The *Schizosaccharomyces pombe* Aurora-related Kinase Ark1 Interacts with the Inner Centromere Protein Pic1 and Mediates Chromosome Segregation and Cytokinesis

Joel D. Levenson, Han-kuei Huang, Susan L. Forsburg, and Tony Hunter*

Molecular and Cell Biology Laboratory, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037

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The chromosomal passenger proteins aurora-B, survivin, and inner centromere protein (INCENP) have been implicated in coordinating chromosome segregation with cell division. This work describes the interplay between aurora, survivin, and INCENP orthologs in the fission yeast *Schizosaccharomyces pombe* and defines their roles in regulating chromosome segregation and cytokinesis. We describe the cloning and characterization of the aurora-related kinase gene *ark1*⁺, demonstrating that it is an essential gene required for sister chromatid segregation. Cells lacking Ark1p exhibit the *cut* phenotype, DNA fragmentation, and other defects in chromosome segregation. Overexpression of a kinase-defective version of Ark1, Ark1-K147R, inhibits cytokinesis, with cells exhibiting an elongated, multiseptate phenotype. Ark1p interacts physically and/or genetically with the survivin and INCENP orthologs Bir1p and Pic1p. We identified Pic1p in a two-hybrid screen for Ark1-K147R interacting partners and went on to map domains in both proteins that mediate their binding. Pic1p residues 925–972 are necessary and sufficient for Ark1p binding, which occurs through the kinase domain. As with Ark1-K147R, overexpression of Ark1p-binding fragments of Pic1p leads to multiseptate phenotypes. We also provide evidence that the dominant-negative effect of Ark1-K147R requires Pic1p binding, indicating that the formation of Ark1p-Pic1p complexes is required for the execution of cytokinesis.

INTRODUCTION

The segregation of replicated chromosomes in mitosis must be precisely coordinated in space and time with the process of cytokinesis. Failure to separate the proper complement of chromosomes into each daughter cell before cytokinesis can result in aneuploidy, which is linked to cell transformation and the development of cancer (for review, see Marx, 2001). The aurora-Ipl1 kinases have received much attention recently for their roles in regulating chromosome dynamics and cytokinesis (for reviews, see Bischoff and Plowman, 1999, and Giet and Prigent, 1999). Ectopic expression of

aurora kinases has been shown to be transforming (Bischoff *et al.*, 1998; Zhou *et al.*, 1998; Giet and Prigent, 1999), and *aurora* amplification or overexpression has been associated with breast (Sen *et al.*, 1997; Zhou *et al.*, 1998; Tanaka *et al.*, 1999; Miyoshi *et al.*, 2001) and colon (Bischoff *et al.*, 1998; Takahashi *et al.*, 2000; Sakakura *et al.*, 2001) tumors. A detailed understanding of how these kinases regulate cell division could thus prove useful in the development of cancer treatments.

Aurora kinases are defined by a high degree of identity in their respective kinase domains and can be subdivided into three classes: auroras A, B, and C (for review, see Nigg, 2001). The aurora-A kinases are regulators of centrosomal duplication and separation and are thus crucial for the formation of the bipolar mitotic spindle (Glover *et al.*, 1995; Roghi *et al.*, 1998; Giet and Prigent, 2000; for review, see Goepfert and Brinkley, 2000). Glover and coworkers identified the founding member of this family in *Drosophila melanogaster* and discovered that cells lacking aurora have circular arrays of chromosomes organized around a monopolar spindle (Glover *et al.*, 1995). Aurora-A kinases localize to centrosomes and are found along mitotic spindles through-

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* Corresponding author. E-mail address: hunter@salk.edu

Abbreviations used: APC, anaphase-promoting complex; BIR, baculoviral inhibitor-of-apoptosis repeat; DAPI, 4,6-diamidino-2-phenylindole; GST, glutathione-S-transferase; HA, hemagglutinin; INCENP, inner centromere protein; KD, kinase-defective; YES, yeast extract with supplements.

out mitosis and into telophase (Kimura *et al.*, 1997; Bischoff *et al.*, 1998; Roghi *et al.*, 1998; Schumacher *et al.*, 1998; Zhou *et al.*, 1998). Depletion of *Caenorhabditis elegans* aurora-A (AIR-1) by RNA interference leads to severe aneuploidy and embryonic lethality (Schumacher *et al.*, 1998a). Although centrosomes are able to separate in this case, they appear abnormal, and the mitotic spindle is disorganized.

Members of the aurora-B subfamily function later in mitosis, with transcript and protein levels peaking after those of aurora-A (for review, see Bischoff and Plowman, 1999). These kinases belong to a class of proteins referred to as chromosomal passengers, which are thought to play key roles in coordinating chromosome segregation with cytokinesis (for review, see Adams *et al.*, 2001). Chromosomal passengers are found along the length of chromosomes in prophase, concentrate at the inner centromere in metaphase, and are left behind at the central spindle during anaphase, where they are thought to function in organizing and activating the cytokinetic machinery. Depletion of aurora-B kinases (Schumacher *et al.*, 1998b; Woollard and Hodgkin, 1999; Kaitna *et al.*, 2000) or expression of dominant-negative versions (Terada *et al.*, 1998) results in late blocks to cytokinesis. Interestingly, the downregulation of aurora-B is required for the polyploidization of human megakaryocytic lineages, which involves multiple rounds of replication in the absence of cytokinesis (Katayama *et al.*, 1998; Kawasaki *et al.*, 2001).

Aurora-B orthologs have been shown to bind to the inner centromere proteins (INCENPs), chromosomal passenger proteins that serve to localize and perhaps activate these kinases (Kim *et al.*, 1999; Adams *et al.*, 2000; Kaitna *et al.*, 2000; Adams *et al.*, 2001). HeLa cells expressing a C-terminally truncated INCENP mutant (INCENP₁₋₄₀₅) mislocalize aurora-B (Adams *et al.*, 2000) and exhibit defects in mitosis and cytokinesis (Mackay *et al.*, 1998). RNA interference experiments have shown that *D. melanogaster* aurora-B is required for the transfer of INCENP from chromosome arms to centromeres and the midbody (Adams *et al.*, 2001). Another passenger protein, baculoviral inhibitor-of-apoptosis repeat (BIR)-1/survivin, is required for the localization of *C. elegans* aurora-B (AIR-2) to chromosomes (Speliotes *et al.*, 2000). Human survivin is reported to bind directly to both aurora-B and INCENP (Wheatley *et al.*, 2001), and INCENP₁₋₄₀₅ inhibits the transfer of survivin from chromosomes to centromeres and the central spindle. Taken together, these data indicate that the initial localization of aurora-B depends on INCENP and/or survivin and that the subsequent migrations of these three passenger proteins are interdependent.

Binding to INCENP and survivin may serve to target aurora-B to its substrates, which include diverse proteins involved in mitosis and cytokinesis (for review, see Giet and Prigent, 1999). The single budding yeast aurora kinase Ipl1p phosphorylates the kinetochore protein Ndc10p and is thought to mediate microtubule-kinetochore attachments (Biggins *et al.*, 1999; Sassoon *et al.*, 1999). Bir1p binds directly to Ndc10p and could target Ipl1p in this case (Yoon and Carbon, 1999). Aurora-B orthologs also phosphorylate serine residue 10 in histone H3 (Hsu *et al.*, 2000; Speliotes *et al.*, 2000; Adams *et al.*, 2001; Giet and Glover, 2001), an event that is required for premitotic chromosome condensation (Wei *et al.*, 1999). Aurora-B family members have also been

implicated in phosphorylating kinesin-like proteins that assemble and stabilize the central spindle (Geiser *et al.*, 1997; Giet *et al.*, 1999; Kaitna *et al.*, 2000; Severson *et al.*, 2000). *C. elegans* aurora-B (AIR-2) binds to the kinesin-like protein ZEN-4, and both AIR-2 and INCENP (ICP-1) are required to recruit ZEN-4 to the central spindle (Kaitna *et al.*, 2000; Severson *et al.*, 2000). There is also evidence that aurora-B is required to recruit the Pavarotti kinesin-like protein in *D. melanogaster* (Giet and Glover, 2001), although these data were recently contradicted by another report (Adams *et al.*, 2001). Finally, it has been reported that rat aurora-B can phosphorylate the myosin II regulatory light chain (Murata-Hori *et al.*, 2000) and might thereby regulate contraction of the actomyosin ring.

Although vertebrates have up to three aurora kinases, budding yeast and fission yeast each possess only one family member. Chan and Botstein discovered the first aurora kinase, budding yeast Ipl1p, in 1993 (Chan and Botstein, 1993), and since then it has been further characterized by others (Biggins *et al.*, 1999; Kim *et al.*, 1999; Wei *et al.*, 1999). Cells bearing a temperature-sensitive allele of *IPL1* exhibit spindle pole defects and severe chromosome missegregation. Ipl1p was also the first aurora kinase shown to associate with an INCENP, Sli15p (Kim *et al.*, 1999). Here, we describe the essential role of the *S. pombe* counterpart, aurora-related kinase 1p (Ark1p), in chromosome segregation, its interactions with the survivin homolog Bir1p, and its binding to the fission yeast inner centromere protein Pic1p. We define the domains in both Ark1p and Pic1p that mediate their binding and provide evidence that their interaction is required for the completion of cytokinesis.

MATERIALS AND METHODS

Yeast Strains and Media

Fission yeast were grown in the rich medium yeast extract with supplements (YES) or in Edinburgh minimal medium with the appropriate supplements (Moreno *et al.*, 1991). Diploids were generated by crossing the haploid strains FY527 (*h⁺ ura4-D18 leu1-32 his3-D1 ade6-M216*) and FY528 (*h⁺ ura4-D18 leu1-32 his3-D1 ade6-M210*). Overexpression studies were carried out in the haploid wild-type strain FY254 (*h⁺ ura4-D18 leu1-32 can1-1 ade6-M210*). The integration construct pJK210-*ark1⁺*-HA3 was linearized with PflMI and used to transform the temperature-sensitive strain FY584 (*h⁺ cdc25-22 ura4-D18 leu1-32 ade6-M216 ark1+HA3::ura4⁺*) to yield JLY55 (*h⁺ cdc25-22 ura4-D18 leu1-32 ade6-M216 ark1+HA3::ura4⁺*).

Cloning, *ark1⁺* Disruption, and Mutagenesis

ark1⁺ was PCR-amplified from genomic DNA using the primers 5'-AGTGGCGCCGCTGATGGTGTTACCTCAAAATG-3' and 5'-TTGAGCGCCGCGCGGAAGATTTCAGAACTTTTGC-3', digested with *NotI*, and cloned into pBluescript-KS⁺ to give pBS-*ark1⁺*. To generate an *ark1⁺* disruption construct, two *ark1⁺* fragments were PCR-amplified from pBS-*ark1⁺* and cloned into pAF1. The first product, encompassing the first 347 bp of *ark1⁺*, was amplified with a T3 primer and 5'-ACGCGTCGACCAATATGAAATTCTCGC-CATTG-3', digested with *SalI*, and cloned into pAF1 to give pAF1-*ark1⁺*-A. The second product, comprising the last 308 bp, was amplified with a T7 primer and 5'-AACTGCAGCCACCTGAAATGG-TGGAGGG-3', digested with *PstI* and *SacI*, and cloned into pAF1-*ark1⁺*-A to give disruption construct pAF1-*ark1⁺*-B. Diploids generated by crossing strains FY527 and FY528 were then transformed with a 2.7-kb *NotI* fragment from pAF1-*ark1⁺*-B. Proper integration

was confirmed by probing Southern blots of *Ban*I-digested genomic DNA with a probe spanning the first 351 bp of *ark1*⁺.

ark1⁺ was subcloned from pBS-*ark1*⁺ into pGEX-KG using *Xba*I and *Sac*I to generate pGST-*ark1*⁺. Constructs linking *ark1*⁺ downstream of the full-strength *nmt1* promoter (Maundrell, 1993) were generated by digesting pBS-*ark1*⁺ with *Not*I and ligating the purified insert into either pSLF172 (Forsburg and Sherman, 1997) or pSGP72. Partial fragments of *ark1*⁺ were PCR-amplified from pBS-*ark1*⁺ using primers engineered with *Not*I sites for cloning into pSLF172. All pSLF172 constructs express proteins in-frame with three C-terminal hemagglutinin (HA) epitope tags, which were detected by immunoblotting with the 12CA5 anti-HA monoclonal antibody (mAb). An insert containing *ark1*⁺::HA3 and the *nmt1* terminator was subcloned from pSLF172-*ark1*⁺ into pJK210 using *Xho*I and *Sac*I to yield pJK210-*ark1*⁺-HA3.

The two-hybrid bait construct pGBT9-*ark1*^{K147R} was generated by PCR-amplifying *ark1*^{K147R} with 5'-CCGGAATTCGTGTACCTCAAAATGTAAACAAC-3' and 5'-TTTCTGCAGGGAAGATTCA-GAACTTTTGCAG-3', digesting with *Eco*RI and *Pst*II, and ligating into pGBT9 (Clontech, Palo Alto, CA). Bait pGBT9-*ark1*^{NT}, comprising the N-terminal 116 residues of Ark1p, was constructed by amplifying an *ark1*⁺ fragment with 5'-CCGGAATTCGTGTACCTCAAAATGTAAACAAC-3' and 5'-TTTCTGCAGCATTCCAATATGAAATTCGCGCC-3' and ligating into pGBT9 with *Eco*RI and *Pst*II. *pic1*⁺ fragments were PCR-amplified with primers engineered with *Eco*RI and *Xho*I sites for ligation into the prey vector pGAD-GH (Clontech).

All mutagenesis was carried out with the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The sequence of each mutant was confirmed by automated sequencing (Applied Biosystems Inc., Foster City, CA).

Spore Germination Assay, 4,6-Diamidino-2-Phenylindole/Calcofluor Staining, and Immunofluorescence

Spo⁺ diploid cells heterozygous for the *ark1*⁺ disruption were grown in YES to an OD₅₉₅ of 0.8 and then allowed to sporulate for 72 h in liquid malt extract (25°C). Cultures were checked for asci and then treated with 2% glutulase overnight at 25°C. Spores were rinsed in yeast nitrogenous bases lacking ammonium sulfate and spun through a 25% glycerol cushion for 10 min at 2000 rpm. After three additional rinses, *ark1*⁺-disrupted his⁺ spores were allowed to germinate overnight at 32°C in Edinburgh minimal medium lacking histidine. Cells were then stained with 4,6-diamidino-2-phenylindole (DAPI) for analysis by fluorescence microscopy. DAPI/calcofluor staining was performed according to previously published methods (Moreno *et al.*, 1991).

Recombinant Proteins and Kinase Assays

Glutathione-S-transferase (GST)-fusion and His-tagged proteins were expressed in *Escherichia coli* and purified as described previously (Leverson *et al.*, 2000). An N-terminal fragment of Bir1p encompassing the first 330 residues was expressed fused to eight N-terminal histidines for purification with Talon metal affinity resin (Clontech). Bir1₁₋₃₃₀ fractions also contained an abundant breakdown product, which peptide sequencing and mass spectrometry revealed to comprise residues 1–208. Kinase assays were performed as follows: GST-Ark1p was bound to 10 µl bed-volume glutathione-agarose in 1 ml PBS for 30 min at 4°C. Beads were washed twice with PBS, three times with kinase wash (20 mM HEPES pH 7.4, 1 mM dithiothreitol), and then incubated for 20 min with or without substrates in kinase wash with 20 mM MgCl₂, 25 µM ATP, and 10 µCi [γ-³²P]ATP. Reactions were stopped by adding equal volumes of 2× SDS-PAGE loading buffer. The samples were boiled and separated on SDS-PAGE gels, which were then dried for autoradiography.

Yeast Two-Hybrid Screens and Direct Pairwise Tests

Budding yeast two-hybrid strain AH109 (Clontech) harboring the *HIS3*, *ADE2*, and *lacZ* reporters downstream of heterologous GAL4-responsive promoter elements was first transformed with pGBT9-*ark1*^{K147R} or pGBT9-*ark1*^{NT} with a standard lithium acetate procedure. Then, 600-ml cultures of these transformants were used for transformation with 50 µg of *S. pombe* Matchmaker cDNA library (Clontech) and plated to minimal medium containing Lys, Ade, Ura, Tyr, Met, and 2.5 or 10 mM 3-amino-1,2,4-triazole. Several His⁺ colonies were picked between days 4 and 8 after plating, restreaked to –Trp–Leu–His–Ade medium, and patched out for β-galactosidase colony-lift filter assays (Matchmaker System User Manual, Clontech). Isolates that tested positive for β-galactosidase activity were cultured in –Leu liquid medium overnight and used to prepare total DNA for PCR amplification and sequencing of library vector inserts.

RESULTS

ark1⁺ Is Required for Sister Chromatid Segregation

Others had previously noted the existence of an aurora kinase gene in *S. pombe* (accession number AL022245.2; Bischoff and Plowman, 1999; Giet and Prigent, 1999). We designed oligonucleotides to PCR-amplify *ark1*⁺ (aurora-related kinase 1, named by I. Hagan, see Morishita *et al.*, 2001) from genomic DNA and went on to disrupt one copy of the gene in a diploid strain. Genomic Southern blotting confirmed that a large portion of *ark1*⁺, encoding kinase subdomains I–VIII, had been replaced by the *his3*⁺ gene (Figure 1A and our unpublished results). His⁺ heterozygotes were allowed to sporulate, and tetrad dissection was carried out on YES. Of 30 tetrads examined, 29 comprised two viable and two nonviable spores, with all of the viable colonies being His[–] (Figure 1B and our unpublished results). The disruption was thus lethal, demonstrating that the *ark1*⁺ gene is essential for viability. As expected, expressing *ark1*⁺ from an episome rescued the lethality of haploid disruptants (our unpublished results).

We next investigated the cause of lethality by performing spore germination experiments. Spores generated from heterozygous *ark1*⁺ disruptants were grown overnight in medium lacking histidine, allowing us to selectively monitor the growth of *ark1*⁺-disrupted cells. Spores germinated, but DAPI staining revealed that most of the resulting cells failed to complete mitosis. A large portion exhibited the *cut* (cell untimely torn) phenotype, with sister chromatids failing to separate before cytokinesis (Figure 1C). This phenotype can be indicative of failures in chromosome condensation, bipolar spindle formation, microtubule-kinetochore attachments, and/or sister chromatid separation/segregation (Yanagida, 1998). We often observed dividing cells with unequal amounts of DNA on either side of the septum, and many cells exhibited stretched or fragmented DNA (Figure 1C). These phenotypes are very similar to the chromosome condensation and segregation defects observed in cells deleted for *bir1*⁺/*pbh1*⁺/*cut17*⁺ (Samejima *et al.*, 1993; Rajagopalan and Balasubramanian, 1999; Uren *et al.*, 1999; and our unpublished results). All of the observed *ark1*⁺ disruption phenotypes are consistent with a role for Ark1p in chromosome segregation.

Figure 1. *ark1⁺* is essential for chromosome segregation. (A) One copy of *ark1⁺* was disrupted in a diploid strain by homologous recombination. The disruption replaces kinase subdomains I–VIII with the *his3⁺* gene. The kinase-encoding region is indicated by a thick line, and restriction sites are indicated as reference points. (B) *ark1⁺/ark1::his3⁺* heterozygotes were sporulated and analyzed by tetrad dissection. Viable:inviable spore ratios of 2:2 were observed on rich medium (YES). Replica plating to minimal medium lacking histidine demonstrated that viable spores possess wild-type *ark1⁺*. (C) Purified spores bearing the *ark1⁺* disruption were allowed to germinate in minimal medium lacking histidine. After 20 h at 32°C, cultures were analyzed by DAPI staining. Cells lacking Ark1p exhibited the *cut* phenotype (arrows) and often showed stretched or fragmented DNA (bottom left panels). Wild-type cells are shown for comparison (top left panels).

Ark1p Alleviates the Slow-Growth Defect of bir1–46 and Phosphorylates Bir1p In Vitro

The inhibitor of apoptosis protein survivin was originally described as a suppressor of programmed cell death, but recent work has shed light on its main role as a mitotic regulator (for reviews, see Reed and Bischoff, 2000, and Silke and Vaux, 2001). In fact, survivin is now known to behave as

a chromosomal passenger protein (Skoufias *et al.*, 2000; Uren *et al.*, 2000; Wheatley *et al.*, 2001). The *C. elegans* survivin homolog BIR-1 has been reported to play essential roles in cytokinesis (Fraser *et al.*, 1999). Like Ark1p, the fission yeast survivin homolog Bir1p (Pbh1p/Cut17p) is an essential mediator of chromosome segregation (Samejima *et al.*, 1993; Rajagopalan and Balasubramanian, 1999; Uren *et al.*, 1999;

Figure 2. Ark1p alleviates the slow-growth phenotype of *bir1-46* and phosphorylates Bir1p *in vitro*. (A) Temperature-sensitive strain *bir1-46* grows more slowly than wild-type cells at 28°C. *bir1-46* cells were transformed with a construct for expressing *bir1*⁺ from its endogenous promoter (squares), one for expressing *ark1*⁺ from the thiamine-repressible *nmt1* promoter (diamonds), or with empty *nmt1* vector alone (circles). Transformed cells were washed extensively in medium lacking thiamine and grown overnight at 28°C to allow for the induction of Ark1p. The cultures were diluted to an OD₅₉₅ of 0.1 and grown in parallel at 28°C. The OD₅₉₅ of each culture was measured at various

time points as a measure of cell division. (B) Wild-type and KD Ark1p were purified as GST-fusion proteins and used to perform *in vitro* kinase assays. GST-Ark1p (WT) incubated alone became autophosphorylated and migrated as multiple bands (lane 1), whereas KD GST-Ark1-K147R migrated as a single band (lane 2). Both proteins were also incubated with purified fragments of Bir1p (Bir1p 1-330 and Bir1p 1-208). GST-Ark1p phosphorylated both fragments of Bir1p (arrows), whereas GST-Ark1-K147R did not. The positions of molecular weight markers (in kDa) are indicated at left. The schematic depicts the domain structure of the Bir1 proteins, with gray boxes representing the two BIR motifs.

Huang *et al.*, in preparation). Cells lacking Bir1p resemble *ark1*⁺-deleted cells, exhibiting the *cut* phenotype and other chromosome segregation defects. We thus investigated the potential links between Ark1p and Bir1p.

Yeast cells bearing the temperature-sensitive allele *bir1-46* grow more slowly than wild-type cells at 28°C and exhibit severe defects in sister chromatid segregation at 34°C (Huang *et al.*, in preparation). Because BIR-1 acts to localize AIR-2 in *C. elegans* (Speliotes *et al.*, 2000), we hypothesized that high levels of Ark1p might rescue the defects associated with *bir1-46*. Indeed, overexpression of Ark1p alleviated the slow-growth phenotype observed at 28°C (Figure 2A). Cells expressing Ark1p from the full-strength *nmt1* promoter grew nearly as well as those expressing wild-type Bir1p and grew much faster than cells transformed with the *nmt1* vector alone. However, Ark1p overexpression was not sufficient to rescue *bir1-46* at 34°C (our unpublished results). Nevertheless, the former result indicates that Ark1p, when produced at sufficient levels, is capable of fulfilling some function that is compromised in *bir1-46* cells.

To determine whether Ark1p could phosphorylate Bir1p *in vitro*, recombinant Ark1p was isolated as a semipurified GST-fusion protein. On incubation with [γ -³²P]ATP, GST-Ark1p was autophosphorylated and could be detected as multiple bands on autoradiographs (Figure 2B, lane 1). Like other aurora family members, GST-Ark1p is able to phosphorylate myelin basic protein but not histone H1 (our unpublished results). GST-Ark1p also phosphorylated a semipurified, His-tagged Bir1p fragment (residues 1-330) and strongly phosphorylated a shorter breakdown product (residues 1-208) that retains the two BIR domains (Figure 2B, lane 3). As a negative control, we constructed a kinase-defective (KD) form of Ark1p, with lysine 147 in kinase subdomain II mutated to arginine (Hanks and Hunter, 1995). When analyzed for kinase activity *in vitro*, GST-Ark1-K147R generated only a single weak autophosphorylated band (Figure 2B, lane 2) and failed to phosphorylate myelin basic

protein (our unpublished results), indicating that the kinase is indeed defective. As expected, GST-Ark1-K147R failed to phosphorylate either fragment of Bir1p (Figure 2B, lane 4).

Overexpression of KD Ark1p Leads to Defects in Cytokinesis

Because KD versions of other aurora family members have been shown to inhibit cytokinesis, we tested whether Ark1-K147R could exert dominant-negative effects *in vivo*. Several versions of Ark1p (Figure 3A) were overexpressed in wild-type strain FY254 from the *nmt1* promoter, which is induced in the absence of vitamin B₁/thiamine (Maundrell, 1993). Each protein was expressed in-frame with three C-terminal HA epitope tags to allow for its detection by immunoblotting. Although HA-tagged Ark1p was capable of rescuing *ark1*⁺-disrupted haploids, the HA-tagged kinase domain alone (Ark1-KIN) was not (our unpublished results). Neither Ark1p nor Ark1-KIN overexpression had any obvious effects on colony formation (Figure 3B). Cells overproducing Ark1-K147R (KD), however, grew very slowly and could only form microcolonies. Microscopic examination revealed that the majority of these cells were elongated and often branched (Figure 3C). Cells overexpressing Ark1-K147R in liquid medium were also elongated, and many possessed multiple division septa (Figure 3C). An unusually large percentage (~40% compared with ~10% in typical wild-type cultures) of cells were singly septated and binucleate. Chromosome segregation appears to occur normally in these cells, and even cells with multiple septa exhibit centrally localized DAPI-staining masses on either side of a given septum. Ark1-K147R thus appears to be acting after chromosome segregation to inhibit cell division in a dominant-negative manner.

Interestingly, a KD mutant lacking the first 116 amino acids (Figure 3A, KIN-KD) did not affect cell growth (Figure 3B), suggesting that the N-terminal, nonkinase domain of

Figure 3. The Ark1-K147R dominant-negative mutant inhibits cytokinesis. (A) Schematic of wild-type and mutant versions of Ark1p expressed from the thiamine-repressible *nmt1* promoter. The catalytic kinase domain (CAT) is indicated as a gray box, and the putative KEN box is shown as a hatched box. Vect, empty *nmt1* vector; Ark1, wild-type Ark1p; KD, KD Ark1-K147R; NT, N-terminal fragment of Ark1p (1–116); KIN, kinase domain of Ark1p (117–384); KIN-KD, KD kinase domain of Ark1p. An immunoblot performed with anti-HA mAb 12CA5 (right) demonstrates that similar levels of wild-type Ark1, the KD kinase domain, and the N-terminal fragment were expressed. Molecular weight markers (in kDa) are indicated at left. (B) Ark1 proteins described in A were overexpressed in wild-type cells grown on solid medium lacking thiamine (–B1). Cells expressing KD Ark1p grew poorly, forming only microcolonies, whereas cells expressing the N-terminal domain (NT) or the KD kinase domain alone (KIN-KD) were unaffected. (C) Cells expressing Ark1-KD on solid medium were elongated and branched (bottom left). The same cells grown in inducing liquid medium possess multiple septa (center) and multiple nuclei (right), as revealed by calcofluor and DAPI staining, respectively.

Ark1p is required for the dominant-negative effect. This region could feasibly serve to mediate protein–protein interactions, and we reasoned that expressing it alone (Figure 3A, NT) might be sufficient to inhibit cytokinesis. NT, however, did not affect cell growth (Figure 3B), although significant levels of the mutant were expressed (Figure 3A). Thus, the KD kinase domain, along with some N-terminal sequence, is required for the dominant-negative effect.

To identify the N-terminal regions that are required for the dominant-negative effect, we constructed a series of truncation mutants, each bearing the K147R mutation (Figure 4A). Each mutant was again expressed from the *nmt1* promoter in wild-type cells. As before, full-length Ark1-K147R exerted a dominant-negative effect, whereas its kinase domain alone (117–384 KD) did not (Figure 4B). Truncation mutant 24–384-KD retained dominant-negative activity, but mutants 48–384-KD and 72–384-KD did not, suggesting to us that residues 24–47 play a crucial role in mediating the effect. However, colony formation was also inhibited by mutant 88–384-KD, demonstrating that residues 88–116 are necessary and sufficient to restore dominant-negative activity to the KD kinase domain.

The inability of 48–384-KD and 72–384-KD to act as dominant-negatives seemed to be a function of residues 48–87, which are absent in 88–384-KD. The amino acids KEN-KRTSNSK (78–87) are conspicuous in this region, because similar N-terminal sequences are found in most of the other aurora family members (see Bischoff and Plowman, 1999). This sequence also resembles the recently described “KEN box” (Pfleger and Kirschner, 2000), which has been shown to act as a recognition sequence for the anaphase-promoting complex (APC) ubiquitin ligase. If the truncations in 48–

384-KD and 72–384-KD were exposing a functional degron, the proteins would no longer exert a negative effect simply because they were unstable. However, 48–384-KD and 72–384-KD bearing the mutations KENKRTSNSK to AAARKRTSNSA were unable to inhibit cell growth (Figure 4B). Moreover, these mutants were stably expressed regardless of whether the KEN box was mutated or not (Figure 4C and our unpublished results). Constitutive expression of full-length Ark1p with the KEN box mutations also had no effect on the ability of cells to form colonies (Figure 4B). Thus, the Ark1p KEN sequence does not appear to be responsible for the failure of 48–384-KD and 72–384-KD to act as dominant-negatives.

Ark1p Levels Remain Constant throughout the Cell Cycle

Although our data indicated that the KEN sequence is not playing a role in regulating Ark1p levels, we nevertheless decided to monitor Ark1p levels throughout the cell cycle. We engineered temperature-sensitive strain *cdc25–22* to express a C-terminally HA epitope-tagged version of Ark1p from its endogenous promoter. The strain was blocked in G2 at 35°C and then released synchronously into mitosis by shifting to 25°C. Samples were taken every 20 min to prepare cell extracts and to determine the percentage of septated cells as a measure of synchrony. Although the cultures progressed synchronously through the cell cycle, anti-HA immunoblotting revealed that Ark1p levels did not vary significantly over the course of our experiment (Figure 4D). Ark1p levels thus appear to remain relatively constant throughout the cell cycle. These data, along with the obser-

Figure 4. Structure-function analysis of the Ark1-K147R dominant-negative mutant. (A) A series of N-terminally truncated Ark1-K147R mutants was tested for the ability to inhibit wild-type cell growth. The residues present in each mutant are indicated at left. To test the role of a putative KEN box, comprising residues 78–87 (hatched box), the KEN-KRTSNK sequence was mutated to AAARKTSNSA (black dots). (B) Ark1 proteins described in A were overexpressed in wild-type cells from the thiamine-repressible *unt1* promoter. Cells expressing KD Ark1p (KD) grew poorly on solid medium lacking thiamine, whereas cells expressing the KD kinase domain alone (117–384-KD) were not affected. Truncation mutant 88–384-KD behaves identically to the full-length KD mutant, indicating that residues 88–116 are sufficient to restore dominant-negative activity to the kinase domain. Mutants 48–384-KD and 72–384-KD have no effect on cell

growth, whereas 24–384-KD again inhibits colony formation. Wild-type cells were also unaffected by the expression of KEN box-mutated versions of 48–384-KD and 72–384-KD. (C) Extracts were prepared from liquid cultures of cells expressing HA-tagged Ark1p or truncation mutants KEN-48–384-KD, KEN-72–384-KD, or 117–384-KD, and then analyzed by immunoblotting with anti-HA mAb 12CA5. Molecular weight markers (in kDa) are indicated at left. (D) Temperature-sensitive strain *cdc25-22* was engineered to express an HA epitope-tagged version of Ark1p from its endogenous promoter. A liquid culture of this strain was blocked in G2 by incubation at 35°C, then released to undergo synchronous cell division at 25°C. Samples were taken every 20 min to prepare cell extracts, which were then analyzed by SDS-PAGE and immunoblotting with anti-HA antiserum. The percentage of septated cells was determined at each time point as a measure of the culture's synchrony.

vation that constitutive expression of Ark1p appears to have no serious consequences (Figure 3B), indicate that total Ark1p levels do not need to be tightly regulated during the cell cycle.

Ark1 Binds to Pic1p, the Fission Yeast Inner Centromere Protein

We next performed a yeast two-hybrid screen in an attempt to identify potential Ark1p regulators, adaptors, and/or substrates. The full-length, KD mutant Ark1-K147R was used as bait in the hope of identifying proteins related to its dominant-negative effect. Because a portion of the N-terminal region of Ark1-K147R is clearly required for its dominant-negative effect (Figure 4), a bait comprising residues 1–116 (pGBT9-NT) was also used. With the Ark1-K147R bait, a screen of $>1.5 \times 10^7$ transformants yielded 42 *his*⁺ *ade*⁺ colonies, 16 of which also scored positive for β -galactosidase activity. DNA sequencing revealed that six of the library clones expressed C-terminal fragments of the *S. pombe* homolog of INCENP, which we will refer to as Pic1p (*S. pombe* inner centromere protein). Four of these clones expressed Pic1p residues 765–1018 in-frame with the GAL4 activation domain, whereas the other two expressed only residues 925–1018 (Figure 5A). The latter piece corresponds to the highly conserved “IN box” previously described in other

INCENP homologues (Adams *et al.*, 2000; Kaitna *et al.*, 2000; Adams *et al.*, 2001). Ark1-K147R binding to Pic1p residues 925–1018 was confirmed in direct two-hybrid pairwise tests (Figure 5A), and an analysis of additional Pic1p fragments revealed that residues 925–972 are necessary and sufficient for Ark1p binding (Figure 5A). No *pic1*⁺ clones were identified in screens that used the pGBT9-NT bait, and consistent with this observation, the N-terminal bait failed to bind the IN box in direct pairwise tests (Figure 5B). A reciprocal experiment in which full-length Pic1p was used as bait demonstrated that Pic1p binds to the Ark1p kinase domain (residues 117–384) but not to the N-terminal extension alone (Figure 5B). The Pic1p-Ark1p kinase domain interaction appeared to be weaker than that observed between Ark1p or the Ark1p kinase domain and the minimal IN box (Figure 5B and our unpublished results). This may be because the IN box is less accessible in full-length Pic1p. In addition, Ark1p residues 88–116, which are required for the dominant-negative effect of Ark1-K147R, could be required for strong binding to full-length Pic1p.

The Pic1p IN Box Acts as a Dominant-Negative Inhibitor of Cytokinesis

Mice homozygous for an *INCENP* disruption die early in embryogenesis with severe defects in chromosome segrega-

Figure 5. Ark1-K147R interacts with the inner centromere protein Pic1p. (A) Two C-terminal fragments of the *S. pombe* inner centromere protein Pic1p were identified as Ark1-K147R binding partners in a yeast two-hybrid screen. The first fragment comprises residues 765-1018, whereas the second includes only residues 925-1018. Both fragments contain the IN box (indicated by a black box), which is highly conserved among INCENPs (Adams *et al.*, 2000). The Ark1-K147R bait (DBD) and various Pic1p fragment prey (AD) constructs were used to transform yeast two-hybrid strain AH109. Cotransformants were streaked to selective medium to test for interactions. Pic1p residues 925-972 were necessary and sufficient for Ark1p binding. (B) Various Ark1p and Pic1p bait (DBD) and prey (AD) constructs were used to transform strain AH109. Cotransformants were streaked to selective medium to test for interactions. Although Ark1-K147R bound to Pic1p 925-1018, the N-terminal domain alone (residues 1-116) did not. In reciprocal experiments, the wild-type kinase domain of Ark1p (residues 117-384) was sufficient to bind full-length Pic1p.

tion, microtubule bundling, and cytokinesis (Cutts *et al.*, 1999). Dominant-negative versions of INCENP have also been described, including the C-terminally truncated mutant INCENP₁₋₄₀₅, which causes defects in chromosome segregation and cytokinesis (Mackay *et al.*, 1998). Another mutant, made by fusing INCENP to the centromere-targeting region of CENP-B, can no longer localize to the midbody late in mitosis and causes defects in cytokinesis (Eckley *et al.*, 1997). The defects caused by both mutants may be a result of improperly targeting aurora-B. We reasoned that overproducing the Pic1p IN box would also have negative consequences, because it is sufficient to bind Ark1p but is unlikely to localize properly. Indeed, fission yeast overexpressing Pic1p fragment 925-1018 exhibited phenotypes very similar to those seen with Ark1-K147R. These cells grew poorly on solid medium (Figure 6A) and were elongated and often branched. Likewise, cells grown in inducing liquid medium were elongated and branched and exhibited multiple septa (Figure 6B). These cells also occasionally exhibited the *cut* phenotype (our unpublished results), which is the primary phenotype of cells deleted for *pic1*⁺ (our unpublished results).

To determine whether Ark1p binding was required for the dominant-negative effect of this fragment, we next overexpressed larger C-terminal fragments of Pic1p with or without the IN box (Figure 6C). As expected, Pic1-765-1018 bound to Ark1p in two-hybrid pairwise tests and exerted a dominant-negative effect on cell growth (Figure 6C). Pic1-765-924, which lacks the IN box, failed to bind Ark1p, and fission yeast overproducing this mutant grew normally (Fig-

ure 6C). To test whether the dominant-negative effect of the IN box depended on its ability to sequester endogenous Ark1p, we next tested whether concomitant overexpression of wild-type Ark1p would alleviate the effect. Indeed, wild-type cells overexpressing both the IN box and Ark1p grew normally on solid medium (Figure 6C) and appeared normal (our unpublished results). Our interaction data and overexpression studies thus strongly indicate that the effects of Ark1-K147R are mediated through stable Pic1p binding. Such an interaction would be likely to compete away wild-type Ark1p, denying it access to crucial substrates.

To further examine this idea, the various KD Ark1p truncation mutants (Figure 4) were used as bait in the two-hybrid system to assess their ability to bind the Pic1p IN box. Although dominant-negative mutants 24-384-KD and 88-384-KD bound to the IN box as well as full-length Ark1p (Figure 6D), the other mutants bound poorly (72-384-KD) or not at all (48-384-KD). The ability of these mutants to confer a dominant-negative effect in wild-type *S. pombe* thus corresponds directly to their ability to bind to Pic1p, indicating that Pic1p-Ark1p complex formation is essential for executing cytokinesis in fission yeast.

DISCUSSION

This work describes the initial characterization of the *S. pombe* aurora-related kinase, Ark1p. We have shown that *ark1*⁺ is an essential gene required for the proper segregation of sister chromatids in mitosis. Cells lacking Ark1p

Figure 6. Ark1p-Pic1p complexes are required for the completion of cytokinesis. (A) Ark1p and Pic1p mutants were overexpressed in wild-type cells. Vect, empty *mtl1* vector; Ark1, wild-type Ark1p; KEN, Ark1p bearing four mutations in the putative KEN box (see text); KD, KD Ark1-K147R; NT, N-terminal fragment of Ark1p (1–116); KIN, kinase domain of Ark1p (117–384); KIN-KD, KD kinase domain of Ark1p; Pic1 IN, Pic1p IN box (925–1018). Transformants isolated on media containing thiamine (B_1) were streaked to medium with (+B1) or without (–B1) thiamine and grown at 32°C for 3 d. Wild-type cells overexpressing the Pic1p Ark1p-binding IN box (925–1018) grew poorly and formed only microcolonies. (B) The Pic1p IN box was overexpressed in wild-type cells grown in liquid medium lacking thiamine. DAPI/calcofluor staining revealed that DNA segregated properly but that many cells failed to complete cytokinesis and were multiply septated. (C) Pic1p fragments 765–1018, 925–1018, and 765–924 were tested in parallel for the ability to bind Ark1-K147R in direct two-hybrid tests (left plate) and to act as dominant-negative mutants when overexpressed in wild-type *S. pombe* (center plate). Only fragments 765–1018 and 925–1018, which possess the IN box, bound to Ark1p and acted as dominant-negatives. Wild-type cells overexpressing the dominant-negative Pic1p IN box (925–1018) were rescued by coexpressing Ark1p, but not by empty vector (right plate). (D) KD Ark1p truncation mutants (left) were used as bait in direct two-hybrid tests

for binding to the Pic1p IN box (residues 925–1018). Yeast transformed with the indicated constructs were assayed for growth on selective media (right) as an indicator of protein–protein interactions. Ark1p mutants that failed to bind Pic1p were incapable of exerting dominant-negative effects.

contain fragmented nuclei and often exhibit the *cut* phenotype, indicating that these cells are defective in some aspect of chromosome condensation and/or migration. We also found that overexpressing a KD version of Ark1p inhibits cell division, indicating that Ark1p plays an additional role in regulating cytokinesis. Ark1p thus functions at multiple points in the cell cycle and may serve to coordinate chromosome segregation with cytokinesis.

Ark1p interacts, functionally and/or physically, with Bir1p and Pic1p. Purified Ark1p phosphorylates N-terminal Bir1p fragments in vitro, and overexpressed Ark1p alleviates the slow-growth phenotype of the *bir1–46* temperature-sensitive strain. Pic1p was identified as an Ark1-K147R interacting protein, and overexpression of its C-terminal Ark1p-binding domain leads to cytokinesis defects identical to those produced by Ark1-K147R. Ark1-K147R truncation mutants that fail to bind Pic1p also fail to act as dominant-negatives, indicating that Ark1p-Pic1p complexes are required for the completion of cytokinesis in fission yeast.

Ark1/Aurora and Bir1/Survivin

We found that *ark1*⁺ overexpression is capable of rescuing the *bir1–46* slow-growth phenotype at 28°C, indicating that these genes are functionally related. Ark1p was also capable of phosphorylating Bir1p in vitro, which suggests that these proteins are capable of interacting at least transiently. Ark1p is unlikely to be the only crucial factor downstream of Bir1p, however, because high levels of Ark1p fail to rescue the temperature-sensitive defects of *bir1–46* at 34°C. Ark1p phosphorylates the N-terminal region of Bir1p, which contains two BIR domains. The single BIR motif of human survivin mediates homo-dimerization (Chantalat *et al.*, 2000; Muchmore *et al.*, 2000; Verdecia *et al.*, 2000), and so Ark1p could serve to regulate Bir1p dimerization. Ark1p might also be required to trigger the movements of Bir1p during mitosis. The transfer of survivin from chromosomes to the centromere, midzone, and cell cortex seems to depend on aurora activity, because these migrations are inhibited by INCENP_{1–405}, which lacks the aurora-binding IN box (Wheatley *et al.*, 2001). Future studies will be aimed at deter-

mining whether Ark1p phosphorylates Bir1p in vivo and what the functional significance of such modifications might be.

Human aurora-B was recently shown to bind, but not phosphorylate, survivin (Wheatley *et al.*, 2001). Conversely, we found that Ark1p is able to phosphorylate Bir1p in vitro but failed to detect Ark1p-Bir1p interactions in our two-hybrid screens or in direct pairwise tests (our unpublished results). It is possible that another, as yet unidentified, protein might be required to bridge, physically and/or functionally, between Ark1p and Bir1p in fission yeast. Survivin orthologs are quite divergent, and although human survivin can partially substitute for *C. elegans* BIR-1 (Speliotes *et al.*, 2000), it is unable to rescue budding yeast deleted for *BIR1* (Li *et al.*, 2000) or fission yeast deleted for *bir1*⁺ (Huang *et al.*, in preparation), indicating that these proteins function in slightly different manners. Like budding yeast Bir1p, fission yeast Bir1p is much larger than survivin and, outside of its N-terminal BIR domains, shows only limited homology to it. The C-terminal extension in budding yeast Bir1p has been shown to mediate association with the spindle apparatus in anaphase (Uren *et al.*, 1999) and also with the kinetochore protein Ndc10p (Yoon and Carbon, 1999). Ndc10p homologues have not been identified in mammals, so it clearly is possible that distinct Bir1 intermolecular interactions have evolved in various organisms. It will be important to determine whether the yeast Bir1 proteins also dimerize and exactly which proteins bind to their various domains in vivo.

Ark1p/Aurora and Pic1p/INCENP

We identified Pic1p in an unbiased yeast two-hybrid screen for Ark1-K147R interacting proteins. C-terminal fragments of Pic1p that include the highly conserved IN box (Adams *et al.*, 2000) were sufficient for Ark1p binding. The corresponding region in murine INCENP is sufficient to bind human aurora kinases from HeLa cell extracts (Kaitna *et al.*, 2000), and a C-terminal piece of *C. elegans* ICP-1 could also bind to AIR-2, albeit weakly. We have further defined the Ark1p-Pic1p interacting domains, demonstrating that Pic1p residues 925–972 are sufficient for Ark1p binding and most likely make contact with the kinase domain (Figures 5 and 6). Overexpressing Ark1p-binding fragment 925–1018 led to cytokinesis defects that were indistinguishable from those seen with Ark1-K147R, whereas overexpressing a fragment that fails to bind Ark1p had no effect. Furthermore, the ability of truncated Ark1-K147R mutants to inhibit cytokinesis required Pic1p binding. These data strongly indicate that wild-type Ark1p-Pic1p complexes are required for the completion of cell division.

What Is the Role of the Ark1 N-Terminus?

Our data indicate that INCENPs probably bind to the highly conserved kinase domains of aurora family members. Outside of the kinase domain, aurora family members exhibit only limited homology. Their N-terminal extensions range from 7 to 162 amino acids (for review, see Giet and Prigent, 1999), and several possess a sequence that resembles the recently described KEN box (Pfleger and Kirschner, 2000). The KEN box was originally identified in the APC activator and substrate Cdc20 (Pfleger and Kirschner, 2000) and acts as a degron that is bound by APC-Hct1/Cdh1 or APC-Cdc20 complexes (Burton and Solomon, 2001; Hilioti *et al.*, 2001; Pfleger *et al.*, 2001; Schwab *et al.*, 2001). Human auro-

ra-A associates with Cdc20 in HeLa cells (Farruggio *et al.*, 1999) and appears to be degraded in an APC-dependent manner (Honda *et al.*, 2000; Walter *et al.*, 2000). Although aurora-A is not stabilized by mutations in putative destruction boxes, the role of the KEN box has not been investigated.

Our studies indicate that Ark1p levels do not need to be tightly regulated. Overexpression of Ark1p does not significantly affect cell growth (Figure 6A), and we failed to detect cell cycle-dependent changes in Ark1p levels (Figure 4C). Although we cannot rule out the possibility that specific subpopulations of Ark1p are targeted for destruction, the KEN sequence does not appear to function as a degron. This may be simply because the Ark1p sequence does not conform closely enough to the KEN box consensus (Burton and Solomon, 2001).

In the course of performing a structure-function analysis of the Ark1-K147R mutant, we identified mutant 88–384-KD as the minimal unit capable of exerting dominant-negative activity. Residues 88–116 must also be appended to the functional kinase domain to rescue *ark1*⁺-disrupted haploids (our unpublished results). Although these residues are not absolutely required for Pic1p binding, our results indicate that they do play an essential role and perhaps mediate interactions with other Ark1p partners and/or substrates. Surprisingly, KD mutants with additional N-terminal sequence (residues 48–87 or 72–87) did not behave as dominant-negatives. This raised the possibility that residues 72–87 function as an autoinhibitory domain, and indeed, mutants 48–384-KD and 72–384-KD failed to bind Pic1p in direct two-hybrid tests, whereas mutant 88–384-KD bound quite strongly. Mutant 24–384-KD also bound Pic1p, indicating that residues 24–47 may function to relieve the inhibitory effect of residues 72–87. Thus, whereas 24–384-KD may represent a fully regulatable Ark1 protein, 48–384-KD and 72–384-KD may be constitutively inhibited, i.e., unable to bind Pic1p.

A recent study of the *Xenopus laevis* aurora-A kinase revealed that its N-terminal extension plays a role in localizing it to centrosomes (Giet and Prigent, 2001). This domain behaves as a dominant-negative mutant in *Xenopus* egg extracts, acting to inhibit bipolar spindle formation and to destabilize assembled spindles. In our studies, overproducing the Ark1p N-terminal domain had no discernible effect on cell growth (Figures 3 and 6), indicating that it probably does not interfere with wild-type Ark1p function.

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