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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Manuscript received June 10, 1997
Accepted for publication November 6, 1997

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^{ts} was hypersensitive to their overexpression. *med1* encodes a homologue of the human Ran-binding protein, RanBP1, and has been renamed *sbp1* (spi1-binding protein). sbp1p binds to spi1p-GTP and costimulates the GTPase-activating protein (GAP)-catalyzed GTPase activity. Cells in which sbp1p is depleted or overproduced phenocopy cells in which the balance between spi1p-GTP and spi1p-GDP is perturbed by other means. Therefore, sbp1p 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 10^7 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-

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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).

spi1p, the *S. pombe* member of the Ran-GTPase family, is 82% identical and 90% similar to human Ran (Matsumoto and Beach 1991). pim1p, the spi1p 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 spi1p for GTP. Inactivation of pim1p by a temperature-sensitive mutation in fission yeast results in a failure of cell cycle progression at the mitosis-to-interphase transition (Sazer and Nurse 1994). Several easily identifiable characteristics distinguish this terminal phenotype: (1) after a normal mitosis, the separated, postanaphase chromosomes are hypercondensed; (2) the nuclear envelope, which normally remains intact during the entire yeast cell cycle, loses its integrity; and (3) the cells have an abnormally wide medial septum (Demeter *et al.* 1995; Matynia *et al.* 1996; Sazer and Nurse 1994). Other defects include disruption of the nucleolus (Tani *et al.* 1995) and accumulation of poly(A)+ RNA in the nucleus (Kadowaki *et al.* 1993). Taken together, these studies establish that multiple processes depend on the proper functioning of the spi1p GTPase system in fission yeast. Still unanswered is the question of whether there is one primary function whose perturbation has pleiotropic effects on multiple nuclear processes and structures, or whether these functions are independently regulated.

The loss of pim1p nucleotide exchange activity would be expected to result in an increase in the abundance of spi1p-GDP relative to spi1p-GTP. Consistent with this prediction is the finding that the lethality and terminal phenotype of cells in which pim1p function is compromised by a temperature-sensitive mutation are also observed in wild-type cells expressing a mutant form of spi1p stabilized in the GDP-bound form (J. Demeter and S. Sazer, unpublished results) and in wild-type cells overproducing the spi1p GAP, rna1p (Matynia *et al.* 1996). Consistent with a model in which both decreased pim1p GEF activity and increased rna1p GAP activity independently increase the spi1p-GDP pool, pim1-d1^{ts} mutant cells display an increased sensitivity to *rna1* overproduction compared to wild-type cells (Matynia *et al.* 1996).

Cells overproducing rna1p die with a terminal phenotype indistinguishable from that of pim1-d1^{ts} 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 spi1p-GTP and spi1p-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 spi1p-GTP and spi1p-GDP might cause a failure of the mitosis-to-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 regulatable *nmt1* promoter (Maundrell 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 spi1p that directly or indirectly perturb the normal balance between spi1p-GDP and spi1p-GTP, (2) downstream components of the spi1p-GTPase pathway that do not directly affect the spi1p-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 spi1p 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*⁺ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 ade6-m210*/*ade6-m216*), and a mutant haploid strain pim1-d1^{ts} (*h*⁻ *leu1-32 ura4-D18 pim1-d1*^{ts}; 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).

***nmt1* promoter regulation:** Gene expression under the control of the *nmt1* promoter (Maundrell 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°, the 60,000 transformants were replica plated onto thiamine-free plates to derepress cDNA expression. Phloxine B dye (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°, 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° for 2 days. These precultures were washed and transferred to thiamine-free EMM medium with supplements at an initial concentration of ~10^5 cells/ml. After 13 hr at 32°, 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− (Stratagene, 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/*Bam*HI 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 60-0-0, provided by Reference Library Database (RLDB) Max Planck Institute for Molecular Genetics, was probed with the *Xho*I/*Bam*HI 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*-Δ1: A 4.0-kb *Sac*I/*Sac*I genomic DNA fragment containing the *sbp1* gene was subcloned from cosmid ICRFc60D0115D (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 *Eco*RV/*Pst*I 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*⁺ *sbp1*::*ura4*⁺/*sbp1 leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 ade6-m210*/*ade6-m216*) was generated by transforming diploid cells (*h*⁻/*h*⁺ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 ade6-m210*/*ade6-m216*) with the 4.4-kb *Sac*I/*Sac*I 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 ($DiOC_6$) 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 Sherwood 1990). To determine the DNA content of each half of the septated cells, cells were briefly digested with Novozyme (0.7 mg/ml) and Zymolyase (0.2 mg/ml) for 15 min at 37° immediately before analysis.

sbp1-Δ1 phenotype characterization: The sbp1-Δ1 heterozygous diploid cells were transformed with pREP41X-*sbp1*, and the transformants were sporulated. Haploid sbp1-Δ1 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*-Δ1 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 $DiOC_6$ and Hoechst 33342 to visualize the nuclear envelope and DNA (Demeter *et al.* 1995).

GTPase assay: Ran-GTP was incubated at 25° for 30 min with fourfold excess of [γ-^{32}P]GTP (30 Ci/mmol) in 20 mm Hepes-NaOH (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 Hepes-NaOH (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-^{32}P]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° for 5 min, then 10 μl of 5 nM *S. pombe* rna1p 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-GTPase binding assay: *E. coli*–produced GST-sbp1p or GST (1 μg) was separated by 10% SDS-PAGE and either transferred to polyvinyldifluoride membrane filters or stained with Coomassie brilliant blue. The filters were incubated with either ^{35}S-labeled Ran-GTPγS or ^{35}S-labeled Ran-GDPβS, 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-spi1p antiserum (Matynia *et al.* 1996).

Amino acid sequence analysis: A BLAST (Altschul *et al.* 1990) search in March 1997 using the National Center for

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-d1^{ts} 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-to-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* and *med2* were cloned three times each, and *med3* was cloned once.

Cells overexpressing *med* genes arrest with terminal phenotypes similar to *rna1* overexpressing cells and pim1^{ts} 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 $DiOC_6$ 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

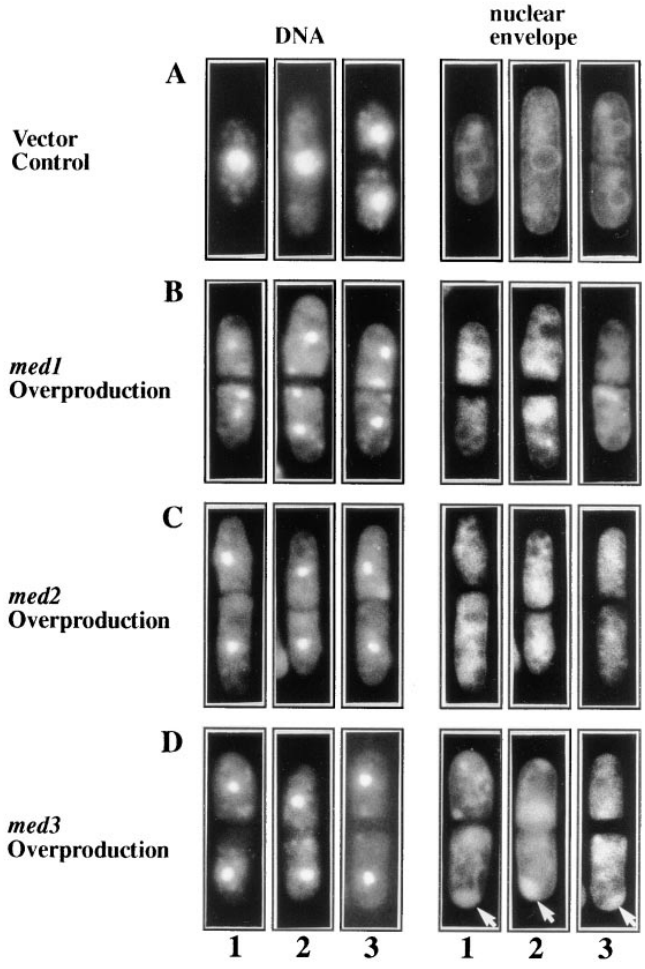

Figure 1.—*med* overexpression arrests cells with a medial septum, condensed chromosomes, and an abnormal nuclear envelope. Live cells were double stained with Hoechst 33342 to visualize the DNA and $DiOC_6$ to visualize the nuclear envelope. Micrographs of (A) wild-type vector-transformed cells at different 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 $DiOC_6$ staining material (arrows, panels 1–3).

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 pim1^{ts}-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 $DiOC_6$ 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).

pim1^{ts} 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-d1*^{ts} 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-d1^{ts} mutant cells to overexpression of *med1*, *med2*, or *med3*. At the *pim1-d1*^{ts} permissive temperature of 25°, wild-type

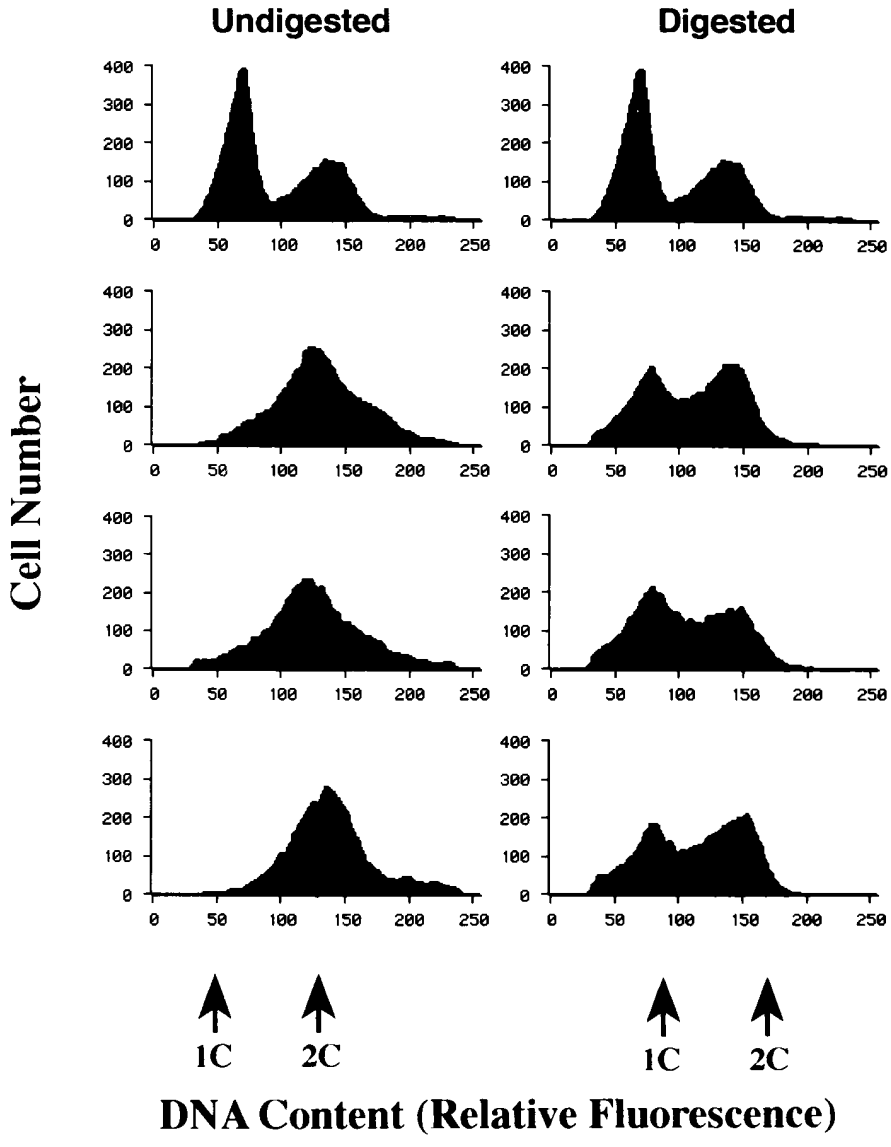

Figure 2.—*med* overexpression arrests cells with an unreplicated genome. FACS analysis of the DNA content of septated, binucleated *med1*, *med2*, or *med3* overexpressing cells (undigested) is 2C (1C per nucleus). Separation of the two daughter cells by treatment with a mixture of Novozyme and Zymolyase (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-d1^{ts} 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-d1^{ts} cells grew equally well at 25° when either *med2* or *med3* were expressed (Figure 3); however, the growth inhibition of pim1-d1^{ts} 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-d1^{ts} 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.

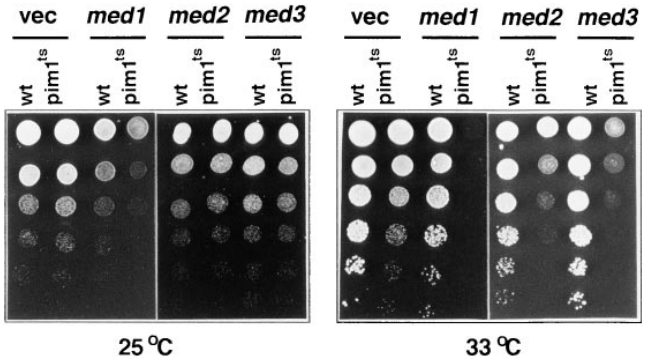

Figure 3.—pim1-d1^{ts} cells are hypersensitive to sublethal *med* overexpression. Wild-type cells or pim1-d1^{ts} cells, transformed with pREP41X vector, pREP41X-*med1*, pREP41X-*med2*, or pREP41X-*med3* plasmids, were grown to midlog phase in thiamine-free liquid media to derepress transcription. Fivefold 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-d1^{ts}*.

The *med1-1* 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* (spi1-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 $\sim 10^5$ 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 enzyme activity, a level 15 times greater than the 2.6 ± 0.6 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 (Beddow *et al.* 1995; Coutavas *et al.* 1993; Lounsbury *et al.* 1994; Schlenstedt *et al.* 1995), GST-sbp1p immobilized on filters bound to Ran-[γ-^{35}S] GTP but not to Ran-[β-^{35}S] 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 spi1p, 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-

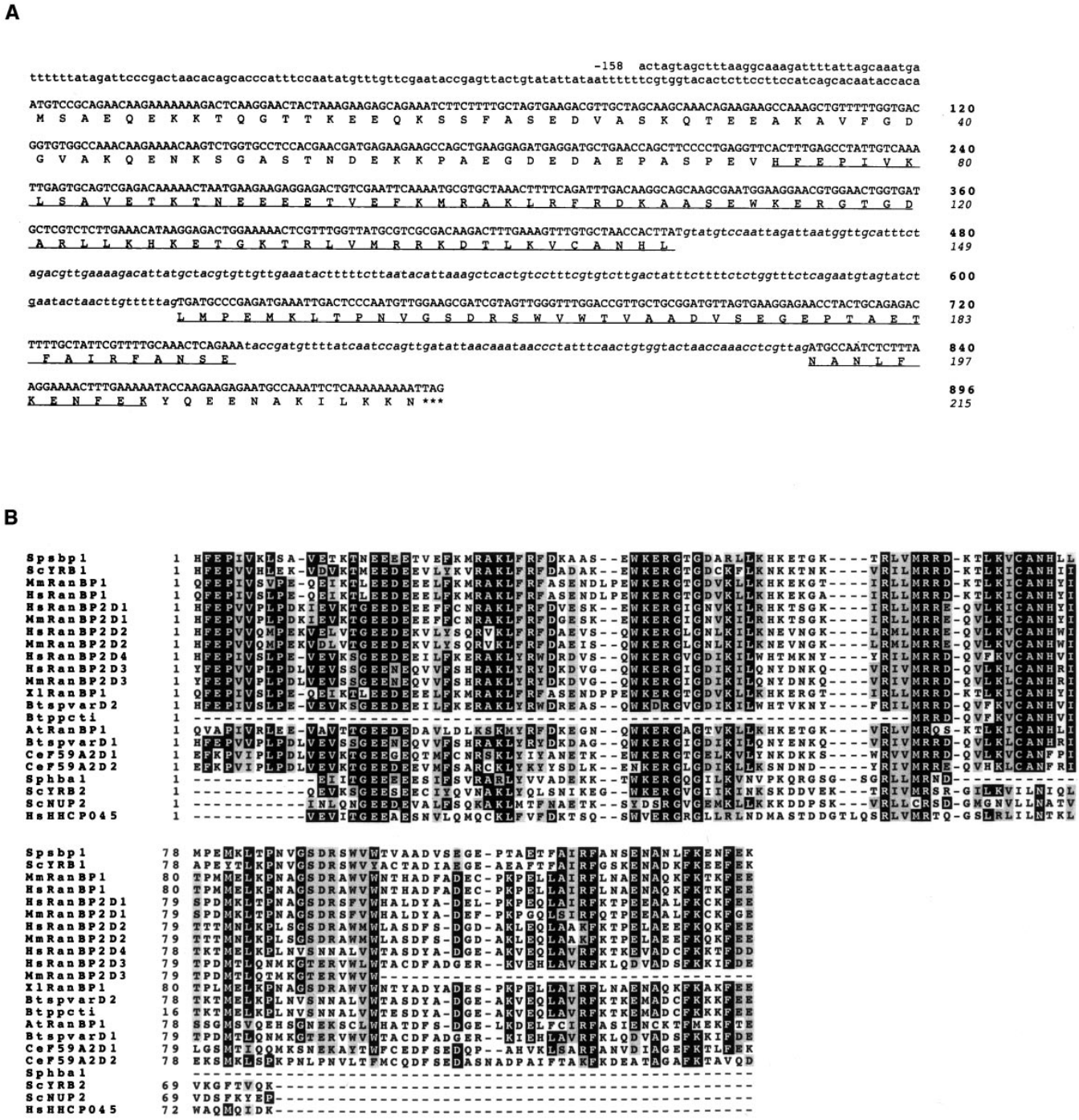

Figure 4.—sbp1p is a structural homolog of the evolutionarily conserved RanBP family of proteins. (A) Gene structure of *S. pombe sbp1*. DNA sequence of *sbp1* and conceptual translation of its protein product. Nucleotides are numbered from the A of the initiating methionine codon as +1. Nucleotides 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; Btppcti, (*B. taurus* peptidyl-prolyl *cis-trans* isomerase) L41692; Btspvar1, (*B. taurus* spliced variant with Ran-BD and cyclophilin domain, domain 1) L41691; AtRanBP1, (*A. thaliana* RanBP1) U62742; CeF59A2-1, (*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; HsHHCP045, (*H. sapiens*-expressed sequence tag HHCP045) M79174.

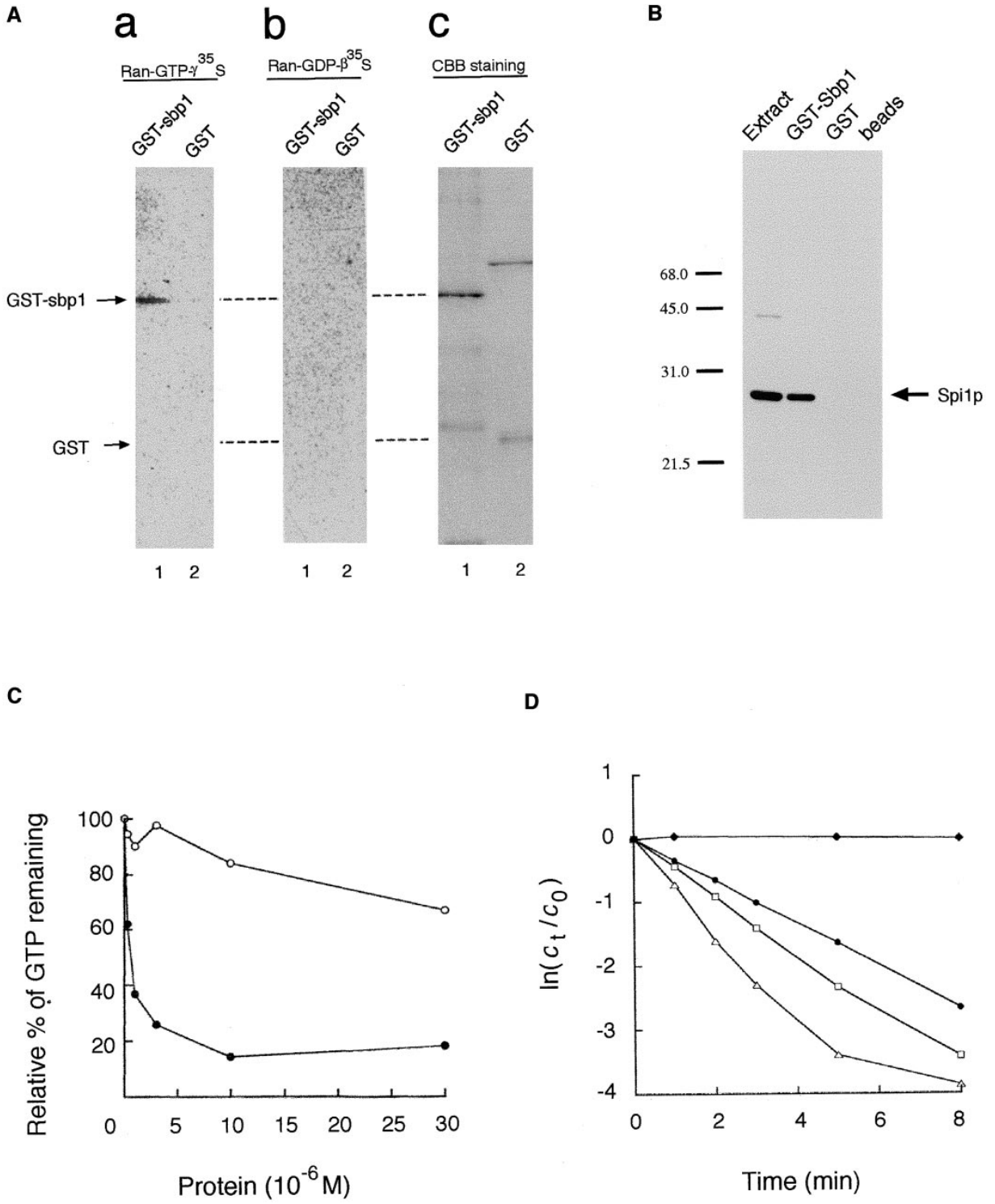

Figure 5.—sbp1p is a functional homolog of the evolutionarily conserved RanBP family of proteins. (A) sbp1p binds specifically to Ran-GTP. *E. coli*-produced GST-sbp1p (lane 1) or GST (lane 2) was separated electrophoretically and either transferred to filters (a and b) or stained with Coomassie brilliant blue (c). The filters were incubated with either ^{35}S-labeled Ran-GTPγS (a) or ^{35}S-labeled Ran-GDPβS (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 antiserum. (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^{32}P]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 $DiOC_6$ (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.

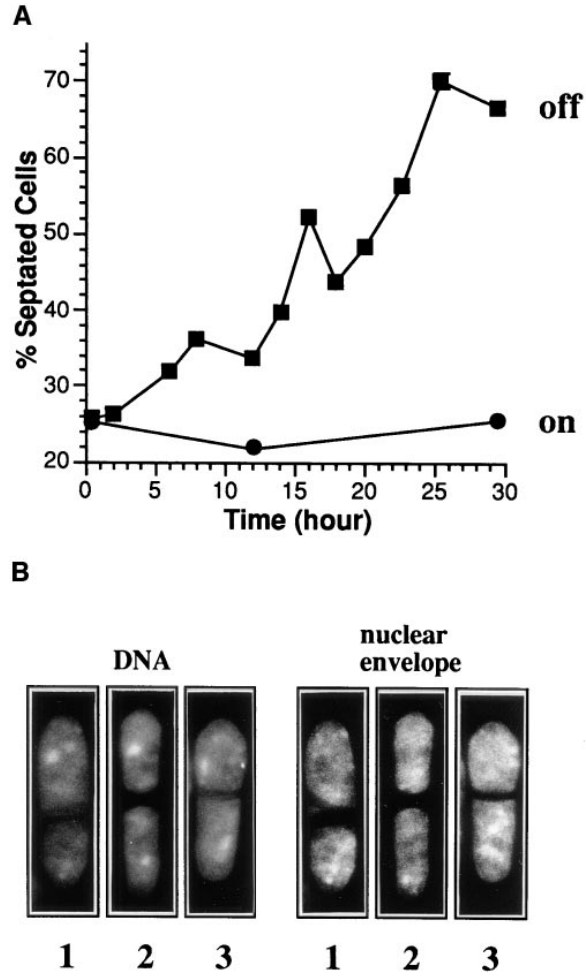

Figure 6.—*sbp1* is an essential gene whose deletion results in the accumulation of cells with a medial septum, condensed chromosomes, and an abnormal nuclear envelope. (A) The inviability of sbp1-Δ1 haploid cells is rescued by expression from pREP41X-*sbp1* but addition of thiamine (at $t = 0$) to repress transcription results in a progressive increase of the percentage of septated cells. (B) After 24 hr, live cells were double stained with Hoechst 33342 to visualize the DNA and $DiOC_6$ to visualize the nuclear envelope.

He, S. Sazer, L. Chang and K. Gould, unpublished results). Cells overexpressing the second class of cDNAs, which includes *mad2* 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 genes, *med1*, *med2*, and *med3*, the overproduction of which causes wild-type cells to exhibit all the phenotypes that were established as criteria for this screen, meaning that they phenocopy both a loss of pim1p GEF activity (Demeter *et al.* 1995; Sazer and Nurse 1994) and an increase in rna1p GAP activity (Matynia *et al.* 1996). Overexpression of *med1*, *med2*, or *med3* results in the accumulation of binucleated cells with hypercondensed chromosomes, abnormal nuclear envelopes, and a medial septum. The terminal overproduction phenotypes of *med1* and *med2* are indistinguishable. Although overexpression phenotypes must be interpreted with caution, the biological relevance of the *sbp1* overexpression phenotype is supported by the observation that it is identical to the deletion phenotype. In addition to the pim1^{ts}-like phenotypes it shares with *med1* and *med2*, *med3* also accumulates $DiOC_6$-staining material, presumably lipid or membranes, in the cytoplasm. Further characterization will be required to determine the nature of this cytoplasmic material. The genetic interaction of *med1*, *med2*, and *med3* with the spi1p GTPase system was established by demonstrating that overproduction of each of these three cDNAs is synthetically lethal with the *pim1-d1*^{ts} mutation, although further biochemical analysis is required to confirm a direct relationship between *pim1* and *med 2* or *med3*.

med1, *med2*, and *med3* are previously uncharacterized fission yeast genes that encode proteins with structural homologs in both mammals and budding yeast. Fission yeast med2p and its homologs have no informative domains, and their function is unknown. Its overexpression phenotype and genetic interaction with *pim1*, however, suggest that *med2* is likely to encode a regulator or target of the GTPase system. *med3* encodes a protein that shares sequence similarity with enzymes known to be involved in lipid metabolism, and its relationship to the spi1p GTPase system is currently under study. It is tempting to speculate that since the pim1-d1^{ts} mutant undergoes nuclear envelope fragmentation, the perturbation in intracellular lipid metabolism caused by *med3* overexpression exacerbates this membrane defect.

In this manuscript, we have focused on the characterization of one of these cDNAs, *med1*, which has the most toxic effect when expressed in pim1-d1^{ts} mutant cells. Because of its sequence similarity with a previously characterized family of proteins named RanBP (Figure 4B), *med1* was renamed *sbp1*. sbp1p is 51.4% identical to the human RanBP and 55.4% identical to the budding yeast homolog Yrb1p. These three proteins share 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 cerevisiae* 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 (Demeter *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 pim1p GEF or the rna1p GAP (Demeter *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 spi1p 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* Yrb2, 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 *hba1* 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 spi1p 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 spi1p 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 *sbp1* 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 spi1p. It remains to be determined if med2p and med3p are regulators or effectors of the spi1p GTPase system, or if they independently influence the mitosis-to-interphase transition.

We thank Ngoctuyen Ong for excellent technical assistance; Wendy Schrober in the Baylor Flow Cytometry Core Facility for FACS analysis; Nadja Pohl and Katarina Hentschel at the Resource Center/Primary Database of the German Human Genome Project, Max Planck Institute for Molecular Genetics, for mapping the *med1* gene; and Tetsuya Taura and Pam Silver (Harvard Medical School and Dana Farber Cancer Institute) for communication of results before publication. This work was funded by grants from the National Institutes of Health to S.S. (GM49119) and the Human Frontier Science Program to S.S., T.N., and H. Ponstingl (RG-0423/95M).

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Communicating editor: F. Winston