were ABP1, SAC6, SLA1, SLA2, SRV2, RVS167, CRN1, and AIP1. Abp1p is an actin-binding protein that localizes to actin patches (Drubin et al., 1990). Sac6p, the yeast fimbrin homologue, bundles actin filaments and localizes to both actin patches and to actin cables (Adams et al., 1989, 1991). Sla1p is an SH3 domain containing protein found in cortical actin patches (Holtzman et al., 1993). Null alleles of the SLA1 gene are synthetic lethal with null alleles of the ABP1 and RVS167 genes (Holtzman et al., 1993; Lila and Drubin, 1997). SLA2 encodes a cortical actin-binding protein and mutant alleles of this gene are synthetic lethal with ABP1 null alleles. Mutations in the SLA2 gene lead to actin and endocytosis defects (Holtzman et al., 1993; Wesp et al., 1997). Srv2p, the yeast homologue of CAP, binds to adenylyl cyclase, Abp1p, and actin monomers (Field et al., 1988; Freeman et al., 1995, 1996). Rvs167p is necessary for a normal actin cytoskeleton morphology and for endocytosis (Bauer et al., 1993; Munn et al., 1995). Yeast coronin (Crn1p) binds tightly to actin and localizes to actin patches (Goode et al., 1999). Aip1p interacts with actin and cofilin, and localizes to actin patches (Rodal,
A.A., J. Tetrault, P. Lappalainen, D.G. Drubin, and D.C. Amberg, manuscript submitted for publication).

Table III summarizes the effects of combining null mutations in the above genes with null mutations in the ARK1 and PRK1 genes. In addition to showing a negative synergism with prk1Δ, ark1Δ also shows a synthetic genetic interaction with sac6Δ. This was the only additional genetic interaction detected involving ARK1. By contrast, prk1Δ, as well as showing negative synergism with ark1Δ and with sac6Δ, shows synthetic genetic interactions with sla2Δ and with abp1Δ. From two separate crosses between different sla2Δ and prk1Δ strains (32 tetrads were dissected), >50% of predicted prk1Δ sla2Δ double-mutant spores were viable. Surviving double mutants were extremely temperature and cold sensitive and could not reliably be streaked to single colonies. Thus, we consider the prk1Δ sla2Δ combination to be lethal.

Discussion

We have identified a putative serine-threonine kinase that, in the yeast two-hybrid system, binds to the evolutionarily conserved, cortical actin-associated protein, Sla2p. We have subsequently assigned the gene name ARK1 (for actin-regulating kinase 1) to this locus.

A rk1p and another S. cerevisiae protein, encoded by the PRK1 gene, have at their NH2 termini predicted serine-threonine kinase domains of ~300 amino acids that are >70% identical. Searches of sequence databases identified six other kinases that are highly similar, within their kinase domains, to Ark1p and Prk1p, but are dissimilar outside the kinase domains. Together, these eight proteins define a new family of serine-threonine kinases, which we have termed the Ark family. Two mammalian members of the Ark family were identified as cyclin-G–associated proteins (Kanaoka et al., 1997; Kimura et al., 1997), although there are no proteins with significant homology to cyclin-G in budding yeast. A third predicted serine-threonine kinase from S. cerevisiae, encoded by the ORF YBR059c, is a member of the Ark family of kinases and has been implicated in the yeast pheromone response pathway (Caponigro et al., 1998). The kinase domain of this protein is less similar to those of Ark1p and Prk1p than they are to each other. Since Ark1p and Prk1p are similar primarily only in their kinase domains, but both regulate the actin cytoskeleton in S. cerevisiae, it is also possible that Ark family kinases present in other organisms have a related role.

Deletion of either the ARK1 or the PRK1 gene individually had no observed consequences with respect to actin distribution, cell morphology, or growth. By contrast, when deletions in the ARK1 and the PRK1 genes were combined, the effect was strongly deleterious. Yeast lacking both Ark1p and Prk1p were sensitive to high and low temperatures, were slow growing, and had severely disrupted actin cytoskeletons. It appeared that the majority of the filamentous actin in these cells was present in large clumps. These actin clumps also contain other proteins normally found in cortical actin patches, suggesting that the clumps result from a loss of actin patch regulation. The clumps are typically not cortical, although electron microscopy (not shown) suggests that they might maintain con-
connection with the cortex. Therefore, the clumps might be formed by the inappropriate aggregation of many cortical patches/patch proteins at the cell cortex. Since cortical actin cytoskeleton proteins seem to be part of the endocytic machinery, actin patch aggregation at the cell cortex might in turn lead to invagination of the cell surface such that large clumps of cortical actin cytoskeleton proteins become inaccurately localized to the cytoplasm. Alternatively, the clumps might form as a result of detachment of patches from the cortex, or they might form as a result of inappropriate nucleation of patch assembly in the cytoplasm. The latter scenario would be possible if patch components are themselves recycled between an endocytic compartment and the plasma membrane, and loss of Ark1p and Prk1p function caused proteins responsible for nucleating patch assembly to be trapped in an endocytic compartment.

The cellular defects caused by loss of both Ark1p and Prk1p are alleviated by expressing wild-type Ark1p or Prk1p, but not by expressing Ark1p or Prk1p carrying a mutation in a residue required for kinase activity. Overexpression of either wild-type kinase results in a number of actin and growth defects, but these defects are absent when kinase-dead Ark1p is overexpressed, and reduced when kinase-dead Prk1p is overexpressed. Thus, the function(s) of Ark1p and Prk1p appear to depend upon their ability to function as kinases.

AArk1 and Prk1 fusion proteins localize to cortical actin patches. These two kinases are the first signaling proteins known to localize to actin patches. Because of their local-
Table III. Synthetic Genetic Interactions Involving ARK1 and PRK1

<table>
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<th>Temperature growth range</th>
<th>Actin organization</th>
<th>Negative growth synergy</th>
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<td>arklΔ</td>
<td>15°C–37°C</td>
<td>Normal</td>
<td>—</td>
</tr>
<tr>
<td>prk1Δ</td>
<td>15°C–37°C</td>
<td>Normal</td>
<td>—</td>
</tr>
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<td>sac6Δ</td>
<td>15°C–34°C</td>
<td>Depolarized patches, fewer cables</td>
<td>—</td>
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<td>slalΔ</td>
<td>15°C–34°C</td>
<td>Fewer, larger patches, depolarized</td>
<td>—</td>
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<td>slalΔ</td>
<td>15°C–30°C</td>
<td>Larger number of patches, depolarized</td>
<td>—</td>
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<td>abplΔ</td>
<td>15°C–37°C</td>
<td>Normal</td>
<td>—</td>
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<td>—</td>
</tr>
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<td>rvs167Δ</td>
<td>15°C–37°C</td>
<td>Delocalized actin</td>
<td>—</td>
</tr>
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<td>Normal</td>
<td>—</td>
</tr>
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<td>aiplΔ</td>
<td>15°C–37°C</td>
<td>Normal</td>
<td>—</td>
</tr>
<tr>
<td>arklΔ prk1Δ</td>
<td>20°C–34°C&lt;sup&gt;\text{a}&lt;/sup&gt;</td>
<td>Severely disrupted: actin “clumps” and depolarized patches, fewer cables</td>
<td>Yes</td>
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<tr>
<td>arklΔ sac6Δ</td>
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<td>Larger number of depolarized patches</td>
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<tr>
<td>prk1Δ sac6Δ</td>
<td>15°C&lt;sup&gt;\text{a}&lt;/sup&gt;–30°C&lt;sup&gt;\text{b}&lt;/sup&gt;</td>
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<td>15°C–37°C</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>prk1Δ abplΔ</td>
<td>15°C–34°C&lt;sup&gt;\text{b}&lt;/sup&gt;</td>
<td>Severely disrupted: actin “clumps” and depolarized patches</td>
<td>Yes</td>
</tr>
<tr>
<td>arklΔ svr2Δ</td>
<td>15°C–37°C&lt;sup&gt;\text{c}&lt;/sup&gt;</td>
<td>Same as svr2Δ</td>
<td>No</td>
</tr>
<tr>
<td>prk1Δ svr2Δ</td>
<td>15°C–37°C&lt;sup&gt;\text{c}&lt;/sup&gt;</td>
<td>Same as svr2Δ</td>
<td>No</td>
</tr>
<tr>
<td>arklΔ rvs167Δ</td>
<td>15°C–37°C</td>
<td>Same as rvs167Δ</td>
<td>No</td>
</tr>
<tr>
<td>prk1Δ rvs167Δ</td>
<td>15°C–37°C</td>
<td>Same as rvs167Δ</td>
<td>No</td>
</tr>
<tr>
<td>arklΔ cmrlΔ</td>
<td>15°C–37°C</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>prk1Δ cmrlΔ</td>
<td>15°C–37°C</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>arklΔ aiplΔ</td>
<td>15°C–37°C</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>prk1Δ aiplΔ</td>
<td>15°C–37°C</td>
<td>Normal</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>\text{a}</sup> Growth was tested at 15, 20, 25, 30, 34, and 37°C.
<sup>\text{b}</sup> Very slow growing at the indicated extremes of temperature.
<sup>\text{c}</sup> 7 out of the 12 predicted double-mutant progeny were inviable.
<sup>\text{d}</sup> Slow growing at the indicated extremes of temperature.

No other synthetic interactions involving ARK1 were observed. By contrast, prk1Δ showed severe negative synergy in combination with sla2Δ and with abp1Δ. prk1Δ and sla2Δ were synthetic lethal. prk1Δ abp1Δ double-mutant cells were large and contained actin clumps very similar to those found in arklΔ prk1Δ cells. abp1Δ cells, like arklΔ and prk1Δ single-mutant cells, are very healthy and normal in appearance. The pronounced synthetic phenotype of the prk1Δ abp1Δ double mutants thus reflects an extreme negative synergism. We conclude that Prk1p and A bp1p contribute to a critical process in a redundant manner, and that A bp1p may function with A rkp1p because both proteins are redundant with Prk1p but not with each other. arklΔ sac6Δ and prk1Δ sac6Δ cells were very large and temperature sensitive, but did not contain actin clumps (data not shown). The genetic interactions with sac6Δ may reflect general additive effects of cytoskeleton mutants rather than specific functional relationships in patch regulation because the sac6Δ mutation shows synthetic effects with a large number of mutant alleles of genes encoding components of the actin cytoskeleton (Holtzman et al., 1993; Botstein et al., 1997; Lila and D rubin, 1997), and because Sac6p is an actin filament bundling and stabilizing protein (A dams et al., 1989, 1991).

A pronounced phenotypic synergy between mutant alleles of two genes can reflect at least two functional relationships between the products of the genes. First, it may indicate that they act in separate pathways that can each perform the same essential function. Second, it may indicate that the two gene products are components of an essential protein complex which can retain functionality in the absence of one, but not both, proteins. abp1Δ, for example, is synthetic lethal with sla2Δ. This could be interpreted to suggest that Sla2p and A bp1p function in separate pathways towards a common, essential process. However, there is evidence suggesting that A bp1p and Sla2p might function as part of the same protein complex. A bp1p interacts with R vs167p and Srv2p via SH3 domain–poly-proline interactions (Lila and D rubin, 1997). R vs167p interacts with Sla2p in a two-hybrid assay (Wesp et al., 1997). Both proteins localize to cortical actin patches, and a specific domain in Sla2p performs an endocytosis function that is redundant with a function performed by A bp1p (Wesp et al., 1997). Thus A bp1p, Sla2p, R vs167p, and Srv2p have the potential to form a complex that may interact with and be regulated by A rkp1p.

Similarly, the genetic interactions involving Prk1p and A rkp1p (summarized in Fig. 9 A) may reflect synergistic contributions to the integrity of a single protein complex, or, alternatively, parallel pathways (Fig. 9 B). The lack of synthetic interactions between arklΔ and sla2Δ, or between arklΔ and abp1Δ, suggests formally that A rkp1p, Sla2p, and A bp1p function in the same pathway. The fact that A rkp1p and Sla2p interact in the two-hybrid assay supports this assessment. However, abp1Δ and sla2Δ are synthetically lethal, as mentioned above. Therefore, a more satisfactory interpretation of the current results would place A rkp1p in a cortical complex together with A bp1p and Sla2p. In the absence of Sla2p, A rkp1p is still capable of localizing to cortical patches, so A rkp1p is obviously capable of interacting with other cortical patch components, possibly via its proline-rich motifs. A bp1p, on the other hand, appears to be required in combination with A rkp1p for localization to cortical patches.

Since A rkp1p and Prk1p both need to be eliminated before significant defects in the actin cytoskeleton are observed, these kinases seem to have redundant functions. To gain deeper insights into the functional relationships between these kinases, we made a series of double mutants between the arklΔ or prk1Δ mutants and mutants of genes encoding actin cortical cytoskeleton proteins. A ny synthetic effects involving the ARK1 or the PRK1 genes are likely to be significant since arklΔ and prk1Δ single-mutant cells are very similar to wild-type cells in terms of appearance and growth. Null mutants in ARK1 or in PRK1 both show synthetic defects in combination with null mutants in the SCA6 gene, encoding yeast fimbrin, an actin filament bundling protein (Table III and Fig. 9 A).
hand, is important for the localization of A rk1p to patches, so perhaps its SH 3 domain is also capable of interacting with the proline-rich motif in A rk1p.

Ark1p, in contrast to ark1Δ, shows synthetic effects with both sla2Δ and with abp1Δ. Therefore, Prk1p may either function in a separate pathway that operates in parallel to the A rk1p pathway to regulate the cortical actin cytoskeleton, or it may impinge upon the same complex in a different manner. The fact that combining null mutations in the SLA 2 and PRK 1 genes leads to more deleterious effects than result from combining null mutations in the ARK 1 and PRK 1, or in the ABP 1 and PRK 1 genes, suggests that Sla2p has additional functions to those that involve A rk1p and A bp1p. A s mentioned above, placing Prk1p in a separate pathway from A rk1p, however, does not exclude the possibility that Prk1p interacts with a potential A bp1p/R vs167p/Srv2p/Sla2p complex. Prk1p localizes to cortical actin patches, and, as with Ark1p, it shows a dependency on A bp1p (but not Sla2p, R vs167p, S ac6p, or Srv2p) for normal localization to these patches, implying that it too may associate with such a complex. However, since deletion of the ABP 1 gene does not result in a phenocopy of the ark1Δ prk1Δ double deletion and does not eliminate the effects of A rk1p or Prk1p overexpression, it is reasonable to assume that localization at a reduced level allowed by interaction with, for example, Sla2p, is sufficient for A rk1p and Prk1p function. Alternately, it might be that localization of these kinases to cortical patches is not required for A rk1p and Prk1p to fulfill their cellular functions.

What might be the phosphorylation targets of A rk1p and Prk1p? Our evidence strongly suggests that both A rk1p and Prk1p are functional kinases in vivo (see above). Other A rk family kinases have also been shown to be functional protein kinases in vitro (K imura et al., 1997). One putative target is Sla2p, because it interacts with A rk1p in the two-hybrid system. Sla2p is a phosphoprotein in vivo, but phosphorylation of Sla2p is not eliminated in ark1Δ prk1Δ cells (data not shown). Results published while this manuscript was under revision indicate that Prk1p regulates by phosphorylation the activity of Pan1p (Zeng and Cai, 1999). Pan1p is a yeast homologue of the mammalian Eps15 proteins that play an important role in endocytosis (W endland and E mr, 1998). Prk1p is also capable of phosphorylating the cortical actin patch protein, Sla1p (Zeng and Cai, 1999). Pan1p is an essential protein, a temperature-sensitive mutation (pan1-4) which causes the appearance of actin clumps similar to those observed in ark1Δ prk1Δ cells under nonpermissive conditions (T ang and C ai, 1996). The similar phenotypes of pan1-4 and ark1Δ prk1Δ cells suggest that Pan1p might be regulated by A rk1p and by Prk1p. Since actin clumps do not appear in ark1Δ or prk1Δ single-mutant cells, Pan1p might be phosphorylated by A rk1p in the absence of Prk1p and vice versa (Fig. 9 B). While our studies, together with those of Zhang and Cai, suggest that both A rk1p and Prk1p play important roles in Pan1p regulation, our genetic analysis (Fig. 9 A) is most consistent with the possibility that Prk1p but not A rk1p regulates Sla1p. We base this conclusion on the observation that null mutations in the PRK 1 gene, but not in the ARK 1 gene, have similar genetic interactions as those exhibited by null alleles of the SLA 1 gene.

A rk1p and Prk1p clearly play a critical role in regulating actin distribution in vivo. This conclusion is based on several different criteria: effects of null mutations, localization, overexpression effects, and genetic interactions with other genes encoding proteins known to be involved in modulating actin distribution in yeast. Elucidation of the signaling pathways in which A rk1p and Prk1p are involved and the identification of upstream and downstream components of those pathways are now important goals.

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