The Identification of cDNAs That Affect the Mitosis-to-Interphase Transition in Schizosaccharomyces pombe, Including sbp1, Which Encodes a spi1p-GTP-Binding Protein

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ABSTRACT

Perturbations of the spi1p GTPase system in fission yeast, caused by mutation or overexpression of several regulatory proteins, result in a unique terminal phenotype that includes condensed chromosomes, a wide medial septum, and a fragmented nuclear envelope. To identify potential regulators or targets of the spi1p GTPase system, a screen for cDNAs whose overexpression results in this terminal phenotype was conducted, and seven clones that represent three genes, named med1, med2, and med3 (mitotic exit defect), were identified. Their genetic interaction with the spi1p GTPase system was established by showing that the spi1p guanine nucleotide exchange factor mutant pim1-d1 was hypersensitive to their overexpression. med1 encodes a homologue of the human Ran-binding protein, RanBP1, and has been renamed sbp1 (spi1p-binding protein). sbp1 binds to spi1p-GTP and costimulates the GTPase-activating protein (GAP)-catalyzed GTPase activity. Cells in which sbp1 is depleted or overproduced phenocopy cells in which the balance between spi1p-GTP and spi1p-GDP is perturbed by other means. Therefore, sbp1 mediates and/or regulates the essential functions of the spi1p GTPase system. med2 and med3 encode novel fission yeast proteins that, based on our phenotypic analyses, are likely to identify additional regulators or effectors of the spi1p GTPase system.

GTPases are molecular switches that adopt different conformations and interact with different regulatory and effector proteins depending upon whether they are bound to GTP or to GDP. Ran-GTPases lack consensus membrane attachment sequences, are predominantly nuclear localized (Bischoff and Ponstingl 1991b), and are very abundant, with an estimated 10^5 molecules per HeLa cell (Bischoff and Ponstingl 1991b). Structural and functional homologs have been identified in eukaryotes, ranging from yeast to man, of the three core components of the Ran GTPase system: the GTPase; the guanine nucleotide exchange factor (GEF), which stimulates the conversion of Ran from the GDP- to GTP-bound state (Bischoff and Ponstingl 1991a); and the GTPase-activating protein (GAP), which stimulates GTP hydrolysis and thus the conversion of Ran from the GTP- to GDP-bound state (Bischoff et al. 1994). Studies in vivo and in vitro have demonstrated that Ran and its regulators influence a variety of nuclear events: cell cycle progression; condensation, decondensation, and transmission of chromosomes; and nucleocytoplasmic transport of protein and RNA (Sazer 1996). The terminal phenotypes that result from perturbation of this system vary among experimental systems (Sazer 1996).

The primary role(s) of the Ran-GTPase system has not been elucidated. Within a particular organism, Ran may have one primary function that indirectly affects a variety of cellular processes. Alternatively, Ran may have multiple independent functions that are mediated by its interaction with a variety of downstream effectors. Consistent with the latter possibility is the discovery of multiple Ran-binding proteins (RanBPs) in both budding yeast and mammalian cells (Beddow et al. 1995; Dingwall et al. 1995; Hartmann and Gorlich 1995; Lounsbury et al. 1994). Like effectors of other GTPases, RanBPs and isolated Ran-binding domains (RanBDs) bind specifically to the GTP-bound form of the GTPase (Beddow et al. 1995; Coutavas et al. 1993; Lounsbury et al. 1994; Schlenstedt et al. 1995). Several RanBPs and isolated RanBDs have also been shown to costimulate the GTPase activity of Ran in vitro in the presence of the GAP, but to stabilize the GTP-bound form of the GTPase in the absence of GAP (Beddow et al. 1995; Bischoff et al. 1995; Lounsbury et al. 1994; Schlenstedt et al. 1995). In mammalian cells, RanBP1 is cyto-
plasmic (Lounsbury et al. 1994; Schlenstedt et al. 1995), whereas RanBP2/Nup358 is localized to the cytoplasmic fibrils emanating from the nuclear pore complex (Wu et al. 1995; Yokoyama et al. 1995). Their different intracellular localizations suggest that these binding proteins are likely to mediate different Ran functions, although both are required for nuclear import (Chi et al. 1996; Hayashi et al. 1995; Lounsbury et al. 1996; Schlenstedt et al. 1995; Yokoyama et al. 1995).

Additional evidence that the Ran-GTPase may have several independent functions is the recent discovery in mammalian cells that there are two populations of the Ran-GAP: one that is soluble and cytoplasmic, as well as another insoluble, post-translationally modified form that is associated with the nuclear envelope (Mahajan et al. 1996; Matunis et al. 1996). One clearly established role for the Ran-GTPase and its associated proteins is the nuclear import of proteins; however, there is mounting evidence of additional roles for this GTPase and its associated proteins: (1) a dominant lethal mutant of human Ran inhibits cell growth but not nuclear protein import (Carey et al. 1996), (2) depletion of Xenopus RCC1 or addition of a dominant mutant of Ran inhibits nuclear envelope growth in vitro but not nuclear protein import (Dasso et al. 1994; Kornbluth et al. 1994), (3) the post-translationally modified form of Ran-GAP associates with mitotic spindles at metaphase (Matunis et al. 1996), (4) a dominant mutant of Xenopus Ran affects cell cycle progression in vitro in the absence of nuclei (Kornbluth et al. 1994), and (5) protein import is normal in budding yeast in which the RanBP YRB2 is deleted (Noguchi et al. 1997; Taura et al. 1997).

spilp, the S. pombe member of the Ran-GTPase family, is 82% identical and 90% similar to human Ran (Matsumoto and Beach 1991). pim1p, the spilp GEF, is a structural (Matsumoto and Beach 1991) and functional (H. Seino, X. He, R. Bischoff, N. Ong, H. Ponstingl, T. Nishimoto and S. Sazer, unpublished results) homolog of the mammalian protein RCC1 that catalyzes the exchange of GDP-bound to spilp for GTP. Inactivation of pim1p by a temperature-sensitive mutation results in a growth defect at the restrictive temperature. Several of these characteristics, including cell cycle arrest with condensed postmitotic chromosomes and a wide medial septum, are also seen in germinated spores in which the rna1 gene has been disrupted. Taken together, these experiments suggest that increasing or decreasing the ratio between spilp-GTP and spilp-GDP by means other than pim1p inactivation causes the same lethal effect, indicating that maintenance of the proper balance between the two forms of the GTPase is necessary for cell viability (Matynia et al. 1996).

Cells overproducing rna1p die with a terminal phenotype indistinguishable from that of pim1-d1ts cells incubated at the restrictive temperature. Several of these characteristics, including cell cycle arrest with condensed postmitotic chromosomes and a wide medial septum, are also seen in germinated spores in which the rna1 gene has been disrupted. Taken together, these experiments suggest that increasing or decreasing the ratio between spilp-GTP and spilp-GDP by means other than pim1p inactivation causes the same lethal effect, indicating that maintenance of the proper balance between the two forms of the GTPase is necessary for cell viability (Matynia et al. 1996).

The mechanism by which an imbalance between spilp-GTP and spilp-GDP might cause a failure of the mitosisto-interphase transition in fission yeast remains to be determined. To elucidate this pathway, we devised a strategy to identify both regulators and effectors of this GTPase system. A cDNA library screen was carried out to isolate clones that are toxic when expressed from the regulable nmt1 promoter (Maundrell et al. 1990) and interfere with the ability of cells to properly complete mitosis. Several classes of cDNAs might be identified using this approach: (1) regulators of spilp that directly or indirectly perturb the normal balance between spilp-GDP and spilp-GTP, (2) downstream components of the spilp-GTPase pathway that do not directly affect the spilp-GTPase but link its functioning to other cellular processes, and (3) components of parallel pathways that regulate the mitosis to interphase transition independently of the spilp system.

MATERIALS AND METHODS

Yeast strains and cell culture: Schizosaccharomyces pombe strains used were a haploid strain (h-leu1-32 ura4-D18 ade6-m216), a
diploid strain (h/ h ^ {1} leu1-32 / leu1-32 ; ura4-D18 / ura4-D18; ade6-m210 / ade6-m216), and a mutant haploid strain pm1-1d1 (h/ h ^ {1} leu1-32 ; ura4-D18; pm1-d15; Sazer and Nurse 1994), all of which are derived from strain 972 (Leupold 1970). Cell culture conditions, media composition, and genetic analyses have been described previously (Moreno et al. 1991).

**nt1 promoter regulation:** Gene expression under the control of the nt1 promoter (Mandrill et al. 1990) in pREP3X or pREP41X (Forsburg 1993) was repressed by the inclusion of 5 μg/ml thiamine in the Edinburgh Minimal Media (EMM; Moreno et al. 1991). To derepress expression, cells were washed three times with thiamine-free EMM and grown in fresh thiamine-free EMM.

cDNA library screen and DNA manipulations: An S. pombe cDNA library (a gift from Bruce Edgar and Chris Norbury) in the pREP3X vector (Forsburg 1993) was transformed into wild-type cells, and transformants were grown on EMM plates with appropriate supplements and thiamine to repress cDNA expression. After 5 days of incubation at 32°C, the 60,000 transformants were replica plated onto thiamine-free plates to derepress cDNA expression. Phloxine B (Sigma, St. Louis, MO), which specifically accumulates in dead cells, was also included in the medium at a final concentration of 5 μg/ml. After a 24-hr incubation at 32°C, the 4500 dark red colonies that were preferentially stained by phloxine B were examined by light microscopy. The 150 transformants that were enriched in septated cells were picked from the replica plates plus thiamine, inoculated into liquid EMM media with supplements plus thiamine, and grown at 32°C for 2 days. These precultures were washed and transferred to thiamine-free EMM medium with supplements at an initial concentration of ~10^6 cells/ml. After 13 hr at 32°C, the cells were fixed in 70% ethanol and stained with 4',6-diamidino-2-phenylindole (DAPI; Moreno et al. 1991) and examined under the microscope to identify transformants enriched in cells with condensed chromosomes.

Plasmids from the transformants were recovered by standard procedures (Moreno et al. 1991) and amplified in Escherichia coli. The cDNA inserts and their restriction fragments were subcloned into Bluescript KS- (Strategene, La Jolla, CA) and sequenced using Sequenase version 2.0 (United States Biochemical, Cleveland, OH). pREP41X-med1, pREP41X-med2, and pREP41X-med3 were constructed by subcloning the Xho/I/BamHI fragments from the pREP3X cDNA library into pREP41X to achieve a lower level of overexpression (Forsburg 1993).

**Two-hybrid screen:** Two-hybrid screening using human Ran as bait was performed as described previously (Yokoyama et al. 1995). The S. pombe Matchmaker cDNA library was purchased from Clontech (Palo Alto, CA). Transformants were selected on 25 mm 3-amino triazole (3-AT) and transferred to fresh 3-AT plates to confirm drug resistance. Plasmids were isolated from the 3-AT-resistant cells and sequenced using standard protocols.

cosmid hybridization and mapping: The S. pombe cosmid library filter 60-0, provided by Reference Library Database (RLDB) Max Planck Institute for Molecular Genetics, was probed with the Xho/I/BamHI fragment of the sbp1 cDNA insert. The coordinates of the positive spots were used to map the sbp1 gene in the S. pombe genome (Hoheisel et al. 1993; Lehrach et al. 1990).

**Construction of the sbp1 deletion strain sbp1-Δl:** A 4.0-kb SacI/SalI genomic DNA fragment containing the sbp1 gene was subcloned from cosmid ICRF600D0115D (provided by Resource Center/Primary Database of German Human Genome Project, Max Planck Institute for Molecular Genetics) into Bluescript KS-, and the 1.4-kb EcoRV/PstI fragment, which encompasses the N-terminal two-thirds of the open reading frame, was replaced with the ura4+ gene. The sbp1 deletion strain (h/ h ^ {1} sbp1:ura4+/sbp1 leu1-32; leu1-32; ura4-D18/ura4-D18; ade6-m210/ade6-m216) was generated by transforming diploid cells (h/ h ^ {1} leu1-32; leu1-32 ura4-D18/ura4-D18 ade6-m210/ade6-m216) with the 4.4-Red2/SalI deletion fragment, and it was confirmed by Southern blot analysis using standard techniques.

**Fluorescence microscopy:** Cells fixed with ethanol were stained with DAPI (Moreno et al. 1991). Living cells were simultaneously stained with Hoechst 33342 and 3,3’-dihexyloxacarbocyanine (DiOC 2) using a previously described procedure (Demeter et al. 1995). Stained cells were examined using an Axioskop fluorescence microscope (Carl Zeiss, Thornwood, NY).

**FACS analysis:** Cell samples were fixed with ethanol, treated with RNase, stained with propidium iodide, and their DNA content was measured by flow cytometry (Sazer and Shewd 1990). To determine the DNA content of each half of the septated cells, cells were briefly digested with Noxyme (0.7 mg/ml) and Zymolyase (0.2 mg/ml) for 15 min at 37°C immediately before analysis.

**sbp1-Δl phenotype characterization:** The sbp1-Δl heterozygous diploid cells were transformed with pREP41X-sbp1, and the transformants were sporulated. Haploid sbp1-Δl cells containing the pREP41X-sbp1 were recovered by germinating the spores on EMM plates lacking uracil to select for the sbp1 disruption, lacking leucine to select for the plasmid, and lacking thiamine to derepress transcription of sbp1. sbp1 cDNA expression from pREP41X was sufficient to rescue the sbp1-Δl lethality. To monitor the phenotype of cells depleted of sbp1p, thiamine was added to repress sbp1 cDNA expression, and cells were periodically fixed in ethanol and stained with DAPI (Moreno et al. 1991) to determine the percentage of septated cells, or living cells were stained with DIOc 2 and Hoechst 33342 to visualize the nuclear envelope and DNA (Demeter et al. 1995).

**GTPase assay:** Ran-GTP was incubated at 25°C for 30 min with fourfold excess of [γ-32P]GTP (30 Ci/mmole) in 20 mm HepesNaOH (pH 7.5), 20 mM EDTA, and 2 mm DTT, then MgCl 2 was added to a final concentration of 50 mm. The buffer was changed to 20 mm HepesNaOH (pH 7.5), 5 mm MgCl 2, 0.05% hydrolyzed gelatin, and 2 mm DTT (reaction buffer) on a Nap 5 column (Pharmacia, Piscataway, NJ). The resulting [γ-32P]GTP-bound Ran was diluted to 3.3 μM with reaction buffer, and 30-μl aliquots were mixed with various concentrations of purified glutathione S-transferase (GST) or GST-sbp1p. The mixture was preincubated at 25°C for 5 min, then 10 μl of 5 nM S. pombe RNA1 was added to start GTPase reaction. After a 3-min incubation, the reaction was stopped by addition of ice-cold stop buffer containing 20 mm Tris/HCl (pH 7.5), 25 mM MgCl 2, and 100 mM NaCl, and was subsequently filtered through nitrocellulose (0.45 mm, BA85; Schleicher & Schuell, Keene, NH). The filters were dried, and the radioactivity remaining with the proteins on the filter was counted in a liquid scintillation counter.

**sbp1p-GTase binding assay:** E. coli-produced GST-sbp1p or GST (1 μg) was separated by 10% SDS-PAGE and either transferred to polyvinylidifluoride membrane filters or stained with Coomassie brilliant blue. The filters were incubated with either 35S-labeled Ran-GTP-γS or 35S-labeled Ran-GDPγS, as described previously (Noguchi et al. 1996; Yokoyama et al. 1995). Protein of crude extracts (66 μg) prepared from wild-type fission yeast or an equal amount of extract incubated with beads bound with either E. coli-produced GST-sbp1p fusion protein or glutathione S-transferase was electrophoresed in SDS-PAGE and immunoblotted with anti-sbp1p antiserum (Matynia et al. 1996).

**Amino acid sequence analysis:** A BLAST (Altschul et al. 1990) search in March 1997 for Schizosaccharomyces pombe cDNAs that affect Mito1
Biotechnology Information's BLAST WWW Server and post-processed by the Human Genome Center, Baylor College of Medicine, using Beauty (Worley et al. 1995) identified 13 proteins that show similarity with the prototypical RanBP, mouse RanBP1 (Coutavas et al. 1993). The predicted protein product of sbp1 was aligned with these 13 proteins using Clustal W version 1.6 (Thompson et al. 1994).

RESULTS

Screen for cDNAs that cause a mitotic exit defect when expressed in wild-type cells: Overproduction of rna1p, the GAP for the spi1p GTPase, results in a terminal phenotype similar to that of the pim1-d1ts mutant (Matynia et al. 1996). Based on this observation, to identify new genes involved in the mitotic exit pathway, a cDNA library expression screen (see materials and methods for details) was initiated to isolate cDNAs that produce effects similar to those of rna1 overexpression. The primary screen was for genes that showed transcription-dependent toxicity, the secondary screen was for overexpression strains that accumulated septated cells, and the tertiary screen was for septated overexpression strains that had condensed chromatin.

Overexpression of one cDNA resulted in cells with multiple septa but without condensed chromatin. This cDNA, renamed sid3, encodes the fission yeast homolog of the budding yeast GTPase Tem1p (Shirayama et al. 1994) and is the subject of another manuscript (M.K. Subramanian, D. McCollum, K.C.Y. Wong, X. He, S. Sazer, L. Chang and K. Gould, unpublished results). sid3 was independently identified as spg1, a high copy suppressor of the septation mutant cdc7 (Schmidt et al. 1997).

In three transformants, cells accumulated with condensed metaphase chromosomes and short mitotic spindles after 13 hr of cDNA transcription. Upon longer transcriptional induction, septation occurred in the absence of nuclear division and spindle elongation, resulting in one anucleate daughter cell and one daughter cell that contained a nucleus with a short mitotic spindle. These three genes, which include components of the spindle checkpoint system, are the subject of another manuscript (He et al. 1997).

The seven other septated strains with hypercondensed chromosomes were binucleate, indicating that they had undergone an apparently normal mitosis but were blocked at the mitosis-interphase transition. The cDNA-containing plasmid from each of these seven strains was isolated and amplified in E. coli. The purified plasmids were transformed back into fission yeast cells to confirm the lethality and terminal phenotype resulting from their overproduction. This collection of cDNAs was temporarily named med (mitotic exit defect). Partial DNA sequence analysis of these seven cDNA clones revealed that they represented three different genes: med1, med2, or med3 were cloned three times each, and med3 was cloned once.

Cells overexpressing med genes arrest with terminal phenotypes similar to rna1 overexpressing cells and pim1ts mutants: The three strains overexpressing med1, med2, or med3 were grown in the absence of thiamine for 13 hr to derepress cDNA transcription, and these living cells were then stained with Hoechst 33342 to visualize the DNA and with DiOC6 to visualize the nuclear envelope, as described previously (Demeter et al. 1995; Figure 1). All three cDNAs caused cells to arrest after mitosis as binucleated cells with highly condensed chromosomes and a medial septum (Figure 1), the width of which increased with time of expression (data not shown).

In wild-type cells (Demeter et al. 1995) and vector-transformed wild-type cells (Figure 1A), the circular

![Figure 1.—med overexpression arrests cells with a medial septum, condensed chromosomes, and an abnormal nuclear envelope. Live cells were double stained with Hoechst 33342 to visualize the DNA and DiOC6 to visualize the nuclear envelope. Micrographs of (A) wild-type vector-transformed cells at different stages of the cell cycle, as well as cells arrested by (B) med1, (C) med2, or (D) med3 overproduction 20 hr after transcriptional derepression. med3-overexpressing cells have a unique accumulation of cytoplasmic DiOC6 staining material (arrows, panels 1–3).](https://example.com/figure1.png)
nuclear envelope surrounds the DNA at all stages of the cell cycle. The nuclear envelope surrounding the chromatin in cells in which transcription of med1, med2, or med3 genes was repressed by thiamine was also clearly delineated (data not shown). When transcription was derepressed for 13 hr, however, the nuclear envelope could no longer be detected surrounding the hypercondensed chromatin in 18–20% of the cells (Figure 1, B–D), and after 18 hr, this percentage increased to 50%. The percentage of pim1ts-like cells increased to 50% when transcription of med1, med2, or med3 was derepressed for 18 hr. Expression of med3 also caused an accumulation of DiOC₆ staining material in the cytoplasm (Figure 1D, arrow).

med cDNA overproduction also caused cells to arrest with an unreplicated genome. An asynchronous population of wild-type fission yeast has a 2C DNA content because daughter cells in G1, each with a 1C DNA content, remain attached to one another after mitosis and because DNA replication is virtually completed by the time of cell separation. The binucleated, med-arrested cells also displayed a 2C DNA content, indicating that the genome of each daughter cell was unreplicated (Figure 2, undigested). Partial digestion of the septum with a mixture of Novozyme and Zymolyase resulted in an accumulation of mononucleated cells with a 1C DNA content, confirming that the genomes of the septated cells are unreplicated in the med1, med2, and med3 overexpression samples (Figure 2, digested).

**pim1ts is hypersensitive to overexpression of med1, med2, or med3:** Expression of rna1, which encodes a known regulator of the spi₁p GTPase system, is synthetically lethal with the pim1-d₁ts mutation (Matynia et al. 1996). To determine whether the med genes are likely to encode additional components of this system, we compared the sensitivity of wild-type and pim1-d₁ts mutant cells to overexpression of med1, med2, or med3. At the pim1-d₁ts permissive temperature of 25°C, wild-type

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**Figure 2**—med overexpression arrests cells with an unreplicated genome. FACS analysis of the DNA content of septated, binucleated med1, med2, or med3 overexpressing cells (undigested) is 2C (1C per nucleus). Separation of the two daughter cells by treatment with a mixture of Novozyme and Zymolyase (digested) results in the accumulation of mononucleated cells with a 1C DNA content. Nitrogen-starved, wild-type haploid cells were used as a control (ctrl) to determine the position of 1C and 2C DNA peaks (arrows).
or mutant cells transformed with med1, med2, med3, or the vector control grew equally well when transcription from the intermediate version of the nmt1 promoter in plasmid pREP41X (Forsburg 1993) was repressed (data not shown). When transcription was derepressed at 25°, however, the pim1-d1ts mutant cells showed growth inhibition caused by expression of med1 but not the vector when compared to wild-type cells (Figure 3). Wild-type and pim1-d1ts cells grew equally well at 25° when either med2 or med3 were expressed (Figure 3); however, the growth inhibition of pim1-d1ts with med2 or med3 overexpression was seen when cells were incubated at the semipermissive temperature of 33° (Figure 3). Expression of all three med genes was more toxic to pim1-d1ts mutant cells than to wild-type cells, but overexpression of med1 was more toxic than that of either med2 or med3.

**med1 encodes the fission yeast structural homolog of RanBP1:** Three med1 cDNA–containing clones, which resulted in identical phenotypes when overexpressed, were identified in the screen. DNA sequencing revealed that two of the clones, med1-1 and med1-2, contained the same 0.7-kb full-length cDNA (GenBank accession number D76431) encoding a predicted protein product of 215 amino acids (Figure 4A). A third cDNA, med1-3, contained the same open reading frame interrupted by a putative intron of 172 bp that was flanked by consensus 3' and 5' splice sites and had stop codons in all three reading frames.

The med1 gene was cloned by colony hybridization, and from the DNA sequence (GenBank accession number D86381), two introns were identified (Figure 4A) based on the presence of consensus splice sites and comparison with the sequences of the med1-1 and med1-2 cDNAs. The first intron was 172 bp and was retained in med1-3 cDNA, which was presumably derived from an RNA splicing intermediate. The second intron was 86 bp and was not retained in any of the cDNA clones.

The med1 cDNA insert was used as a probe to screen an ordered fission yeast cosmid library (Lehrach et al. 1990) to determine the chromosomal location of the med1 gene. The five positive cosmids ICRF c60E087D, ICRF c60D0115D, ICRF c60C048D, ICRF c60B0335D, and ICRF c60A052D mapped to chromosome II between rad11 and mei3.

The predicted med1p amino acid sequence is 54% identical and 67% similar to human RanBP1, and 57% identical and 70% similar to the budding yeast RanBP1 homolog YRB1 (Figure 4B). The highest region of similarity lies in the RanBD (Figure 4A, underlined), which is highly conserved among a large family of proteins (Figure 4B). Several of these proteins have been shown to bind to the Ran family of GTPases (Figure 4B). Based on these sequence similarities, we have renamed med1 according to the mammalian nomenclature. It will subsequently be referred to as sbp1 (splicing binding protein 1).

**Fission yeast sbp1 encodes a functional homolog of RanBP1:** The first indication that sbp1p was a functional homolog of RanBP1 was its identification in a two-hybrid screen for fission yeast proteins that bind to human Ran. The Y190 strain, which harbors pAS-Ran and has previously been used for two-hybrid screening of a human cDNA library (Yokoyama et al. 1995), was transformed with an S. pombe cDNA library. From ~10⁶ transformants, 56 colonies survived selection on 25 mm 3-AT, and 31 of these retained this resistance when transferred to fresh 3-AT plates. cDNA-containing plasmids were isolated from 18 of these colonies and sequenced. Six were found to encode a protein identical to sbp1p. When assayed in quadruplicate for β-galactosidase activity, a representative sbp1-containing clone (the transformant bearing pFTBP112 and subsequently renamed Y190Ran) had 37.8 ± 8.7 units/mg protein of the transformant bearing only the vector pGAD-DH. These results are consistent with there being a direct protein–protein interaction between sbp1p and human Ran. To test whether this interaction was specific for the GTP-bound form of the GTPase, as has been shown for other RanBD-containing proteins (Beddoz et al. 1995; Cout avas et al. 1993; Louns bury et al. 1994; Schlen st et al. 1995), GST-sbp1p immobilized on filters bound to Ran-[β-35S] GTP but not to Ran-[γ-35S] GDP (Noguchi et al. 1996; Yokoyama et al. 1995), whereas the GST control bound to neither (Figure 5A).

The ability of sbp1p to bind to sp1p, the fission yeast Ran homolog, was tested by incubating S. pombe cell extracts with either GST-sbp1p or GST immobilized on beads. The proteins that bound to the beads were separated by electrophoresis, transferred to a mem-

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**Figure 3.—pim1-d1ts cells are hypersensitive to sublethal med overexpression.** Wild-type cells or pim1-d1ts cells, transformed with pREP41X vector, pREP41X-med1, pREP41X-med2, or pREP41X-med3 plasmids, were grown to midlog phase in thiamine-free liquid media to derepress transcription. Fivefold serial dilutions of equal numbers of cells were spotted on thiamine-free minimal media plates and incubated at either 25°, the permissive temperature, or 33°, the semipermissive temperature of pim1-d1ts.
Figure 4.—sbp1p is a structural homolog of the evolutionarily conserved RanBP family of proteins. (A) Gene structure of S. pombe sbp1. DNA sequence of sbp1 and conceptual translation of its protein product. Nucleotides are numbered from the A of the initiating methionine codon as +1. Nucleotides corresponding to the two introns are in small italics. Amino acids are numbered in italics. The domain with highest similarity to other RanBPs is underlined. (B) Alignment of sbp1p to other proteins containing RanBDs. Multiple alignments were performed with proteins containing significant similarity to mouse Ran BP1. Dark shading indicates 60% or greater amino acid identity, and light shading indicates 60% or greater amino acid similarity. GenBank accession numbers are as follows: Sp sbp1, (S. pombe sbp1) D86381; MmRanBP1, (Mus musculus RanBP1) X56045; HsRanBP1, (Homo sapiens RanBP1) X83617; ScYRB1, (S. cerevisiae YRB1) Z33503; HsRanBP2, (H. sapiens RanBP2) D42063; XlRanBP1, (Xenopus laevis RanBP1) Y09128; MmRanBP2, (M. musculus RanBP2) X87337; Btspvar2, (Bos taurus spliced variant with Ran-BD and cyclophilin domain, domain 2) L41691; Btspvar1, (B. taurus spliced variant with Ran-BD and cyclophilin domain, domain 1) L41691; AtRanBP1, (Arabidopsis thaliana RanBP1) U62742; CEF59A2, (Caenorhabditis elegans clone F59A2, domain 1) Z34801; CEF59A2-2, (C. elegans clone F59A2, domain 2) Z34801; Sphba1, (S. pombe hba1) U38783; ScYRB2, (S. cerevisiae YRB2) Z38060; ScNUP2, (S. cerevisiae NUP2) X69964; HHCP045, (H. sapiens expressed sequence tag HHCP045) M79174.
Figure 5.—sbp1p is a functional homolog of the evolutionarily conserved RanBP family of proteins. (A) sbp1p binds specifically to Ran-GTP. E. coli-produced GST-sbp1p (lane 1) or GST (lane 2) was separated electrophoretically and either transferred to filters (a and b) or stained with Coomassie brilliant blue (c). The filters were incubated with either 35S-labeled Ran-GTP γS (a) or 35S-labeled Ran-GDP βS (b). (B) sbp1p binds to spi1p. A crude extract prepared from wild-type fission yeast (lane 1), or an equal amount of extract incubated with beads bound with either E. coli-produced GST-sbp1p fusion protein (lane 2) or GST (lane 3) was electrophoresed, transferred to filters, and immunoblotted with anti-spi1p antiseraum. (C) sbp1p coactivates the rna1p-stimulated GTPase activity of Ran. Ran-GPT was incubated with rna1p and either GST (open circles) or GST-sbp1p (closed circles) in the indicated amounts, and the GTPase activity was monitored by determining the percent of remaining GTP bound to Ran.
brane, and the presence of spi1p was determined by immunoblotting using the previously described anti-spi1p antiserum. GST-sbp1p, but not GST alone, bound spi1p (Figure 5B).

sbp1p was also tested for its ability to stimulate the low endogenous GTPase activity of spi1p. Using a previously described in vitro assay (Bischoff et al. 1995), varying amounts of GST-sbp1p or GST were incubated with [γ-32P]GTP and the GAP rna1p. GST-sbp1p, but not GST alone, was able to costimulate the GAP-catalyzed GTPase activity of spi1p by three- to fivefold, depending on the protein concentration (Figure 5C).

sbp1 is essential for viability in fission yeast: A diploid strain in which one copy of the sbp1 (med1) open reading frame was replaced with the ura4 gene was constructed (see materials and methods), and tetrad analysis was carried out to determine if sbp1 was essential for viability. No tetrad contained more than two viable spores, and the viable spores were all ura-, indicating that these cells contained the wild-type copy of sbp1. To confirm that sbp1 and no other essential gene was disrupted in this strain, the sbp1-Δ1 diploid was transformed with the LEU2-based plasmid pREP41X-sbp1, in which the sbp1 cDNA is under control of the medium strength nmt1 promoter because expression from the full-strength promoter in pREP3X is toxic. Transformants that were leu+ were obtained, and the diploid was sporulated. All of the ura- spores that were capable of germination and colony formation were also found to be leu+, indicating that spores in which the endogenous sbp1 gene had been disrupted could grow only in the presence of a plasmid-borne copy of sbp1. The rescue was shown to be dependent on the transcription of sbp1 because ura- leu- colonies were present when the nmt1 promoter was derepressed, which led to sbp1 transcription, but not when transcription was repressed by the presence of thiamine.

The phenotype of sbp1-Δ1 cells was examined by growing these cells in liquid minimal media in the absence of thiamine to derepress transcription of the plasmid-borne copy of sbp1 and then inhibiting promoter activity by the addition of thiamine to the media. Cells were examined periodically after promoter repression to monitor the development of the terminal phenotype. The percentage of terminally arrested cells, determined by the presence of a medial septum, increased steadily between 5 and 29 hr (Figure 6A). After 24 hr, the DNA and nuclear envelope morphology was examined by staining the living cells with Hoechst 33342 and DiOC6 (Figure 6B). The cells look strikingly similar to cells harboring a temperature-sensitive mutation in pim1 (Demeter et al. 1995; Sazer and Nurse 1994), harboring a null allele of rna1 (Matynia et al. 1996), and to cells overexpressing either rna1 (Matynia et al. 1996) or sbp1 (Figure 1B): they are binucleated, have condensed chromosomes, a fragmented nuclear envelope, and a medial septum.

DISCUSSION

Based on the complex phenotype of fission yeast cells in which the balance between the GTP- and GDP-bound forms of the spi1p protein is expected to be perturbed by decreased GEF activity (Sazer and Nurse 1994) or increased GAP activity (Matynia et al. 1996), we have designed and carried out a screen for cDNAs that cause similar phenotypic consequences when overexpressed. Using a fission yeast cDNA library, the transcription of which is controlled by the thiamine regulatable nmt1 gene promoter (Maundrell 1990), we have identified three categories of cDNAs.

One category, represented by a single cDNA, causes cells to arrest with multiple septa when overexpressed (M.K. Subramanian, D. McCollum, K.C.Y. Wong, X.
Cells overexpressing the second class of cDNAs, which includes med2 and other components of the spindle checkpoint system, have some but not all characteristics of cells in which the spi1p system is perturbed (He et al. 1997). The third category of cDNAs includes seven clones that identify three different proteins sharing significant sequence similarity in a region of the protein previously termed the RanBD (Beddow et al. 1995) or RanBP1 motif (Hartmann and Gorlich 1995), with a large group of proteins from several eukaryotic organisms, some but not all of which have been shown to bind specifically to the GTPase in its GTP-bound state (Figure 4B).

The functional similarity between the fission yeast, budding yeast, and mammalian BP1 proteins was established by demonstrating that, like RanBP1 (Bischoff et al. 1995) and Yrb1p (Schlenstedt et al. 1995), sbp1p specifically binds to the GTPase in its GTP-bound form and acts as a costimulator of the spi1p-GTPase activity in the presence of the spi1p-GAP protein rna1p. We propose that overproduction of sbp1p, the costimulator of the spi1p-GTPase, causes a perturbation in the ratio of Ran-GTP to Ran-GDP similar to that caused by inactivation of the GEF or overproduction of the GAP. In all three of these conditions, cells die with identical terminal phenotypes.

While this work was in progress, an Arabidopsis thaliana cDNA encoding a structural homolog of sbp1p was cloned on the basis of the abnormal phenotype it produced when overexpressed in wild-type fission yeast cells (Xia et al. 1996). It has also been found that the Saccharomyces cerevisia and mammalian homologs of sbp1p, Yrb1p, and RanBP1, respectively, are also toxic when overproduced in cells carrying temperature-sensitive mutations in their respective Ran-GEFs (Hayashi et al. 1995), and that YRB1 overexpression increases the frequency of chromosome instability in wild-type cells (Ouspenski et al. 1995). These results are consistent with our finding that overexpression of sbp1p causes lethality when overexpressed in fission yeast. This overexpression, however, causes a specific cell cycle arrest after mitosis that is phenotypically identical to that seen when the balance between the GTP- and GDP-bound pools of spi1p is perturbed by other means (Demet et al. 1995; Matynia et al. 1996; Sazer and Nurse 1994).

There are multiple RanBPs and RanBD-containing proteins in several organisms. For example, mammalian cells have Ran-GTP-binding proteins, RanBP1 and RanBP2, with similar biochemical properties (Beddow et al. 1995; Bischoff et al. 1995) but different intracellular localizations: RanBP1 is primarily cytoplasmic (Lounsbury et al. 1994; Schlenstedt et al. 1995), whereas RanBP2 is localized to the cytoplasmic filaments of the nuclear pore complex (Wu et al. 1995; Yokoyama et al. 1995). This suggests the possibility that each may independently mediate a different function of the GTPase system. We have shown that overproduction or depletion of sbp1p in fission yeast phenocopies perturbation of the GTPase system through either the spi1p GEF or the rna1p GAP (Demet et al. 1995; Matynia et al. 1996; Sazer and Nurse 1994). Our characterization of sbp1p function in vivo indicates that it affects all of the essential functions of the spi1p GTPase system. This suggests that sbp1p regulates or in-
fluences the nucleotide-bound state of the spi1 GTPase and/or that it mediates the essential functions of the GTPase system.

Other RanBD-containing fission yeast proteins may mediate a subset of spi1 GTPase functions. The gene that encodes hba1p, the only other known RanBD-containing fission yeast protein, was cloned by virtue of its ability to confer resistance to Brefeldin A when overexpressed in wild-type cells (Turi et al. 1996). hba1p is the first RanBD-containing protein shown to be nuclear localized, setting it apart from RanBP1, RanBP2, and Yrb1p, all of which are cytoplasmic. hba1p is 25% identical and 64% similar to S. cerevisiae Yrb2b, which was recently identified as a RanBD-containing protein based on its DNA sequence (Taura et al. 1997) and by virtue of its interaction with Ran in a two-hybrid screen (Noguchi et al. 1997). The phenotypes of null mutants of hba1p and yrb2 differ from those of other known components of the fission yeast or budding yeast GTPase systems, indicating that these proteins may influence some but not all of their downstream targets.

In our initial characterization, we were unable to demonstrate a significant physical interaction between hba1p and spi1p using either two-hybrid or spi1p-GTP overlay assays (Turi et al. 1996). Using more sensitive assays, however, Yrb2p has been shown to have a weak interaction with the spi1 homolog, Gsp1p (Noguchi et al. 1997; T. Taura and P. Silver, personal communication), suggesting that the same may be found for hba1p as well. dis3p, a previously cloned and characterized fission yeast protein implicated in mitotic progression, also binds to spi1 and Ran, but lacks the consensus RanBD. The specificity of binding by dis3p also differs from the previously characterized RanBD proteins in that it binds to the nucleotide-free form of the GTPase (Noguchi et al. 1996).

In summary, from a cDNA overproduction screen designed to identify regulators and/or downstream targets of the spi1 GTPase system, we have isolated three genes, med1, med2, and med3, which cause a cell cycle arrest after mitosis identical to that resulting from an imbalance between spi1-GTP and spi1-GDP. med1, renamed spb1 because of its homology to the mammalian Ran-binding protein RanBP1, encodes a protein that binds specifically to the GTP-bound form of the GTPase and costimulates the rna1p catalyzed GTPase activity of spi1. It remains to be determined if med2p and med3p are regulators or effectors of the spi1 GTPase system, or if they independently influence the mitosisto-interphase transition.

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LITERATURE CITED


Hoheisel, J. D., E. Maier, R. Mott, L. McCarthy, A. V. Grigoriev et al., 1993 High resolution cosmids and P1 maps spanning the 14 Mb genome of the fission yeast S. pombe. Cell 73: 109–120.


Wu, J., M. J. Matunis, D. Kraemer, G. Blobel and E. Cout avas, 1995 Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. J. Biol. Chem. 270: 14209–14213.


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