Direct Activation of the Fission Yeast PAK Shk1 by the Novel SH3 Domain Protein, Skb5*

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The p21-activated kinase (PAK) homolog Shk1 is essential for cell viability in the fission yeast Schizosaccharomyces pombe. Roles have been established for Shk1 in the regulation of cell morphology, sexual differentiation, and mitosis in S. pombe. In this report, we describe the genetic and molecular characterization of a novel SH3 domain protein, Skb5, identified as a result of a two-hybrid screen for Shk1 interacting proteins. S. pombe cells carrying a deletion of the skb5 gene exhibit no discernible phenotypic defects under normal growth conditions, but when subjected to hypertonic stress, become spherical in shape and growth impaired. Both of these defects can be suppressed by overexpression of the Shk1 modulator, Skb1. The growth inhibition that results from overexpression of Shk1 in S. pombe cells is markedly suppressed by a null mutation in the skb5 gene, suggesting that Skb5 contributes positively to the function of Shk1 in vivo. Consistent with this notion, we show that Skb5 stimulates Shk1 catalytic function in S. pombe cells. Furthermore, and perhaps most significantly, we show that bacterially expressed recombinant Skb5 protein directly stimulates the catalytic activity of recombinant Shk1 kinase in vitro. These and additional data described herein demonstrate that Skb5 is a direct activator of Shk1 in fission yeast.

p21-activated kinases (PAKs)1 comprise a highly conserved family of serine/threonine kinases that are regulated by the p21 G proteins Cdc42 and Rac, but not by other small G proteins, such as Ras and Rho (1). PAKs share a common structural organization consisting of a C-terminal catalytic domain and a substantial N-terminal regulatory domain that typically comprises at least half the length of the protein. The p21-binding site is invariably located in the N-terminal regulatory domain of PAKs. Diverse functions have been attributed to PAKs in eukaryotic organisms, including roles in regulation of cytoskeletal organization and cellular morphology (2–5), apoptosis (6, 9, 13) in vertebrates. Indeed, the biological functions attributed to PAKs in eukaryotes match or exceed those attributed to the similarly conserved mitogen-activated protein kinase cascades, with which, in some cases, PAKs have been shown to functionally interact (6, 8, 14).

The fission yeast Schizosaccharomyces pombe possesses two known PAKs, Shk1 (2) (also known as Pak1 (3) and Orb2 (11)) and Shk2 (15) (also known as Pak2 (16)). Shk1 is essential for viability of S. pombe cells and has been shown to play roles in the regulation of cell morphology, sexual differentiation, and mitosis (2, 3, 10, 11, 17). The cellular functions of Shk1 are virtually indistinguishable from those of Cdc42 (2, 18, 19), and various molecular and genetic data suggest that Shk1 is a critical effector for Cdc42 in fission yeast (2, 3, 15). A second positive modulator of Shk1, Skb1, was identified by our laboratory from a two-hybrid screen for Shk1-interacting proteins (17). Skb1 functions as a dosage-dependent mitotic inhibitor in S. pombe, and this function is at least partially dependent on Shk1 (10). Unlike Shk1 and Cdc42, Skb1 is not required for viability or mating of S. pombe cells (17). The second known fission yeast PAK, Skb2, is also nonessential, and genetic analyses suggest that it is largely redundant in function with Shk1 (15, 16).

In this report, we describe the genetic and molecular characterization of skb5 (for skb1 kinase binding protein 5), a gene encoding a novel SH3 domain protein that interacts with Shk1 in vivo and in vitro. We present genetic evidence for functional interaction between Skb5 and Shk1 in S. pombe and biochemical evidence that Skb5 is a direct stimulator of Shk1 catalytic function. Our results provide what is to our knowledge the first example of direct activation of a PAK by an SH3 domain protein.

EXPERIMENTAL PROCEDURES

Yeast Strains, Manipulation, Genetic Analyses, and Two-hybrid Assays—S. pombe strains used in this study were SP870 (h** ade6–210 leu1–32 ura4-D18) (from D. Beach), SP870D (h** ade6–210 leu1–32 ura4-D18 ura10-D18) (from V. Jung), CHP428 (h+ ade6–210 his7–366 leu1–32 ura4-D18) (from E. Chang), SP421NT (h** ade6–210 leu1–32 ura4:D18::adhid-cdc42N17) (2), SPSKS5 (h+ ade6–210 leu1–32 ura4-D18 skb5::ura4) (see below), 137 (h+ leu1–32 ura4-D18 wee1::ura4). The Saccharomyces cerevisiae two-hybrid tester strain used was L40 (MATa ade2 his3 leu2 trp1lys2::lexA-HIS3 ura3::lexA-lacZ) (20). Standard yeast culture media and genetic methods were used (21, 22). S. pombe cultures were grown on either rich medium (YE-A) or synthetic minimal medium (EMM) with appropriate auxotrophic supplements (21). S. cerevisiae cultures were grown on either rich medium (YEA) or synthetic minimal medium (EMM) with appropriate auxotrophic supplements (22). Yeast were transformed by the lithium acetate procedure (22). The skb5::ura4 strain SPSKB5 was constructed by transformation of SP870D with a 2.6-kb NdeI skb5::ura4 fragment from the plasmid pBSISkb5::ura4. Diploid transformants carrying a single disrupted and a single wild-type copy of skb5 were identified by Southern blot analysis and skb5::ura4 transformants were...
isolated by tetrad dissection. The liquid assay for β-galactosidase activity was performed as described previously (22).

Plasmids—The two-hybrid plasmids pGADGH (for expression of GAD fusions) and pB7T116 and pVJL11 (for expression of LBD fusions) have been described previously (19, 20, 23, 24). The plasmids pLBDshk1, pLBDshk1 (308–658), pLBDras1, pGADGHbyr2, and pLBDlamin have also been described before (2, 17, 19, 24). The S. pombe-Zeb/cheria cdi/ shuttle vector pAAUCM and pART1CM were used for high level expression of coding sequences from the S. pombe Adam1 promoter (2). pREPs (25) was used for expressing coding sequences from the S. pombe nmt1 promoter. pAAUCMshk1, pAAUCMshk1 N118, pART1CMshk1 N118, pART1CMshk1, pAAUGSTshk1, and pAAUGSTshk1 N118 have been described previously (2, 10, 17). pTrch-Ha-Ras has also been described previously (26). pTrchHshk1 was made by cloning BamHI-SalI fragment of Shk1 into the BamHI-XhoI sites of pTrchB. The polynucleotide chain reaction was used to amplify the full-length Shkb5 protein coding sequence for cloning into pAAUCM, pART1CM, pREPs, pGADGH, and prP295, producing pAAUCMs5b5, pART1CMSkb5, pREPS1kb5, pGADGHs5b5, and pP295s5b5, respectively. The primer pair SKB5KOP3 (5′-AGAAGTCTACGAGAAGAAGCGG) and SKB5KOP6 (5′-CAAGCT-GAGAATTCTCACATTGCGTGTACATCA) was used to amplify a 0.95-kb fragment of the 5′-end of skb5. This fragment was then digested with HindIII and EcoRV and cloned into HindIII and HindIII sites of pBluescriptII, producing pBSItslkb5-3. The primer pair SKB5KOP7 (5′-ATATGGTGGAGCTCAGTGCAA) and SKB5KOP8 (5′-ACTCAGGCT-TCATTCACACAGCAT) was used to amplify a 0.38-kb fragment of the 5′-end of skb5, which was digested with ScaI and HindIII and cloned into the corresponding sites of pBSItslkb5-3 generating pBSItsskb5KO. This pBSItsskb5KO was digested with HindIII and ligated with a 1.8-kb HindIII fragment of the urd gene to produce pBSItsskb5:urd4. Shk1 (1–380) and Skb5 (1–88) protein coding sequences were both obtained by polynucleotide chain reaction and then cloned into pVJL11 and pGADGH, respectively, generating pVJL11shk1 (1–380) and pGADGHs5b5.

Kinase Assays and Coprecipitation Experiments—For kinase assays and coprecipitation experiments using proteins purified from S. pombe cell lysates, S. pombe cells expressing GST fusion proteins, either alone or in combination with CMSKsh5, were grown in EMM to about 107 cells/ml, washed with yeast lysis buffer, and resuspended in yeast lysis buffer before lysing using glass beads as described previously (15). GST fusion proteins were precipitated from yeast cell lysates using glutathione-agarose beads as recommended by the manufacturer (Amersham Pharmacia Biotech). For coprecipitation experiments, immunoblots were performed and visualized using an anti-GST antibody (Pierce) and anti-c-Myc antibody 9E10 (27). Kinase assays of purified GST proteins were carried out at 30 °C in 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM MgCl₂, 1 mM dithiothreitol, 0.05% Triton X-100, and 10% glycerol, using Centriprep-5 concentrators (Millipore). Kinase assays of purified GST proteins were carried out at 30 °C in 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM MgCl₂, 1 mM dithiothreitol, 0.05% Triton X-100, and 10% glycerol, using Centriprep-5 concentrators (Millipore). Kinase reactions were terminated after 20 min by addition of 4 × SDS-PAGE sample buffer (Novex) and boiling for 5 min prior to resolving on a 4–15% SDS-PAGE gradient gel (Bio-Rad) and subsequent autoradiography.

RESULTS AND DISCUSSION

We previously described a two-hybrid screen for identification of Shk1-interacting proteins (17). Partial cDNAs corresponding to two distinct S. pombe genes were identified as a result of this screen, skb1, which has been described previously (17), and skb5 (previously referred to as skb2 (17)), which we describe in this report. The full-length skb5 gene was cloned by a hybridization screen of an S. pombe genomic library. Sequence analysis revealed that the skb5 gene (GenBank™ accession number AF192549) contains an uninterrupted open reading frame encoding a small protein, 140 amino acids in length, with a predicted molecular mass of approximately 16 kDa (Fig. 1A). The Skb5 protein contains a single C-terminal SH3 domain (amino acids 87–136) and a highly acidic N terminus. BLAST searches of the nucleic acid and protein sequence data bases revealed that Skb5 shares structural homology with an unpublished S. cerevisiae gene, Nbp2 (GenBank™ accession number D43693) and 1–137 of chicken c-Src. Identical amino acids are indicated by black boxes with white text and similar amino acids by gray boxes with black text.

![Figure 1](image-url)

**Fig. 1.** Sequence analysis of the skb5 gene and alignment of the predicted Skb5 protein with related proteins from budding yeast and chicken. A, nucleotide sequence and translation of the skb5 gene. The SH3 domain of the Skb5 protein is indicated by black boxes with white text. In-frame stop codons are indicated by the asterisks. B, alignment of amino acids 1–136 of Skb5 with amino acids 1–166 of S. cerevisiae Nbp2 (GenBank™ accession number D43693) and 1–137 of chicken c-Src. Identical amino acids are indicated by black boxes with white text and similar amino acids by gray boxes with black text.
significant. We first expressed Skb5 as a c-Myc epitope-tagged protein (CMSkb5) in fission yeast to determine whether it coprecipitates with full-length Shk1, as well as the original Shk1 two-hybrid bait protein, Shk1ΔN118, which were each fused to glutathione S-transferase (GST-Shk1 and GST-Shk1ΔN118, respectively). Shk1ΔN118 corresponds to the originally published Shk1 protein sequence (2), which was truncated by 118 amino acids due to a sequencing error. Shk1ΔN118 can substitute for full-length Shk1 protein in *S. pombe* cells. CMSkb5 coprecipitated with GST-Shk1 and GST-Shk1ΔN118 from *S. pombe* cell lysates, but did not coprecipitate with GST (Fig. 2), demonstrating that Skb5 and Shk1 proteins form a complex in *S. pombe*.

An skb5 null (skb5Δ) mutation was generated in which most of the skb5 coding sequence was replaced by the *ura4* gene (Fig. 3A). Unlike shk1Δ mutants, skb5Δ mutants were viable and exhibited no obvious phenotypic defects under normal growth conditions in either rich or minimal media (data not shown). skb5Δ mutants were also indistinguishable from wild-type cells when grown at either 20 or 36 °C, indicating that the skb5Δ mutation does not cause cold or temperature sensitive phenotypes, respectively (data not shown). Recent studies in our laboratory have demonstrated that Cdc42, Shk1, and Skb1 are each required for normal response to hypertonic stress in *S. pombe*. *S. pombe* mutants expressing a dominant inhibitory mutant allele of *cdc42* are inviable and cells deficient in Shk1 expression are growth impaired when cultured in hypertonic medium. skb1Δ mutants, which are normally only modestly shorter than wild-type cells, become ellipsoidal in shape when subjected to hypertonic stress. These observations prompted us to investigate whether the growth or morphology of skb5Δ mutants is affected by hypertonic stress. Indeed, we found that skb5Δ mutants grew slower than wild-type cells and became ellipsoidal in morphology when grown in 1.5 M KCl (Fig. 3, B and C), demonstrating that Skb5, like Cdc42, Shk1, and Skb1, is required for normal growth and morphology of *S. pombe* cells in hypertonic medium.

Additional genetic analyses were performed to further establish a functional link between Skb5 and Skh1. We determined that overexpression of Shk1 is significantly less inhibitory to the growth of skb5Δ cells than to wild-type cells (Fig. 3D), providing evidence that Skb5 might be required for normal Shk1 function in *S. pombe*. We found further that Skb5 overexpression was inhibitory to the growth of *S. pombe* cells expressing a dominant inhibitory mutant allele of *cdc42*, *cdc42T17N*, and that this growth defect could be suppressed by overexpression of Shk1ΔN118 (Fig. 3E). Shk1ΔN118 was used for this experiment because, for reasons that at present are unclear, overexpression of full-length Shk1 is highly toxic to cells expressing *Cdc42T17N*. Interestingly, we observed that wild-type cells that overexpressed Skb5 were spheroidal in morphology (data not shown), suggesting that Skb5 overexpression may have a dominant inhibitory effect with respect to function of the Cdc42/Shk1 morphological control pathway in *S. pombe*, possibly due to sequestration of Shk1 from proper interaction with its other regulators and/or targets. This idea is consistent with the observation that overexpression of Skb5 was inhibitory to growth of cells expressing *Cdc42T17N* and that overexpression of Shk1ΔN118 could suppress this growth defect. Although overexpression of Shk1 could not suppress hypertonic stress-induced growth or morphological defects of the skb5Δ mutant (data not shown), these defects were suppressed by overexpression of the Shk1 modulator Skb1 (Fig. 3, B and C). These various genetic data provide strong evidence that Skb5 and Shk1 interact functionally in *S. pombe*, with Skb5 possibly functioning as a positive regulator of Shk1.

Biochemical experiments were performed to establish whether Skb5 regulates the catalytic function of Shk1. *S. pombe* cells expressing GST or GST-Shk1ΔN118, both with and without co-overexpression of Skb5, were lysed and GST and GST-Shk1ΔN118 proteins purified from the resulting cell lysates. Kinase assays were then performed to measure the ability of GST-Shk1ΔN118 to both autophosphorylate and phosphorylate MBP, GST-Shk1ΔN118 was used for this experiment, because we found that a GST fusion of the full-length Shk1 protein was expressed at a substantially higher level in cells that overexpressed Skb5, making it impossible to obtain meaningful comparisons of Shk1 kinase activity between cells that overexpressed Skb5 and cells that did not. As shown in Fig. 4A, the phosphorylation of MBP by GST-Shk1ΔN118 was significantly greater for protein isolated from cells that overexpressed Skb5 than from cells that did not, while autophosphorylation of GST-Shk1ΔN118 was only slightly increased for protein isolated from cells that overexpressed Skb5 (Fig. 4A). No detectable kinase activity was observed for the GST samples. These results suggest that Skb5 stimulates Shk1 catalytic activity in vivo and that it might do so without substantially affecting Shk1 autophosphorylation.

We next asked whether Skb5 directly stimulates Shk1 catalytic function by determining whether recombinant Skb5 pro-

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2 S. M., unpublished results.

3 P. Yang, R. Pimental, H. Lai, and S. Marcus, manuscript in preparation.

4 P. Yang and S. Marcus, unpublished results.

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### Table I

**Two-hybrid interactions between Skb5 and Shk1 proteins**

| LBD fusions | GAD fusions | Bry2 |
|-------------|-------------|------|
| Shk1        | 1676.70     | 873.50 |
| Shk1(308–658) | 0.13       | 0.13  |
| Shk1(1–380) | 285.40      | 1747.20 |
| Ras1        | 0.26        | 0.29  |

Values indicate the β-galactosidase activity detected between pairs of LBD and GAD fusion proteins using the quantitative liquid β-galactosidase assay (see "Experimental Procedures"). The SH3 domain of Skb5 comprises amino acids 87–136. The regulatory domain of Shk1 comprises amino acids 1–380, while the catalytic domain consists of amino acids 381–658. Values are expressed as Miller units and represent the average activity detected for at least two independent transformants.
tein purified from bacterial cells directly stimulates the catalytic function of bacterially expressed Shk1 protein. Skb5 was purified in recombinant form as a GST fusion protein (GST-Skb5), while Shk1 was purified as a polyhistidine-tagged protein (His6-Shk1) (Fig. 4B). GST-Skb5 and His6-Shk1, as well as the control proteins GST and His6-Ha-Ras, were mixed together in various combinations and assayed for kinase activity. Strikingly, we observed that GST-Skb5 strongly stimulated the ability of His6-Shk1 to phosphorylate MBP, but without stimulating Shk1 autophosphorylation (Fig. 4C). This result demonstrates that Skb5 directly stimulates Shk1 catalytic function in vitro. The observed decrease in His6-Shk1 autophosphorylation indicates that Skb5 might be involved in the regulation of Shk1 activity in vivo. Therefore, these results provide evidence for a direct role of Skb5 in the activation of Shk1, which could contribute to understanding the molecular mechanisms underlying Shk1 function in cellular processes.
Direct Activation of Shk1 by the SH3 Domain Protein, Skb5

In summary, we have described the genetic and molecular characterization of a novel SH3 domain protein, Skb5, that positively regulates the function of the fission yeast PAK Shk1. Skb5 clearly plays only an auxiliary role in regulating the overall function of Shk1. While the shk1Δ mutation is lethal to *S. pombe* cells, skb5Δ cells lack any discernible phenotypic defects under normal growth conditions. Even under conditions of hypertonic stress, skb5Δ mutants, while spheroidal in shape, are only modestly inhibited for growth. It is possible that Skb5 plays a more significant cellular role under environmental conditions that we have not tested or that Skb5 is functionally redundant with another protein(s) (e.g., another Skb5-related protein). Alternatively, it is conceivable that the proper regulation of cell morphology under conditions of hypertonic stress does not represent an essential function of Shk1. Although its cellular role, as presently defined, is potentially modest, the identification and characterization of Skb5 is significant, because it represents what is to our knowledge the first example of an SH3 domain protein capable of directly stimulating the catalytic activity of a purified PAK.

Previous studies have demonstrated interactions between mammalian PAKs and SH3 domain proteins, specifically, the SH2/SH3 adaptor protein Nck (29) and the Cdc42/Rac guanine nucleotide exchange factor proteins α- and β-PIX (30, 31). Nck and PIX proteins are thought to function in recruiting PAKs to Cdc42 and/or Rac complexes in the cell, where the PAKs are subsequently activated by the p21 G proteins (29, 30). A mutated form of PIX lacking its guanine nucleotide exchange factor domain was capable of stimulating the kinase activity of a PAK immune complex in *vitro*, suggesting that PIX also has Cdc42/Rac-independent PAK stimulatory function (32). However, since these experiments used PAK immune complexes, they did not provide an indication of whether PIX function is sufficient to directly stimulate PAK catalytic function, as other proteins in the immune complex may have been required for this activity. The founding member of the PAK family, the budding yeast Ste20 kinase (7, 33), has also been shown to form complexes with the SH3 domain protein Bem1 (34), although the functional nature of this interaction has not yet been defined. Based on these previously described findings and those presented in this report, it is reasonable to speculate that functional regulation by SH3 domain proteins represents a broadly conserved primordial feature of eukaryotic PAKs that has been modified and reiterated through the course of evolution to provide multiple mechanisms for PAK activation, thereby allowing these kinases to regulate numerous and diverse cellular processes. Moreover, our results raise the exciting possibility that PAKs in higher organisms might, in some cases, be directly activated by SH3 domain proteins. This potential alternative mechanism for PAK activation may ultimately help to explain how PAKs are functionally regulated, such that they can be targeted to perform the diverse cellular functions to which they have been linked in eukaryotic organisms.

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