The Kelch Repeat Protein, Tea1, Is a Potential Substrate Target of the p21-activated Kinase, Shk1, in the Fission Yeast, Schizosaccharomyces pombe*

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The p21-activated kinase (PAK) homolog, Shk1, is a critical component of a multifunctional Ras/Cdc42/PAK complex required for viability, polarized growth and cell shape, and sexual differentiation in the fission yeast, Schizosaccharomyces pombe. Substrate targets of the Shk1 kinase have not previously been described. Here we show that the S. pombe cell polarity factor, Tea1, is directly phosphorylated by Shk1 in vitro. We demonstrate further that Tea1 is phosphorylated in S. pombe cells and that its level of phosphorylation is significantly reduced in cells defective in Shk1 function. Consistent with a role for Tea1 as a potential downstream effector of Shk1, we show that a tea1 null mutation rescues the Shk1 hyperactivity-induced lethal phenotype caused by loss of function of the essential Shk1 inhibitor, Skb15. All phenotypes associated with Skb15 loss, including defects in actin cytoskeletal organization, chromosome segregation, and cytokinesis, are suppressed by tea1Δ, suggesting that Tea1 is a potential mediator of multiple Shk1 functions. S. pombe cells carrying a weak hypomorphic allele of shk1 together with a tea1Δ mutation exhibit a cytokinesis defective phenotype that is significantly more severe than that observed in the respective single mutants, providing evidence that Shk1 and Tea1 cooperate to regulate cytokinesis. In addition, we show that S. pombe cells carrying the orb2-34 allele of shk1 exhibit a pattern of monopolar growth similar to that observed in tea1Δ cells, suggesting that Shk1 and Tea1 may regulate one or more common processes involved in the regulation of polarized cell growth. Taken together, our results strongly implicate Tea1 as a potential substrate-effector of the Shk1 kinase.

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p21-activated kinases (PAKs)1 are highly conserved serine/threonine kinases that bind to and, in some cases, are stimulated by the Rho-type GTPases, Cdc42, and Rac (reviewed in Refs. 1 and 2). PAKs have been implicated in the regulation of diverse processes in eukaryotic organisms, including actin cytoskeletal organization (3–5), microtubule dynamics (6–8), cell morphology, and motility (9, 10, 12), growth factor-induced signaling pathways (10, 13–15), cell cycle control (16–18), neurological function (19), stress response (20, 21), and apoptosis (16, 22). However, the underlying mechanisms by which PAKs contribute to the regulation of these various processes are not well understood. We are using the rod-shaped fission yeast, Schizosaccharomyces pombe, as a model organism for studying PAK function and regulation. Two PAK family kinases, Shk1 (Pak1 and Orb2) (3, 10, 18) and Shk2 (Pak2) (23, 24), have been identified in S. pombe. Shk1 is an essential protein required for polarized growth and cell shape, proper cell cycle control, normal actin and microtubule cytoskeletal organization, and sexual differentiation in S. pombe (3, 7, 8, 10, 17, 25–27). Loss of Shk2 function results in no discernable phenotypic defects under normal growth conditions and genetic experiments indicate that it is largely redundant in function with Shk1 (23, 24). Genetic and molecular data indicate that Shk1 is an effector for the single Cdc42 GTPase homolog in S. pombe (3, 10, 23). Both proteins are essential for viability, cell polarity, and normal mating response (3, 10, 25). Cdc42 and Shk1 function downstream of the single fission yeast Ras homolog, Ras1, which is also required for normal morphology and mating of S. pombe cells, but which unlike Cdc42 and Shk1, is not required for cell viability (3, 10, 26, 28, 29). Ras1 interacts with Cdc42 via GTP-dependent complex formation with the presumptive Cdc42 guanine nucleotide exchange factor, Scd1 (26). Like Ras1, Scd1 is required for normal cell shape and mating, but not viability of S. pombe cells.

In addition to Cdc42, Shk1 function is positively modulated by at least 3 other nonessential proteins, Scd2 (26, 27), Shk1 (17, 29), and Skb5 (30). Scd2 binds directly to Scd1, Cdc42, and Shk1, and appears to function as a scaffold that positively modulates protein-protein interactions between Scd1 and Cdc42 and between Cdc42 and Shk1 (10, 26). Like ras1Δ and scd1Δ mutants, scd2Δ cells are viable but ovoid in shape and mating defective. Skb1 and Skb5 appear to be specialized modulators of Shk1 function in that they are required for

1 The abbreviations used are: PAK, p21-activated kinases; GST, glutathione S-transferase; HA, hemagglutinin; DAPI, 4′,6-diamidino-2-phenylindole.
proportion maintenance of cell polarity under hyperosmotic conditions but not under normal growth conditions (30, 31). Shk1 is negatively regulated by a highly conserved WD repeat protein, Shk15 (7). Loss of Shk15 function is lethal and results in severe morphological, cytoskeletal, mitotic, and cytokinesis defects. Shk15 loss results in hyperactivation of the Shk1 kinase and can be suppressed by mutations that partially inactivate Shk1 (7). Thus, phenotypes resulting from loss of Shk15 function can be attributed to Shk1 hyperactivity. A mammalian homolog of Shk15 can substitute for its counterpart in S. pombe cells and inhibits PKA activity in mammalian cell cultures, indicating that Shk15-related protein functions have been substantially conserved through evolution (7, 32).

While substantial progress has been made toward elucidating mechanisms of Shk1 regulation in S. pombe, downstream targets of the Shk1 kinase have not yet been described. Previous studies have demonstrated that Shk1 is a regulator of actin cytoskeletal organization in S. pombe (3, 18, 33). New divided S. pombe cells grow initially in a monopolar fashion from their "old ends." Subsequently, via a process referred to as new end takeoff (NETO), they initiate growth from the new end created by the cell division and continue to grow in a bipolar fashion until the onset of mitosis (34). Cortical F-actin patches are localized to the growing ends of S. pombe cells, initially to the old ends, then to both ends after NETO (35). Loss of Shk1 function, either by deletion of the shk1 gene or by overexpression of a kinase defective Shk1 mutant protein, results in the formation of spheroidal cells that exhibit a largely random distribution of cortical F-actin patches (3, 10).2 By contrast, S. pombe cells rendered hyperactive in Shk1 function as a result of loss of function of the essential Shk1 inhibitor, Shk15, are often branched and/or hyperelongated, and exhibit hyperaccumulations of F-actin in the cytoplasmic space, septum-forming region, and/or cell tips (7). An interesting hypomorphic allele of shk1, orb2-34, was identified by Verde et al. (18, 33). S. pombe cells carrying the orb2-34 mutation are viable but monopolar for growth and likewise localize cortical F-actin patches only to the single growing cell tip (36). Experiments analyzing the localization of cortical cell markers in orb2-34 cells suggest that Shk1 may play a role in the process whereby fission yeast cells recognize their ends as growth (36).

In addition to regulating F-actin in cytoskeletal organization, Shk1 is also required for proper organization of both the interphase microtubule cytoskeleton and the mitotic microtubule spindle in S. pombe cells (8). As microtubules play an important role in the establishment of cell polarity in S. pombe (37, 38), it is possible that the regulation of polarized growth by Shk1 involves both actin and microtubule-dependent processes.

Consistent with its roles as a regulator of cytoskeletal organization and cell polarity, the Shk1 protein has been shown to localize to the cell ends, septum-forming region, and microtubules in S. pombe cells (8). This pattern of subcellular localization overlaps with that of other known regulators of polarized growth in S. pombe, including the klc1 repeat protein, Tea1 (37). While not deleterious to cell growth, loss of Tea1 results in defects in actin and microtubule cytoskeletal organization and cell morphology (33, 37). Similar to S. pombe mutants carrying the orb2-34 allele of shk1, cells carrying a null mutation in the tea1 gene exhibit a monopolar growth defect (37). We and other investigators recently showed that a null mutation in the tea1 gene causes growth inhibitory phenotypes when combined with loss of function mutations in genes encoding components of the Ras/Cdc42/Shk1 complex in S. pombe, including ras1, sed1, and shk1 (8, 39). These findings raised the possibility that the Ras/Cdc42/Shk1 pathway might interact functionally with Tea1. In this article, we provide evidence that Tea1 is a potential downstream substrate target of the Shk1 kinase.

EXPERIMENTAL PROCEDURES

Yeast strains, Manipulation, and Genetic and Cytological Analysis—S. pombe strains used for this study were the wild-type strain SP870 (h<sup>+</sup> ade6-210 leu1-32 ura4-D18) (from D. Beach), nmt1-shk15 (h<sup>+</sup> skb15:ura4::nmt1-shk15-ADE2 ade6-210 leu1-32 ura4-D18) (7), nmt1-shk1-K415R (h<sup>+</sup> skb15::ura4::nmt1-shk1-K415R-ADE2 ade6-210 leu1-32 ura4-D18) (8), SP90TEA1 (h<sup>+</sup> tea1::ura4 ade6-210 leu1-32 ura4-D18) (gift from E. Chang), nmt1-shk15-tea1Δ (h<sup>+</sup> skb15::ura4::nmt1-shk1-ADE2 tea1::ura4 ade6-210 leu1-32 ura4-D18) (this study). Standard yeast culture media and genetic methods were used (40, 41). S. pombe cultures were grown in either YEAO (0.5% yeast extract, 3% dextrose, 75 mg/liter adenine, 75 mg/liter uracil), or synthetic minimal medium (EMM) with appropriate supplements (40). Yeast were transformed by the lithium acetate procedure (41). F-actin (rhodamine-phallolidin), DNA (DAPI), and cell wall (calcofluor) staining of S. pombe cells were performed as described (40).

Plasmids—The oligonucleotide primers 5′-AAATCACTGCTGCTGCTGCGCCCTA-3′ and 5′-AGAGAAGATCTGCTGAGAGATCGGATTTGAGGAGAT-3′ were used to amplify the glutathione S-transferase (GST) protein coding sequence using the plasmid pRPl29 (42) as a template. The PCR product was digested with XhoI and BglII then ligated to XhoI-BamHI digested pREP4X (43), producing pREP4XGST, which was used for expressing GST fusion proteins from the thiamine repressible nmt1 promoter (43) in S. pombe cells. pREP3XTea1, which was used for overexpressing tea1 from the nmt1 promoter, was a gift from Eric Chang (Baylor College of Medicine). The oligonucleotide primers 5′-TGTTGAGATCCCTCTAAGGGAATGC-3′ and 5′-TATAGACTCATCGTGAATTTACATGTG-3′ were used to amplify the tea1 protein coding sequence by PCR using a template plasmid (42) as a template. The tea1 PCR product was digested with BamHI and SacI for cloning into the corresponding sites of pREP4XGST and the BglII and SacI sites of pREP4XHA (31), thus producing pREP4XGST-Tea1 and pREP4XHA-Tea1, respectively, and by BamHI and EcoRI for cloning into the BamHI and Smal sites of pREP29 to generate the plasmid pGST-Tea1. pREP4XGST-Tea1 and pREP4XHA-Tea1 were used for expressing GST-Tea1 and triple hemagglutinin (HA)-Tea1, respectively, from the nmt1 promoter in S. pombe, while pGST-Tea1 was used to express GST-Tea1 in bacteria. pTrcHisShk1 has been described and was used to express polyhistidine-tagged Shk1 (His<sub>6</sub>-Shk1) in bacterial cells (30).

Preparation of Yeast Cell Lysate, Immunoprecipitations, and In Vivo Kinase Assays—S. pombe cells transformed with pREP4XHA or pREP4XHA-Tea1 were grown in EMM containing 50 μM thiamine (EMM + thi) to mid-log phase, washed twice with EMM lacking thiamine (EMM-thi), and incubated for 24 h prior to the preparation of cell lysates as described (23). HA immunocomplexes were isolated by incubating yeast lysate (1 mg of total protein) with 5 μl of anti-HA monoclonal antibody 12CA5 ascites (44) and 25 μl of packed protein A agarose beads (Roche Applied Science), which had been preequilibrated with YLB, on an orbital rotator for 2 h at 4 °C. Immune complexes were washed three times with YLB and once with kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 1 mM dithiothreitol). Kinase reactions were performed by resuspending 12.5 μl of packed immunocomplex beads with 25 μl of kinase buffer containing 20 μM ATP, 0.4 μCi/μl of [γ-<sup>32</sup>P]-ATP (6000 Ci/mmol), and ~1 ng/μl His<sub>6</sub>-Shk1 (in control reactions, His<sub>6</sub>-Shk1 was replaced by an amount of His<sub>6</sub>-SKB1 that was equimolar). The 12.5 μl of kinase buffer was used to maximize (EMM-thi), then subcultured into EMM-thi and grown for 24 h prior to the preparation of cell lysates as described (23). HA immunocomplexes were isolated by incubating yeast lysate (1 mg of total protein) with 5 μl of anti-HA monoclonal antibody 12CA5 ascites (44) and 25 μl of packed protein A agarose beads (Roche Applied Science), which had been preequilibrated with YLB, on an orbital rotator for 2 h at 4 °C. Immune complexes were washed three times with YLB and once with kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 1 mM dithiothreitol) 32P labeling experiments In Vivo 32P Labeling Experiments—In vivo 32P labeling experiments in vivo were performed as previously described (30).

In Vivo 32P Labeling Experiments—In vivo 32P labeling experiments for the analysis of Tea1 phosphorylation in S. pombe cells were per-
Shk1 Directly Phosphorylates Tea1 in Vitro—Previous studies have shown that Tea1 is associated with high molecular weight protein complexes in S. pombe cells (37, 46). To investigate whether Tea1 or Tea1-associated proteins are phosphorylated by the Shk1 kinase in vitro, we constructed the plasmid pREP4XHA-Tea1 for expressing HA-tagged Tea1 protein (HA-Tea1) in S. pombe cells. HA-Tea1 complexes were immunoprecipitated from S. pombe cell lysates (Fig. 1A) and the immune complexes were incubated in kinase reactions containing His6-Shk1, which was purified from bacterial cell lysates and incubated in vitro kinase reactions with bacterially expressed His6-Shk1. We observed that GST-Tea1, but not GST, was phosphorylated in kinase reactions containing His6-Shk1 but not in reactions lacking His6-Shk1 (Fig. 1B). The level of Shk1 kinase activity, as measured by autophosphorylation, was not markedly affected by GST-Tea1 in these experiments (Fig. 1B). These results demonstrate that Tea1 is a direct substrate of the Shk1 kinase.

Tea1 Is Phosphorylated in S. pombe Cells in a Shk1-dependent Fashion—Experiments were performed to determine whether Tea1 is phosphorylated in S. pombe cells and, if so, whether its phosphorylation is dependent on Shk1. The plasmid pGST-Tea1 was constructed for expressing GST-Tea1 fusion protein from the nmt1 promoter (43). Wild-type S. pombe cells expressing either GST or GST-Tea1 were metabolically labeled with [32P]orthophosphate. [32P]-labeled cells were lysed, and GST fusion proteins were purified from the cell lysates and subjected to SDS-PAGE and autoradiography. As shown in Fig. 2A, GST-Tea1 but not GST, was labeled by [32P] in vivo, thus demonstrating for the first time that Tea1 is phosphorylated in S. pombe cells.

To determine whether Tea1 phosphorylation in vivo is dependent on Shk1, we expressed GST-Tea1 in an S. pombe mutant in which the endogenous shk1 gene is replaced by a sequence from which a kinase deficient form of Shk1, Shk1K415R, is expressed from a weak allele of the nmt1 promoter (8, 27, 43). Wild-type and shk1K415R cells expressing GST-Tea1 were labeled with [32P] and the degree of [32P] incorporation into GST-Tea1 in each strain was determined. As shown in Fig. 2B, the degree of [32P] labeling observed for GST-Tea1 was significantly greater in wild-type S. pombe cells than expressed proteins to determine whether Tea1 is directly phosphorylated by Shk1. The plasmid pGST-Tea1 was constructed for expressing GST-Tea1 in bacterial cells. GST-Tea1 protein was purified from bacterial cell lysates and incubated in in vitro kinase reactions with bacterially expressed His6-Shk1. We observed that GST-Tea1, but not GST, was phosphorylated in kinase reactions containing His6-Shk1 but not in reactions lacking His6-Shk1.

RESULTS

SHK1 Directly Phosphorylates Tea1 in Vitro—Previous studies have shown that Tea1 is associated with high molecular weight protein complexes in S. pombe cells (37, 46). To investigate whether Tea1 or Tea1-associated proteins are phosphorylated by the Shk1 kinase in vitro, we constructed the plasmid pREP4XHA-Tea1 for expressing HA-tagged Tea1 protein (HA-Tea1) in S. pombe cells. HA-Tea1 complexes were immunoprecipitated from S. pombe cell lysates (Fig. 1A) and the immune complexes were incubated in kinase reactions containing His6-Shk1, which was purified from bacterial cell lysates and incubated in vitro kinase reactions with bacterially expressed His6-Shk1. We observed that GST-Tea1, but not GST, was phosphorylated in kinase reactions containing His6-Shk1 but not in reactions lacking His6-Shk1 (Fig. 1B). The level of Shk1 kinase activity, as measured by autophosphorylation, was not markedly affected by GST-Tea1 in these experiments (Fig. 1B). These results demonstrate that Tea1 is a direct in vitro substrate of the Shk1 kinase.

Fig. 1. Shk1 directly phosphorylates Tea1 in vitro. A, S. pombe wild-type and nmt1-shk1K415R cells transformed with pREP4XHA or pREP4XHA-Tea1 were grown in EMM containing 50 μM thiamine to mid-log phase, then subcultured into phosphate-free EMM supplemented with 1 mM phosphate and grown for about 3.5 generations. Cells were then resuspended at 2 × 10^6 cells/ml in fresh EMM containing 50 μM phosphate. 5 ml of each cell suspension was incubated with 1 μCi of [32P]orthophosphate (104 Ci/mmol) (NEX054, PerkinElmer Life Sciences Tea1 Shk1, HA control (Control) and HA-Tea1 immune complexes were incubated in kinase reactions either with or without His6-Shk1 protein, as indicated, then resolved by SDS-PAGE followed by autoradiography. HA-Tea1 was more heavily phosphorylated in kinase reactions containing His6-Shk1 than in reactions lacking His6-Shk1. C, GST (−10 μg), GST-Tea1 (−1 μg), and His6-Shk1 (−25 ng) fusion proteins purified from bacterial cells were incubated alone or together in in vitro kinase reactions as indicated and resolved by SDS-PAGE (6 or 12% gels) followed by autoradiography. GST-Tea1 but not GST was directly phosphorylated by Shk1. Shk1 autophosphorylation was not markedly affected by Tea1.
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**Fig. 2.** Tea1 phosphorylation is dependent on Shk1 function in *S. pombe* cells. A, wild-type *S. pombe* cells expressing either GST or GST-Tea1 were labeled with [γ-32P]orthophosphate and then lysed as described (“Experimental Procedures”). Lysates were incubated with glutathione-agarose beads to purify GST fusion proteins, which were resolved by SDS-PAGE. GST-Tea1 samples were resolved on 6% SDS-PAGE gels, while GST samples were resolved on 12% gels. The gels were stained with Coomassie Brilliant Blue to visualize GST and GST-Tea1 (left panels) and exposed to film for autoradiography (right panels). [γ-32P] was incorporated into GST-Tea1 but not into GST. B, wild-type and nmt1-skb15K415R cells expressing GST-Tea1, as well as untransformed wild-type cells (Control) were labeled with [γ-32P] and analyzed for GST-Tea1 phosphorylation as described in A. The degree of [γ-32P] incorporation into GST-Tea1 was markedly greater in wild-type cells than in nmt1-skb15K415R cells.

in *shk1K415R* cells. These results demonstrate that Shk1 is required for Tea1 phosphorylation *in vitro* and, when considered together with the above results showing that Tea1 is directly phosphorylated by Shk1 *in vitro*, implicate Tea1 as a potential Shk1 substrate in *S. pombe*.

**Genetic Evidence Implicating Tea1 as an Effector of Shk1 Function in S. pombe**—The above molecular data implicate Tea1 as a potential downstream target of the Shk1 kinase. We reasoned that if Tea1 is a mediator of Shk1 function, then loss of Tea1 function might suppress phenotypic defects resulting from Shk1 hyperactivity. We therefore determined whether phenotypes associated with loss of the essential Shk1 inhibitor, Skb15, are affected by a null mutation in the tea1 gene. For our analyses, we utilized an *S. pombe* strain, nmt1-skb15, in which the skb15 gene is conditionally expressed from the thiamine-repressible nmt1 promoter (7). The nmt1-skb15 strain is indistinguishable from wild-type *S. pombe* cells on media lacking thiamine. However, on thiamine-containing media, the nmt1-skb15 strain is inviable and recapitulates phenotypes of the skb15 null mutant (7). We constructed an nmt1-skb15 tea1Δ mutant and compared its growth characteristics to that of wild-type, nmt1-skb15, and tea1Δ strains in the presence and absence of thiamine. Strikingly, we found that at 35 °C, the nmt1-skb15 tea1Δ mutant grew about as well as wild-type *S. pombe* cells on thiamine-containing media (Fig. 3A). In contrast, the nmt1-skb15 mutant was inviable under the same conditions (Fig. 3A). nmt1-skb15 tea1Δ cells did not grow on thiamine-containing media at 25 or 30 °C, indicating that the tea1Δ mutation is a temperature-dependent suppressor of Skb15 loss (data not shown). The temperature dependence of the suppression of loss of skb15 by tea1Δ is consistent with our previous observation that tea1Δ causes a temperature sensitive growth inhibitory phenotype when combined with a mutation that partially inactivates Shk1 (8). Thus, the effects of the tea1Δ mutation on both loss and gain of Shk1 function are temperature-dependent.

When grown in thiamine-containing medium, nmt1-skb15 cultures display a variety of morphological defects, including a significant frequency of multiseptated, highly branched, severely deformed, and greatly enlarged and/or elongated cells (Fig. 3B, panel ii) (7). The frequency of enlarged cells, multiseptated cells, and cells with elongated branches was markedly lower in nmt1-skb15 tea1Δ cultures than in nmt1-skb15 cultures (Fig. 3B, panel iii). However, the nmt1-skb15 tea1Δ mutant still exhibited a fairly significant frequency of T-shaped and otherwise morphologically aberrant cells similar to that observed in tea1Δ cultures (Fig. 3B, panel iv), but which were not observed in cultures of wild-type *S. pombe* cells (Fig. 3B, panel i). Indeed, nmt1-skb15 tea1Δ cells were for the most part indistinguishable from tea1Δ cells from a morphological standpoint.

**Fig. 3.** The tea1 null mutation rescues the lethality caused by loss of the essential Shk1 inhibitor, Skb15. A, wild-type, nmt1-skb15, tea1Δ, and nmt1-skb15 tea1Δ cells were streaked onto EMM, EMM containing 50 μg thiamine (EMM + thi), or YEAU plates and incubated at 35 °C (YEAU plates for 2 days and EMM plates for 4 days). YEAU partially inhibits the nmt1 promoter, presumably because of thiamine contained in yeast extract. The nmt1-skb15 mutant was strongly inhibited for growth on EMM + thi and YEAU plates, whereas the nmt1-skb15 tea1Δ, tea1Δ, and wild-type strains grew well on these media. B, photomicrographs of wild-type (i), nmt1-skb15 (ii), tea1Δ (iii), and nmt1-skb15 tea1Δ (iv) cells grown in YEAt at 35 °C for 40 h. See text for description of results.
Circled cell about 40 h and stained with rhodamine phalloidin to visualize F-actin. 

In addition, a small percentage of cells (about 1%) exhibit dense 

relations of F-actin throughout much or most of the cytoplasmic 

cultures results in severe abnormalities in F-actin organization 

the logical analyses were performed to assess the degree to which 

culture (Fig. 6, A and B). A similar low frequency of multiseptated 
cells was also observed in nmt1-shk1K415R cultures (Fig. 6B), but not in shk1 mutant strains that are less severely impaired for Shk1 function, such as orb2-34 (data not shown). 

A role for Shk1 in cytokinesis was previously suggested from 

Evidence That Shk1 and Tea1 Cooperate to Regulate Cytokinesis in S. pombe—In our microscopic analyses of tea1 cultures, we noticed a low frequency of cells (<2%) with multiple septa (Fig. 6, A and B). 

Tea1 Potentially Mediates Multiple Shk1 Functions—Cyto-

logical analyses were performed to assess the degree to which the tea1Δ mutation suppresses cytoskeletal, mitotic, and cytokinesis defects resulting from loss of Skb15 function. Thiamine-induced repression of skb15 expression in nmt1-skb15 mutant cultures results in severe abnormalities in F-actin organization (7). Most notably, these include cells exhibiting dense accumulations of F-actin throughout much or most of the cytoplasmic space, the septum-forming region, and/or the cell tips (Fig. 4B). 

In addition, a small percentage of cells (about 1%) exhibit dense 

cable-like and/or circular F-actin structures (see circled cell in 
panel B and cells in small panels to right of panel B). We found that the tea1Δ mutation strongly suppressed F-actin defects of the nmt1-skb15 mutant at 35 °C (Fig. 4C). Indeed, the nmt1-skb15 tea1Δ mutant was phenotypically similar to the tea1Δ mutant in that interphase cells of both strains exhibited a primarily monopolar pattern of cortical F-actin localization (Fig. 4, C and D). 

In addition to defects in F-actin organization, nmt1-skb15 cultures exhibit a high frequency of cells (>40%) with significant hyperaccumulations of cell wall material (chitin-rich deposits), particularly at the septum-forming region (Fig. 4, E and F) (7). As in the case of actin cytoskeletal defects, we found that the tea1Δ mutation strongly suppressed cell wall/septum hypersynthesis defects of the nmt1-skb15 mutant. The degree of suppression was not as complete as was observed for sup-
pression of F-actin defects. However, in this regard, we ob-
erved that the tea1Δ mutant also exhibited a low frequency of cell wall defects similar to those observed in the nmt1-skb15 mutant (Fig. 4F). These results implicate Tea1 as a mediator of Shk1 functions involved in regulating septum and cell wall biogenesis in S. pombe cells. 

nmt1-skb15 cells also exhibit severe mitotic defects in thia-
microscope. These defects were not observed in nmt1-skb15 tea1Δ cultures, as judged by mi-

oma containing media (7). In comparison to wild-type S. pombe 
cells (Fig. 5A), mitotic spindles in nmt1-skb15 cells are often hyperelongated and/or attached to multiple fragments of chromosomal material (Fig. 5B) (7), the latter phenotype being indicative of defective nuclear segregation. These defects were not observed in nmt1-skb15 tea1Δ cultures, as judged by mi-

sults strongly suppressed the 
aneuploid phenotype of the nmt1-skb15 mutant (Fig. 5D). 

These data suggest that Tea1 may also share a role with Shk1 in the regulation of mitotic processes in S. pombe cells. 

Evidence That Shk1 and Tea1 Cooperate to Regulate Cytoki-

nmt1-shk1K415R tea1Δ (7). As in the case of other 

phenotypic defects associated with the loss of Shk1 function, we found that the tea1Δ mutation strongly suppressed the aneuploid phenotype of the nmt1-skb15 mutant (Fig. 5D). 

Evidence That Shk1 and Tea1 Cooperate to Regulate Cytoki-

nmt1-shk1K415 tea1Δ cultures (see panel F). F, quantitation of the percentage of cells exhibiting hyperac-
cumulation of cell wall material in wild-type, nmt1-skb15, tea1Δ, and nmt1-skb15 tea1Δ strains. 

Taken together, the above results demonstrate that cell growth and morphological defects caused by loss of Skb15 func-
tion are dependent on the presence of Tea1. As Skb15 is an inhibitor of Shk1 and genetic and molecular data suggest that phenotypes associated with loss of Skb15 function are atrib-
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Tea1 Potentially Mediates Multiple Shk1 Functions—Cyto-

logical analyses were performed to assess the degree to which the tea1Δ mutation suppresses cytoskeletal, mitotic, and cytokinesis defects resulting from loss of Skb15 function. Thiamine-induced repression of skb15 expression in nmt1-skb15 mutant cultures results in severe abnormalities in F-actin organization (7). Most notably, these include cells exhibiting dense accumulations of F-actin throughout much or most of the cytoplasmic space, the septum-forming region, and/or the cell tips (Fig. 4B). 

In addition, a small percentage of cells (about 1%) exhibit dense
In light of the above findings, we decided to reexamine shk1Δ cells for cytokinesis defective phenotypes. As shk1 is an essential gene, we generated an S. pombe culture enriched for shk1Δ cells by inducing a shk1Δ/lskh1::ura4 diploid culture to undergo sporulation. We then transferred the sporulated culture to fresh minimal medium lacking uracil to induce spore germination and to select for the growth of shk1::ura4 cells (shk1Δ spores germinate as round cells that typically undergo from one to a few rounds of cell division before arresting growth and eventually lysing, Ref. 10). Microscopic analysis of DAPI-stained cells revealed that nearly 60% of shk1Δ cells contained at least one septum and two nuclei, in contrast to wild-type S. pombe cultures, in which only about 20% of cells were septated (Fig. 6D). The percentage of septated shk1Δ cells may have actually been higher, as in some cells, the septum may have been parallel to the viewing plane and, therefore, not detectable. Significantly, we observed that more than 20% of septated shk1Δ cells actually had multiple septa (Fig. 6D), which is a clear indication of a cytokinesis defective phenotype. These results are consistent with the observations of Ottillie et al. (3), which suggested that Shk1 might be required for a late stage(s) of the cytokinesis program in S. pombe.

FIG. 5. Suppression of mitotic defects in nmt1-skb15 cells by the tea1Δ mutation. Mitotic spindle and associated DNA in a wild-type S. pombe cell (A) and an nmt1-skb15 mutant cell (B) from cultures grown in YEPA at 35 °C for 40 h. The spindle (red) was visualized by indirect immunofluorescence of microtubules, while DNA (blue) was visualized by direct fluorescence using DAPI. Spindles were frequently found to be hyperelongated in mitotic nmt1-skb15 cells and often attached to multiple fragments of chromosomal material. These defects were not observed in wild-type, tea1Δ, or nmt1-skb15 tea1Δ cultures (data not shown). C, fluorescence photomicrograph of nmt1-skb15 cells stained with DAPI to visualize DNA. A high degree of aneuploidy was apparent in nmt1-skb15 cultures but not in wild-type (inset panel and panel D) or tea1Δ cultures (panel D). A low frequency of aneuploidy was observed in nmt1-skb15 tea1Δ cultures (panel D). D, quantitation of the percentage of aneuploid cells in wild-type, nmt1-skb15, tea1Δ, and nmt1-skb15 tea1Δ cultures.

Analysis of the Monopolar Growth Defect of S. pombe Cells Carrying the orb2-34 Mutant Allele of shk1—S. pombe mutants carrying the orb2-34 allele of shk1 are unable to activate a bipolar phase of growth after cell division, as is the case for cells carrying a deletion of the tea1 gene. tea1Δ cells have been shown to exhibit an interesting pattern of monopolar growth in which one daughter cell typically initiates growth from its old end after cell division, while the other daughter cell initiates growth from the new end created by the cell division (46). As the growth characteristics of the orb2-34 mutant have not been described in detail, we performed time-lapsed photomicroscopy to determine whether this mutant exhibits a pattern of monopolar growth similar to that of tea1Δ cells. Whereas in the case of wild-type S. pombe cells, both daughter cells invariably initiated growth from the old cell end after cell division, for the orb2-34 mutant, we observed in the vast majority of cases (97%) that one daughter cell initiated growth from the old cell end, while the other daughter cell initiated growth from its new end (Fig. 7, A–C). In the case of wild-type cells, the majority of daughter cells (89%) proceeded to activate NETO and grew bifidiarily until the next cell division, whereas orb2-34 cells never initiated a bipolar phase of growth (Fig. 7C). These observations demonstrate that the orb2-34 mutant exhibits a pattern of mo-
The growth patterns of wild-type and orb2-34 S. pombe cells. The growth patterns of 19 wild-type cells and 35 orb2-34 mutant cells were determined by time lapsed photomicroscopy (15-min intervals) of cells grown on YE agar beds at 25 °C. A, representative photomicrographs showing the progression of growth of a pair of wild-type S. pombe daughter cells (top panels) and a pair of orb2-34 daughter cells (bottom panels). Arrows indicate growing ends of the cells. Time (min) is indicated at the bottom right side of each panel. B, graph showing quantitation of the percentage of wild-type cells (black bars) and orb2-34 cells (gray bars) exhibiting the 3 patterns of postmitotic end growth evaluated in the analysis (left). Both daughter cells initiate growth from their old ends; middle, one daughter initiates growth from its old end, the other from its new end; right, both daughter cells initiate growth from their new ends). We observed no instances in which both daughter cells initiated growth from their new ends for either wild-type or orb2-34 cells. C, schematic representation showing the growth patterns of wild-type (left) and orb2-34 (right) S. pombe cells.

DISCUSSION

An important goal in understanding the function of Ras1/Cdc42/PAK complex in fission yeast is to identify the pertinent substrates of the PAK kinase. In this article, we have provided evidence that the kelch repeat protein, Tea1, is a potential substrate target of the PAK kinase, Shk1. Both proteins regulate cell polarity and are localized to the cell ends, septum, and microtubules. Tea1 was found to exist as a phosphoprotein in S. pombe cells and its phosphorylation was substantially dependent on the presence of Shk1. Tea1 was also directly phosphorylated by Shk1 in vitro, suggesting that it is likely to be a direct substrate of Shk1 in S. pombe cells. Genetic interactions support this molecular interaction and place Tea1 downstream of Shk1. First, a null mutation in the tea1 gene strongly suppresses phenotypes of S. pombe cells rendered hyperactive for Shk1 function as a consequence of loss of the essential Shk1 inhibitor, Skb15. Second, a strain carrying the tea1 null mutation together with a mutation that severely attenuates Shk1 function causes a synthetic interaction. Additional evidence suggesting that Shk1 and Tea1 may regulate one or more common processes involved in the regulation of cell polarity comes from our observations that S. pombe cells carrying a non-lethal hypomorphic allele of shkl, orb2-34, exhibit a pattern of monopolar growth that strongly resembles that resulting from the tea1Δ mutation. Although our attempts to coprecipitate Shk1 and Tea1 proteins from S. pombe cells have not been successful, it is possible that these proteins associate only transiently, as might be expected for a protein kinase-substrate interaction. Nevertheless, our molecular and genetic data provide strong evidence for an interaction between the Shk1 and Tea1 proteins.
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S. pombe cells have been found at focal adhesions (49) as well as ring canals, which are actin-based structures derived from arrested cleavage furrows (50). PAKs have likewise been shown to associate with focal complexes (51) and, more recently, to the cleavage furrow in animal cells (6). Furthermore, recent work in the amphibian, Xenopus laevis, has provided evidence that PAKs, in addition to their established roles as actin cytoskeletal regulators, also contribute to the regulation of microtubule dynamics in animal cells, as is the case in fission yeast (6). It will be of great interest to determine whether Tea1-like kelch repeat proteins mediate functions of PAKs involved in regulating actin and/or microtubule-dependent processes in higher eukaryotes.

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The Kelch Repeat Protein, Tea1, Is a Potential Substrate Target of the p21-activated Kinase, Shk1, in the Fission Yeast, *Schizosaccharomyces pombe*

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