p21-activated kinases (PAKs) bind to and are activated by Rho family GTPases such as Cdc42 and Rac. Since these GTPases play key roles in regulating cell polarity, stress responses, and cell cycle progression, the ability of PAK to affect these processes has been examined. We previously showed that fission yeast pak1+ encodes an essential protein that affects mating and cell polarity. Here, we characterize a second pak gene (pak2+) from Schizosaccharomyces pombe. Like the Saccharomyces cerevisiae proteins Cla4p and Skm1p, fission yeast Pak2p contains a N-terminal pleckstrin homology domain in addition to a p21-binding domain and a protein kinase domain that are common to other members of the PAK family. Unlike pak1+, pak2+ is not essential for vegetative growth or for mating in S. pombe. Overexpression of the wild-type pak2+ allele suppresses the lethal growth defect associated with deletion of pak1+, and this suppression requires both the pleckstrin homology- and the p21-binding domains of Pak2p, as well as kinase activity. A substantial fraction of Pak2p is associated with membranous components, an association mediated both by the pleckstrin homology- and by the p21-binding domains. These results show that S. pombe encodes at least two pak genes with distinct functions and suggest that the membrane localization of Pak2p, directed by its interactions with membrane lipids and Cdc42p, is critical to its biological activity.

Signal transduction by small GTPases of the Rho family has become the focus of intensive investigation in recent years. Rho GTPases (in mammals, Cdc42, Rac, and Rho) regulate a variety of signaling pathways in higher eukaryotes, including those that affect the organization of cortical actin, stress responses, gene transcription, and cell cycle progression (1). In budding and fission yeasts, Cdc42p has been implicated in the generation and maintenance of cell polarity. However, the mechanisms by which Cdc42p and other Rho family GTPases regulate these processes are poorly understood.

Several candidate effectors for Rho GTPases have been identified recently, including a variety of protein kinases, GTPase activating proteins, and other proteins that lack obvious enzymatic function. Among these candidate effectors, the p21-activated serine/threonine kinases (PAKs) are currently the best characterized (2). PAKs bind to activated (GTP-bound) forms of Cdc42 and Rac, but not Rho, and are activated as a result of this binding. Like Cdc42 and Rac, PAKs stimulate stress-activated protein kinases (3–6). Moreover, dominant interfering forms of PAK block stress-activated protein kinase activation by Cdc42 and Rac1, suggesting that PAKs may be effectors for these GTPases in stress signaling pathways. The role of PAKs in mediating other Cdc42/Rac functions, such as cytoskeletal rearrangements and G2 progression, is less clear. In mammalian fibroblasts, expression of activated Pak1 induces changes in focal complex formation and F actin distribution similar but not identical to those induced by Rac1 and Cdc42 (7, 8). Therefore, PAKs may activate multiple pathways, including those that affect gene transcription and those that affect actin dynamics.

To better define the targets and functions of PAK, we and others have examined PAKs in yeast. Three PAK homologs have been characterized in budding yeast. The PAK homolog Ste20p is required for pheromone-induced G1 arrest and activation of the transcription factor Ste12p as well as for certain specialized morphogenic processes, such as formation of mating projections and filamentous growth (9–11). A second PAK-like kinase, Cla4p, has been shown to regulate formation of the bud neck and may also have other functions that are partially redundant with Ste20p (12). A third kinase of this class, Skm1p, is dispensable for normal growth and mating but yields a multibudded phenotype when overexpressed, suggesting a role in cell morphogenesis (13). Unlike Ste20p or mammalian PAKs, both Cla4p and Skm1p contain a pleckstrin homology (PH) domain near the N terminus. The functional significance of this extra domain is not known, although it seems likely that it serves to target these kinases to appropriate locations within membrane structures.

We recently characterized a PAK-like protein in S. pombe. This kinase, known as Pak1p or Shk1p, is essential for viability and mating and may play important roles in the generation and/or maintenance of cell polarity (14, 15). Expression of pak1+ in S. cerevisiae cells deleted for STE20 relieves the mating defect associated with this mutation, indicating that fission yeast pak1+ is to some degree functionally homologous to budding yeast STE20. During the isolation of pak1+ clones, we uncovered a second PAK homolog (pak2+). The protein encoded by this gene, like budding yeast Cla4p and Skm1p,
contains an N-terminal PH domain. In this study, we report the biochemical and functional characterization of Pak2p.

MATERIALS AND METHODS

Strains and Media—S. pombe cells were grown in complex medium, YEA (16), or in yeast extract plus adenine Edinburgh minimal medium (EMM) supplemented as required (17). The haploid strains CH428 (MATa ade2-1 ura4-53 his3-6 ade6-M121), and CH429 (his1-112 leu1-32 his7-366 ade6-M216) were used to generate diploid cells used for gene disruption. The wild-type strain 972 (h+) was used. S. cerevisiae strains used in this work were the mating tester strain DC17 (MATa his1) (9), W303-1A (MATa ade2 leu2 trp1 his3 can1) and the W303-1A derivatives YEL206 (MATa ste20-3::TRP1) (18), M96007–1D (MATa ade2-1 ura4-53 his3-6 ade6-M216), STE20A-2::URA3/STE20 clau4::TRP1/CLA4), and YEL252-1A (MATA clau4::TRP1). The STE20 and CLA4 genes in these strains were deleted as previously described (9, 10). The ste20–4 mutant allele was isolated from a randomly mutagenized STE20 plasmid library by screening for mutants that were capable of mating at room temperature but sterile at 37 °C and then integrated into the genome by homologous recombination.

Isolation of cDNA Clones—The primers 5′-CCG-GAT-CAT-TNG-CNA-T/A-C/T-A-A/G/C-A/G/A-T/G-A-A3′ and 5′-CCG-AAT-TNC-GGN-GG/G/(CT)-TCN-CC/C(T)/CA/A/G-T/ATC-AT/3′ were used to amplify an ~500-base pak-like gene fragment from an S. pombe cDNA library (14). This PCR product was used to probe the library as described previously (14). Most positive clones contained partial or full-length pak1*, but one contained a related sequence that we termed pak2*. The sequence of this clone appeared to represent a partial cDNA, since the predicted protein product lacked a recognizable N-terminal p21-binding domain, which is present in Pak1p and other PAKs. While we attempted to isolate a full-length cDNA, the complete pak2* gene was uncovered during the course of a comprehensive international S. pombe genome sequencing effort. We obtained this clone (ICRFc1F5) from the Imperial Cancer Research Fund Reference Library (21). The genomic pak2* clone was identical to our cDNA clone, but it encoded in addition a potential N-terminal PH domain and a p21-binding domain.

Plasmids—The pak2* coding sequence (~1.8 kilobases kb) was amplified by PCR using Deep Vent polymerase (New England Biolabs) from a cDNA (ICRFc1F5) containing the pak2* clone. The PCR product was subcloned as a SmaI/Acc651 fragment into the hemagglutinin (HA) epitope-tagging vector pJ3H (22). This vector was constructed and transformed with the S. pombe cDNA library (14), we isolated a second, pak-like gene fragment. We

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## RESULTS

Isolation of a Second PAK Homolog from Fission Yeast—During the course of cloning pak1* from a S. pombe cDNA library (14), we isolated a second, pak-like gene fragment. We
were unable to obtain a full-length clone of this gene from this library, but the entire gene was subsequently sequenced as part of a comprehensive fission yeast genome sequencing effort. The sequence of the complete gene clearly indicates that it is a member of the PAK family; thus we have termed it pak2.1 (Fig. 1A).

The pak2.1 gene potentially encodes a protein of 589 amino acids. The domain structure of the predicted Pak2p protein closely resembles that of two S. cerevisiae proteins, Cla4p and Skm1p (2). Like these molecules, the predicted Pak2p protein contains a PH domain at the N terminus, followed by a p21-binding domain, and a serine/threonine protein kinase domain that occupies the C-terminal half of the protein (Fig. 1B).

Unlike many other PAK homologs, S. pombe Pak2p does not contain an acid-rich region C-terminal to the p21-binding domain or notable proline-rich regions. Outside of the conserved PH, p21-binding, and kinase domains, Pak2p is not similar to any other members of the PAK family.

**Pak2p Kinase Activity Is Stimulated by Cdc42p**—To confirm that Pak2p is, in fact, a p21-activated kinase, we immunoprecipitated HA-tagged Pak2p from S. pombe that overexpress this protein. Pak2p immunoprecipitates were then assayed for protein kinase activity in the presence of GTP-loaded, wild-type Cdc42p; a constitutively active (V12) form; a dominant-negative (N17) form; or a control protein. Both wild-type and constitutively active Cdc42p markedly stimulated Pak2p ki-
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**FIG. 2. Activation of Pak2 by Cdc42p.** HA-tagged Pak2p was immunoprecipitated using polyclonal anti-HA (Santa Cruz Biotechnology) from *S. pombe* cells transformed with the thiamine-regulated expression vector pREP5X-HA-pak2 (top). A portion of the immunoprecipitates were immunoblotted with monoclonal anti-HA (12CA5, Babco), while the remainder (bottom) were assayed for protein kinase activity toward myelin basic protein in the presence of the indicated forms of recombinant, GTP-loaded Cdc42p.

nase activity, as reflected in increased phosphorylation of myelin basic protein in an in vitro kinase assay and in the generation of “upshifted” Pak2p bands on anti-HA immunoblot (Fig. 2). In contrast, dominant negative Cdc42p had no effect on basal kinase activity. These data confirm that Pak2p, like Pak1p and other members of the PAK family of protein kinases, can be activated by Cdc42p.

**Pak2p Interacts with Activated Cdc42p—**To establish that the p21-binding domain in Pak2p is functional, we carried out interaction-trap assays (27). Co-expression of LexA-Pak1p or LexA-Pak2p with activation domain-Cdc42p resulted in activation of the lacZ reporter (Table I) as well as LEU2 (not shown). These reporters were not activated when the yeast were grown on dextrose-containing media, which prevents induction of Cdc42p expression from the activation domain vector (not shown), or when a nonspecific bait (RB7) was used and the yeast were grown on galactose-containing media (Table I). The interaction among Pak1p, Pak2p, and Cdc42p was greatly increased when a constitutively activated (V12) form of Cdc42p was used and abolished when an inactive (N17) (not shown) or an effector domain (A35) mutant was used. These results indicate that, like Pak1p, Pak2p can interact with Cdc42p within cells. Furthermore, the Pak2p/Cdc42p interaction is enhanced by mutations that activate Cdc42p and requires an intact effector domain. As with mammalian Pak1 (7), replacing two conserved histidine residues within the p21-binding domain of Pak2p ablates binding to Cdc42p. Neither a mutation that abolishes kinase activity nor deletion of the PH domain, however, measurably affects binding of Pak2p to Cdc42p in the interaction trap assay. In all cases, expression of both baits and interactors was confirmed by immunoblot (Fig. 3).

**pak2** Is Not An Essential Gene—**We and others have previously established that pak1 is essential for viability and is likely to play a role in mating as well (14, 15). To address the functions of the related pak2 gene, we used the *ura4* marker to disrupt the coding sequence of one copy of this gene in diploid cells. After confirming the presence of the disrupted allele by Southern blot (not shown), the appropriate diploids were sporulated and tetrad dissections were carried out. Of 14 tetrads dissected, 12 gave rise to four viable colonies, while the remaining two yielded three colonies. These tetrads that yielded four colonies displayed 2:2 segregation for uracil auxotrophy. These results indicate that pak2 is not required for viability. Haploids containing the disrupted pak2 allele were isolated and tested for growth rate at 20, 30, and 36 °C; viability under stress (e.g., heat shock, osmotic stress, nutrient-deprivation); morphology; and mating competence (not shown). None of these tests revealed a phenotype for pak2 disruption.

**pak2** Overexpression Can Partially Replace pak1 Function—**To assess whether pak2 and pak1 have overlapping functions, we first asked if high level expression of pak2 could allow cells to remain viable in the absence of pak1. pak1/pak1:his7 diploids were transformed with a thiamine-regulated expression plasmid (pREP3X) bearing either no insert or various forms of fission yeast pak1 or pak2, or budding yeast CLA4. The cells were made to sporulate by plating on maltose, and the resulting tetrads were then dissected and plated to complete medium. Diploid cells transformed with the control vector gave rise to two viable and two dead spores (12 tetrads examined), as expected for disruption of an essential gene such as pak1. Expression of pak1 from the pREP3X vector rescued pak1:his7 haploids; three or four spores germinated from most of the tetrads derived from pak1/pak1:his7 (pREP3X-pak1) diploids, one or two of which bore the pak1:his7 allele. That some spores failed to germinate can probably be attributed to the low copy number of the episomal expression vector pREP3X, which may not be present in sufficient numbers to distribute to all four spores. ΔN-pak1, which lacks the p21-binding domain and which we have previously shown to encode an unstable form of Pak1p (14), also supported the growth of pak1:his7+ haploids but only when expressed at high levels (i.e. on medium lacking thiamine) (Fig. 4B).

We found that overexpression of pak2 could also suppress the

| pak2p → ΔPak2p | Cdc42p | Cdc42pV12 | Cdc42pV12,A35 | Interaction |
|----------------|--------|-----------|---------------|-------------|
| Pak1           | 16.4 ± 2.4 | 55.0 ± 7.3 | 1.4 ± 0.4 | 1.3 ± 0.5 |
| Pak2           | 13.0 ± 2.9 | 48.7 ± 7.2 | 1.6 ± 0.4 | 1.3 ± 0.3 |
| ΔPH-Pak2       | 13.2 ± 2.0 | 46.2 ± 6.6 | 1.3 ± 0.3 | 1.8 ± 0.5 |
| Pak2 LL        | 1.8 ± 0.5 | 1.6 ± 0.9 | 1.2 ± 0.5 | 1.8 ± 0.6 |
| Pak2 KA        | 11.6 ± 2.7 | 44.7 ± 7.6 | 1.3 ± 0.3 | 1.1 ± 0.2 |
| RB7            | 1.1 ± 0.3 | 1.3 ± 0.2 | 1.6 ± 0.5 | 1.2 ± 0.5 |

ΔPH-Pak2, Pak2 lacking amino acids 1–125; Pak2 LL, Pak2 H136L,H140L; Pak2 KA, Pak2 K343A.

**FIG. 3. Expression of fusion proteins in interaction trap assay.** LexA fusion protein baits in pEG202 were co-transformed with the indicated activation domain fusion vectors plus the lacZ reporter pSH18–34 into *S. cerevisiae* EGY48. Protein expression was induced by growing the transformants on galactose-containing media. Expression was monitored by immunoblot using either anti-LexA or anti-HA antibodies.

**LexA fusion** | Cdc42p | Cdc42pV12 | Cdc42pV12,A35 | Interaction |
|----------------|--------|-----------|---------------|-------------|
| Pak1           | 16.4 ± 2.4 | 55.0 ± 7.3 | 1.4 ± 0.4 | 1.3 ± 0.5 |
| Pak2           | 13.0 ± 2.9 | 48.7 ± 7.2 | 1.6 ± 0.4 | 1.3 ± 0.3 |
| ΔPH-Pak2       | 13.2 ± 2.0 | 46.2 ± 6.6 | 1.3 ± 0.3 | 1.8 ± 0.5 |
| Pak2 LL        | 1.8 ± 0.5 | 1.6 ± 0.9 | 1.2 ± 0.5 | 1.8 ± 0.6 |
| Pak2 KA        | 11.6 ± 2.7 | 44.7 ± 7.6 | 1.3 ± 0.3 | 1.1 ± 0.2 |
| RB7            | 1.1 ± 0.3 | 1.3 ± 0.2 | 1.6 ± 0.5 | 1.2 ± 0.5 |

ΔPH-Pak2, Pak2 lacking amino acids 1–125; Pak2 LL, Pak2 H136L,H140L; Pak2 KA, Pak2 K343A.
growth defect associated with loss of pak1+ function (Fig. 4B). Suppression was not evident when thiamine was included in the growth medium (which reduces expression from the pREP3X vector), indicating that viability in pak1Δ cells requires high levels of pak2+ expression (data not shown). This conclusion is also supported by the finding that cells transformed with pREP81-pak2−, which has a mutated thiamine-responsive promoter that yields expression levels that are only about 1% of pREP3X-pak1+ (23), did not suppress pak1 disruptants (data not shown). Interestingly, suppression of pak1Δ lethality was not apparent in cells expressing pak2 mutants lacking either functional PH or p21-binding domains or in cells expressing kinase-dead pak2 (Fig. 4B), despite adequate expression of these mutant proteins (Fig. 4C). These results indicate that the PH and p21-binding domains, as well as intact kinase function, are required for pak2+ to suppress the growth defect associated with loss of pak1+. Like pak2−, CLA4 from S. cerevisiae can also suppress the pak1+ deletion. We have not tested if the PH and p21-binding domains of Cla4p are required for this suppression.

We further assessed the ability of pak2+ to replace pak1+ function using mating and sporulation as end points. Both cdc2− and pak1− have been implicated in the mating process in fission yeast; cells overexpressing defective forms of these genes fail to mate normally (14, 15). When grown on thiamine-deficient media, pak1bΔ cells supported by Pak1p, ΔN-Pak1p, Pak2p, or Cla4p mate normally with wild-type partners, and sporulation of diploids is not notably affected (Table II). Support by plasmids encoding ΔN-Pak1p, Pak2p, and Cla4p required high levels of expression, since mating function was poor in cells grown in the presence of thiamine. In contrast, in crosses involving two mutant cells (i.e. crosses in which both partners bear the pak1::his7 allele, supported by a pREP3X-based plasmid), only Pak1p and ΔN-Pak1p, but not Pak2p or Cla4p, efficiently supported mating. Therefore, high levels of Pak2p can fully compensate for Pak1p’s vegetative functions but only partially compensate for its sexual functions. These results suggest that Pak1p and -2p can share certain substrates that are required for viability, but not for mating.

**Subcellular Localization**—Because we found that the PH and p21-binding domains of Pak2p are required for its ability to suppress the growth defect of pak1Δ cells, we assessed the subcellular distribution of wild-type Pak2p and of Pak2p mutants lacking a functional PH or p21-binding domain. Exponentially growing cells expressing these proteins were fractionated into low speed (500 g) and high speed (10,000 × g) pellets and supernatants, which were analyzed by immunoblot for the presence of Pak2p (Fig. 5). The low speed pellet (P1) largely consists of unbroken cells, nuclei, and large organelles, while the high speed supernatant (S1) contains both cytosol and membranous elements (28). The high speed pellet (P2) is comprised largely of plasma membrane, and the high speed supernatant contains cytosol and secretory granules. Wild-type Pak2p is found almost equally in all four fractions, indicating that both membrane-bound and cytosolic pools of this protein are present in the cell. Surprisingly, both the ΔPH and the p21-binding domain Pak2p mutants had similar subcellular distributions, indistinguishable from that of the wild-type enzyme. However, a mutant lacking function of both domains (ΔPH-Pak2p H136L,H140L), was primarily cytosolic (>80%, as assessed by densitometry of immunoblots). These data indicate that the PH and p21-binding domains both contribute to the association of
TABLE II

Suppression of the mating defect in pak1Δ cells

| Mated cells |  | Mating |  |
|-------------|--|--|---|
| WT×WT       |  | 50.8±6.4 | 51.1±5.4 |
| pak1::his7/pak1::his7 × WT |  | 33.8±4.6 | 55.3±6.4 |
| pak1::his7/pak2::his7 × WT |  | 3.3±2.7 | 55.8±6.6 |
| pak1::his7/pak1::his7 × pak2::his7 |  | 3.0±1.3 | 52.1±6.7 |
| pak1::his7/pak1::his7 × CLA4A |  | 3.5±2.4 | 57.7±5.6 |

WT, wild-type S. pombe, strain CHF428 or CHP429.

*WITH thiamine* | *Without thiamine* |
|-----------------|------------------|
| 50.8±6.4 | 51.1±5.4 |
| 33.8±4.6 | 55.3±6.4 |
| 3.3±2.7 | 55.8±6.6 |
| 3.0±1.3 | 52.1±6.7 |
| 3.5±2.4 | 57.7±5.6 |

ChIP429 strain.

**FIG. 5. Immunoblot analysis of fractionated S. pombe cell lysates.** 10 ml of CHF428 cells transformed with the indicated pREP3X-Pak2 expression vectors were grown at 30 °C, in the presence of thiamine (not shown) or absence of thiamine, in leucine-deficient media to mid-log phase. The cells were then lysed with glass beads, and protein extracts were spun at 500 × g to produce pellet (P) and supernatant (S) fractions; the supernatant fractions were subsequently spun at 10,000 × g. Equal volumes of protein extract were fractionated by 10% SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and immunoblotted with anti-HA antibodies. This result is indicative of two independent experiments.

Pak2p with membranes.

**Trans-species Complementation—** As Pak2p is structurally homologous to Cla4p, we asked whether it is also functionally homologous. Like pak2Δ, overexpression of CLA4Δ was able to suppress the loss of viability associated with pak1Δ deletion in S. pombe (Fig. 4), as well as restore mating function (Table II). To more fully explore this issue, we did the reciprocal experiments in clαΔ or ste20Δ S. cerevisiae cells. In ste20Δ cells, expression of transgenic STE20 or pak1Δ restores mating, as shown previously (Fig. 6 and Ref. 14). Interestingly, we found that multicopy expression of CLA4Δ also restores mating competence, suggesting that high levels of CLA4Δ can overlap functions in a morphogenetic pathway (12). Although CLA4Δ and STE20 have previously been shown to share overlapping functions in a morphogenetic pathway (12), to our knowledge CLA4Δ has not previously been shown to substitute for STE20 in the mating pathway. Unlike CLA4Δ, expression of S. pombe pak2Δ did not restore mating to ste20Δ cells. Protein expression for all transgenes was verified by immunoblot (not shown); thus, Pak2p failed to complement Ste20p and Cla4p despite adequate expression.

Deletion of CLA4Δ results in misshapen cells (Fig. 7A). Expression of transgenic CLA4Δ from an episomal plasmid restores normal morphology (Fig. 7B), but expression of either pak1Δ or pak2Δ does not. In fact, prolonged expression of pak1Δ exacerbates the morphologic defect (Fig. 7C), yielding large, rounded cells that grow poorly (not shown).

In budding yeast, simultaneous deletion of STE20 and CLA4Δ results in a vegetative growth defect (12). To analyze whether pak1Δ or pak2Δ is able to complement this defect, the diploid strain YEL252, heterozygous for ste20Δ::URA3/S. pombe clα4Δ::TRP1/CLA4Δ, was transformed with the multicopy plasmid pYES2 carrying either pak1Δ or pak2Δ, respectively, or, as positive controls, with the multicopy plasmids pVTU-STE20 and pVL21 (10) carrying either STE20 or CLA4Δ, respectively. The diploid transformants were then sporulated and dissected for tetrad analysis. We dissected more than 30 tetrads for each transformant. We found no viable double mutant spores in tetrads transformed with either pak1Δ or pak2Δ but found 16 double mutant spores transformed with the STE20 plasmid and 13 double mutant spores transformed with the CLA4Δ plasmid. Putative pak1Δ or pak2Δ transformants predicted to be double ste20Δ clα4Δ mutants germinated and produced aberrantly shaped buds but finally lysed after prolonged incubation.

To further examine whether pak1Δ or pak2Δ is able to complement the growth defect of ste20Δ clα4Δ double mutant cells, we transformed strain M96607–1D, which is deleted for CLA4Δ and carries a temperature-sensitive mutation in STE20. Cells were transformed at the permissive temperature (room temperature) and then analyzed for growth at the restrictive temperature (37 °C). We found that mutant cells transformed with the STE20 or CLA4Δ plasmids were able to grow at 37 °C, whereas transformants with pak1Δ or pak2Δ plasmids stopped growing and underwent lysis at this temperature (not shown).

Taken together, these data indicate that while pak1Δ com-
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pombe (strain YEL252–1A) deleted for CLA4 and transformed with the galactose-inducible expression plasmids pYES2 (a), pDH129 (a CLA4 expression vector) (9) (b), pYES2-pak1+ (c), or pYES2-pak2+ (d). Transformants were grown in selective raffinose (2%) medium to mid-exponential phase at 30 °C, diluted 20-fold in the same medium, and, after the addition of 4% galactose, grown for 8 h at 30 °C. Photographs of fixed cells were then taken with a 100× objective by Nomarski optics and are representative for a large number of cells investigated. Scale bar, 20 μm.

DISCUSSION

In this report, we characterize a second Pak-like protein in S. pombe. Like Pak1p, Pak2p binds to activated Cdc42p and affects cell morphology when overexpressed. However, unlike pak1+ and pak2+, is not essential for cell viability. pak2-null cells exhibit normal vegetative growth rates, mating capability, and survival under stress. High level expression of pak2+ suppresses lethality associated with loss of pak1+, indicating a degree of functional overlap between these proteins. The PH and p21-binding domains, as well as kinase function, are required for the growth of cells lacking the pak1+ gene. Trans-species complementation tests in S. cerevisiae show that pak2+ suppresses neither the mating nor the morphologic defects in STE20- and CLA4-null cells, respectively, nor the growth defect in cells deleted for both STE20 and CLA4. Thus, Pak2p, while similar in structure to budding yeast Cla4p, appears to be functionally distinct. Pak2p may instead represent a homolog of the recently described S. cerevisiae protein Skm1p (13). Like pak2+ in S. pombe, disruption of SKM1 in S. cerevisiae evokes no obvious phenotype, and overexpression leads to aberrant morphology. These results indicate that Pak2p may regulate distinct signaling pathways from Cdc42p in fission yeast.

In budding yeast, the p21-binding domain of Ste20p is dispensable for mating but is required for the vegetative functions of this protein and for filamentous growth (10, 29). Mutant Ste20p, lacking the p21-binding domain, is diffusely localized throughout the cell, whereas wild-type Ste20p is concentrated at emerging bud tips during vegetative growth and at shmoo tips in cells arrested with α-factor. The kinase activity of the wild-type and mutant forms of Ste20p is comparable, indicat-
ing that binding to Cdc42p is not absolutely required for activation of this kinase in vitro. These findings suggest that binding to Cdc42 is important for proper localization of Ste20p and that proper localization is required for the biological function of Ste20p during vegetative growth but not for mating.

In contrast to Ste20p in budding yeast, the p21-binding domain of Pak1p is dispensable both for vegetative growth and for mating in S. pombe. Does this indicate that the consequences of the Cdc42p/Pak1p interaction in fission yeast differ from that of Cdc42p/Ste20p in S. cerevisiae? Unlike wild-type Pak1p, ΔN-Pak1p (which lacks the p21-binding domain) is only capable of supporting the growth of such cells when expressed at high level (i.e., on thiamine-deficient medium). This behavior can be interpreted in several ways. For example, at high levels of expression, small amounts of AN-Pak1p may localize to the required site(s) of action, i.e., to areas occupied by Cdc42p, although no targeting sequence is present. This scenario is especially plausible if deletion of the N terminus activates Pak1p, since N-terminal truncations of S. cerevisiae Ste20p activate that kinase (9, 30). Second, Pak1p may contain a second, weak, and hitherto unrecognized, binding site for Cdc42p. This site may contribute to physiologically significant amounts of Cdc42p binding under conditions of overexpression. However, we have been unable to identify such a cryptic binding site either by in vitro or by interaction trap binding assays. Finally, unlike Ste20p in S. cerevisiae, Pak1p localization may not be required for its vegetative functions in S. pombe. In this interpretation, the requirement for high level expression may merely reflect the fact that the ΔN-Pak1p protein is unstable (14).

Overexpression of pak2” partially suppresses the lethal growth defect associated with loss of pak1”. Unlike Pak1p, the N terminus of Pak2p, containing the PH and the p21-binding domains, are required for this suppression. PH domains are often found in proteins that affect cytoskeletal function and are thought to direct proteins to certain membrane compartments through their ability to bind phospholipids (31–33). The removal of this domain from Pak2 may cause this protein to mislocalize from its normal site(s) of action, perhaps diminishing or precluding interaction with activators, such as Cdc42p, or key downstream targets for its kinase activity. It is interesting to note that the PH domain of Cla4p has also recently been shown to be required for its biological function (34). While ΔPH-Pak2p can bind Cdc42p in an interaction trap assay (Table 1), it is possible that the association of these two proteins is diminished in vivo in S. pombe cells. This interpretation implies that, unlike Pak1p, localization of Pak2p may be crucial for at least some of its biological functions. Disabling the p21-binding domain of Pak2p has a similar effect; the Cdc42-binding minus mutant also fails to suppress the growth defect of pak1-null cells. This finding supports the notion that proper subcellular localization is important to the function of Pak2p.

Perhaps Pak2p is inactive in the absence of association with Cdc42p, whereas Pak1p has a higher basal kinase activity. In this scenario, the presence of the PH and p21-binding domains are necessary because together they direct interaction with Cdc42p, which is required for Pak2p activation.

cdc42 is an essential gene in fission yeast, involved in the regulation of cell polarity as well as the mating response. The effectors used by Cdc42p to regulate these processes are unknown. Because Pak1p and Pak2p bind to and are activated by Cdc42p, they represent attractive candidates in mediating one of more of Cdc42p’s functions. Although the terminal phenotype of pak1-null cells resembles those seen in cells bearing cdc42 disruption, overexpression of pak1” does not suppress the lethal growth defect associated with loss of cdc42 (14). The failure of pak1” to suppress loss of cdc42 could mean that Cdc42p has other effectors that are required for viability or that Pak1p must be activated by Cdc42p to function effectively. We believe the first of these scenarios to be more likely than the second, since a pak1 mutant, encoding a constitutively activated form of Pak1p (Pak1pT539E), also fails to suppress loss of cdc42”.

Could Pak2p represent such a second effector? Like pak1”, overexpression of alleles encoding either wild type or an activated form of Pak2p fail to suppress loss of cdc42”. Although we have not tested whether Pak1p and Pak2p together mediate those functions of Cdc42p that are required for viability, this seems unlikely, since both proteins would be expected to compete for common binding sites on Cdc42p and thus would not be activated simultaneously. Therefore, we expect that S. pombe Cdc42p has other effectors that are vital to its vegetative functions. A similar conclusion has been drawn regarding Cdc42p function in S. cerevisiae. In this organism, ste20 cl4 mutant cells still nucleate and polarize actin (12). In addition, schmoo morphogenesis and actin repolarization occur normally in cells expressing a Ste20p mutant unable to bind Cdc42p (10, 29). Mammalian Cdc42 and the related protein Rac are also thought to have multiple effectors, including PAKs and other p21-binding proteins (2), Rho kinase (35, 36), and proteins of unknown function, such as POR1 (37). Consistent with this notion, expression of activated forms of human or rat Pak1 in murine fibroblasts recapitulate some, but not all, of the functions of Cdc42 and Rac (7, 8). In addition, mutant forms of Cdc42 and Rac1 that are unable to bind PAK nevertheless retain the ability to reorganize actin and to drive cell cycle progression (35, 36). Thus, the weight of evidence from diverse organisms is that Cdc42 and Rac, like the related protein Ras, recruit an array of effector molecules that mediate their biological functions.

Among the known functions of fission yeast Cdc42p are activation of the Byr2p/Byr1p/Spk1p mating cascade and maintenance of cell polarity. Does either Pak1p or Pak2p have a role in mediating these signaling pathways? In budding yeast, the Pak1p homolog Ste20p activates a protein kinase cascade, apparently through a direct phosphorylation of Ste11p (18). In mammalian cells, PAKs 1 and 3 have also been shown to stimulate stress-activated kinase cascades, although direct activation of Ste11p homolog (i.e., a Mekk) has not been demonstrated (4–6, 38). The existing evidence for a connection between PAKs and the mating protein kinase cascade in fission yeast is indirect, based on the ability of inactivated forms of these kinases to interfere with mating. Although we have not yet been able to document an interaction between either Pak1p or Pak2p and the Mekk homolog Byr2p, we consider it likely that Pak1p and/or Pak2p mediates activation of the mating kinase cascade by Cdc42p, given the high degree of similarity in the design of the mating molecular machinery in S. cerevisiae and S. pombe. Whether fission yeast PAKs also mediate other functions of Cdc42p is less clear, but, in the case of Pak1p, this kinase clearly has important targets other than Byr2p, since disruption of pak1” results in lethality, not just sterility. While these vital Pak1p targets are unknown, some are likely to be shared in common with Pak2p, since overexpression of the latter kinase suppresses pak1Δ lethality. Since overexpression of Pak1p or Pak2p in S. pombe yields a characteristic morphologic defect, it is possible that these kinases play a role in Cdc42p-mediated actin reorganization.

The relationship between fission yeast Pak1p and -2p is reminiscent of, but not identical to, that of budding yeast Ste20p and Cla4p. Both pairs of kinases share certain functions.

3 J. Chernoff, unpublished observations.
in mating and cell morphogenesis but also play unique roles in the cell. Given the inability of Pak2p to complement Cla4p function, it is possible that Pak2p is more closely related to the recently characterized, PH-domain containing kinase, Skn1p (13). Clearly, the multiplicity and conservation of Pak-like kinases among diverse eukaryotes as well as their effects on regulating such basic processes as mating and actin polarization warrant further investigation to define the precise functions and targets of these proteins.

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Note Added in Proof—The pak2 gene has been cloned independently by Yang et al. (Yang, P., Kansra, S., Pimental, R. A., Gilbreth, M., and Marcus, S. (1998) J. Biol. Chem. 273, 18481–18489), who named the gene shk2. Their results are consistent with and complement the results presented in this paper.

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