Abstract. The microtubule cytoskeleton plays a pivotal role in cytoplasmic organization, cell division, and the correct transmission of genetic information. In a screen designed to identify fission yeast genes required for chromosome segregation, we identified a strain that carries a point mutation in the SpRan GTPase. Ran is an evolutionarily conserved eukaryotic GTPase that directly participates in nucleocytoplasmic transport and whose loss affects many biological processes. Recently a transport-independent effect of Ran on spindle formation in vitro was demonstrated, but the in vivo relevance of these findings was unclear. Here, we report the characterization of a Schizosaccharomyces pombe Ran GTPase partial loss of function mutant in which nucleocytoplasmic protein transport is normal, but the microtubule cytoskeleton is defective, resulting in chromosome missegregation and abnormal cell shape. These abnormalities are exacerbated by microtubule destabilizing drugs, by loss of the spindle checkpoint protein Mph1p, and by mutations in the spindle pole body component Cut11p, indicating that SpRan influences microtubule integrity. As the SpRan mutant phenotype can be partially suppressed by the presence of extra Mal3p, we suggest that SpRan plays a role in microtubule stability.

Key words: Ran GTPase • microtubules • chromosome segregation • mitosis • fission yeast

Introduction

Accurate duplication of chromosomes and the subsequent precise segregation of the sister chromatids into two daughter cells are essential processes in every eukaryotic cell cycle. The faithful inheritance of genetic information is a prerequisite for the survival of an organism and many diseases are associated with genetic instability (Cahill et al., 1999).

The microtubule cytoskeleton, as the major component of the mitotic spindle, is essential for the precise separation of the duplicated sister chromatids before cytokinesis.

Upon commitment to mitosis in most animal cells, the extensive interphase microtubule array, composed of long, relatively stable microtubules, is disassembled rapidly and reorganized into an elliptical bipolar spindle consisting of shorter and much more dynamic microtubules. The dynamic instability model predicts that microtubules have polymerization (growth) and depolymerization (shrinkage) phases and that switching between these two phases occurs with specific frequencies (Kirschner and Mitchison, 1986). The factors regulating microtubule dynamics can be divided broadly into two groups: proteins with a stabilizing role, such as microtubule-associated proteins (MAPs),1 and proteins like XKCM1 and Op18 that have a microtubule destabilizing function (for reviews see Walczak, 2000; Andersen, 2000). Structural and regulatory proteins required for the rapid transition from the interphase microtubule cytoskeleton to the mitotic spindle structure have not been defined fully, but the importance of altered microtubule dynamics thought to result from the activation of the protein kinase complex M phase promoting factor has been extensively documented (for reviews see Walczak, 2000; Andersen, 2000).

In the fission yeast Schizosaccharomyces pombe a similar reorganization of the microtubule cytoskeleton occurs at the onset of mitosis (for review see Hagan, 1998): the cytoplasmic microtubules disassemble and the mitotic microtubules are nucleated in the nucleus from the centromere equivalents, the spindle pole bodies (SPBs), to generate a bipolar spindle.

We and others have identified several factors needed for chromosome segregation and spindle function by isolating mutants that display increased loss of a nonessential chromosome and are hypersensitive to microtubule destabilizing drugs (Takahashi et al., 1994; Fleig et al., 1996).
data presented here show that Spi1p is one of the proteins required for chromosome transmission fidelity in fission yeast as a mutant allele of spi1+ was identified in this screen. spi1+ codes for the fission yeast Ran GTPase, an evolutionarily conserved essential GTPase of the Ras superfamily (for review see Sazer, 1996). Association with effector and regulatory proteins is dependent on the conformational state of Ran, which is a consequence of being bound to either GDP or GTP. Loss of Ran GTPase function affects many biological processes, but it is generally accepted that the primary function of the Ran GTPase cycle is in nucleocytoplasmic transport (Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998). Several observations have pointed to a possible role of the Ran GTPase cycle in chromosome segregation and microtubule regulation, but it has remained unclear whether this was caused directly by perturbation of multiple downstream effector pathways or indirectly by transport defects (for review see Sazer and Dasso, 2000). The finding of a human centrosomal Ran–GTP binding protein involved in microtubule nucleation (Nakamura et al., 1998) and the demonstration that Ran-GTP in vitro regulates microtubule spindle assembly in Xenopus M phase extracts in a transport-independent manner (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999) support a role for Ran in the regulation of bipolar spindle formation. However, a direct influence of Ran on spindle microtubule arrays in vivo has not been demonstrated previously and the mechanism by which Ran might regulate spindle assembly has remained unclear. Here, we report the characterization of a fission yeast Ran mutant, Spi-25p, that causes microtubule defects in vivo. Strains that regulate spindle assembly has remained unclear. Here, we demonstrated previously and the mechanism by which Ran-GTP affects many biological processes, but it is generally accepted that the primary function of the Ran GTPase cycle is in chromosome segregation and microtubule regulation, but it has remained unclear whether this was caused directly by perturbation of multiple downstream effector pathways or indirectly by transport defects (for review see Sazer and Dasso, 2000). The finding of a human centrosomal Ran–GTP binding protein involved in microtubule nucleation (Nakamura et al., 1998) and the demonstration that Ran-GTP in vitro regulates microtubule spindle assembly in Xenopus M phase extracts in a transport-independent manner (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999) support a role for Ran in the regulation of bipolar spindle formation. However, a direct influence of Ran on spindle microtubule arrays in vivo has not been demonstrated previously and the mechanism by which Ran might regulate spindle assembly has remained unclear. Here, we report the characterization of a fission yeast Ran mutant, Spi-25p, that causes microtubule defects in vivo. Strains carrying the defective spi1-25 allele have no transport defect but show defects in early spindle formation and function, have an activated spindle checkpoint, and are hypersensitive to microtubule destabilizing drugs. Interestingly, moderate overexpression of Mal3p, the fission yeast member of the evolutionarily conserved microtubule-associated EB1 family (Beinhauer et al., 1997), can partially rescue spi1-25 defects. The role of Ran with regard to spindle formation will be discussed in this context.

Materials and Methods

Media and Strains

The genotypes of strains used in this study are listed in Table I. The S. pombe strains YP1022, YP1022a, UFY135, and UFY250 have been described (Fleig et al., 1996; Beinhauer et al., 1997). UFY250 was backcrossed three times with YP1022a or YPK246 resulting in strains UFY250R and UFY25CX, respectively. The strains carrying cut11 alleles or cut11+–GFP were a gift from the J.R. McIntosh lab (West et al., 1998). The mph1+ null, mad2 null, and pin1-d1 strains have been described (Sazer and Nurse, 1994; He et al., 1997, 1998b). The GFP-papl+ expressing plasmid (Toone et al., 1998) was integrated into a wild-type strain, generating strain SS767. All double mutant strains were identified by tetrad analysis.

Strains were grown in rich (YE5S) or Edinburgh minimal medium (EMM) with appropriate supplements (Moreno et al., 1991). Sensitivity to thiabendazole (TBZ) was monitored at 24°C for nuclear protein export) or 15 min after addition of 0.8 mM hydrogen peroxide (to test for nuclear protein export) or 15 min after addition of 0.8 mM hydrogen peroxide (to test for nuclear protein export).

Microscopy

Photomicrographs of cells were taken with a ZEISS Axioskop or AxioplanII. Immunofluorescence images were processed as described previously (Beinhauer et al., 1997). For determination of morphological defects, a minimum of 250 cells were scored microscopically; for the Cut11-GFP localization three different cultures with a total of 600 counted cells were analyzed. Processing of cells for immunofluorescence microscopy was carried out essentially as described (Hagan and Hyams, 1988). For tubulin staining, we used the primary monoclonal antitubulin antibody TATI (Woods et al., 1989) followed by FITC-conjugated goat anti-mouse antibodies (EY labs). SBPs were stained using AP2 affinity-purified anti-Sad1p primary and Cy3-conjugated secondary sheep antirabbit antibodies (Sigma-Aldrich) (Hagan and Yanagida, 1995).

Identification of mal25-1 Multicopy Suppressors and Linkage Analysis

Strain UFY25CX was transformed with an S. pombe genomic bank (Barbet et al., 1992) and Ura+ transformants were selected by incubation for 50 h at 24°C, followed by replica plating onto 7 µg/ml TBZ plates. From the 44,450 transformants, plasmids were isolated (Moreno et al., 1991) from the 21 surviving colonies and processed as described (Beinhauer et al., 1997). Two genomic DNA inserts were found several times: the spi1+ (Matsumoto and Beach, 1991) and mal3+ (Beinhauer et al., 1997) genes were isolated five and four times, respectively. To determine allelism between mal25-1 and spi1+, the kanR marker conferring resistance to the antibiotic G418 was inserted 6.28 kb away from the spi1+ open reading frame (ORF) via PCR-based gene targeting (Bahler et al., 1998). This strain, UFY156, was crossed with UFY25CX, and the resultant spores were analyzed via random spore analysis. After establishment of linkage, the spi1+ ORF in strain UFY25CX was sequenced. Genomic DNA was PCR amplified using oligonucleotides 5’-CTCTCAGTTGTGTTAG-GTGC-3’ and 5’-CTATGTGTTACCAAGTCT-3’ that flank the spi1+ ORF.

Recreation of mal25-1 Mutation In Vitro and Cloning into Expression Vectors

The 5’ end of the spi1+ ORF from strain UFY25CX was amplified by PCR using the following primer pair: 5’-GGATGGACCTACATACCCAAAGTTGCAAATATC-3’ (italized characters represent AvaII restriction site 132 bp downstream of the start codon; underlined characters represent mutated codon to generate spi1-25; underlined bold character represents base change to give mutated codon; bold and italicized characters represent sequence homologous to the spi1+ genomic template) and 5’-GGATGACCTACATACCCAAAGTTGCAAATATC-3’ (underlined characters represent NdeI restriction site; underlined and bold characters represent start codon; bold characters represent sequence homologous to the 5’ end of the spi1+ genomic template). This PCR product was cleaved Avai-II-NdeI to generate the 5’ end of the spi1+ ORF. The 5’ end was obtained by cleaving a spa clone (spi1+ spa1-CHY2; Sazer, unpublished data) with Avai-I-BamHI. The 5’ and 3’ fragments were both cloned into NdeI-BamHI cut pAS1 (Harper et al., 1993) resulting in 5’-spai. Next, the sequenced spi1p-pAS1 was cleaved with NdeI-BamHI and the spi1+ ORF was subcloned into pETXHA (Eilledge et al., 1992) to create spi1-25/pETXHA and into pTrchHis (Invitrogen) to create His-spi1-25/pTrchHis. The XhoI-HA-spi1-25-BamHI insert from the pETXHA-spi1-25 construct was subcloned into XhoI-BamHI cut pREP9X vector for expression of HA-spi1-25 in S. pombe.

Nuclear Protein Transport Assay

Nuclear protein transport was tested in strains with either an integrated copy of the gene encoding the GFP-tagged Pap1p protein (Toone et al., 1998) or a GFP-tagged LacZ construct fused to the SV40 nuclear localization signal (NLS) (Demeter, J., and S. Sazer, unpublished data), constructed by subcloning the NLS-GFP-LacZ containing fragment from the Saccharomyces cerevisiae plasmid pPS17 (Lee et al., 1996) into pREPAX. spi1+ and wild-type cells were grown to midlog phase at 25°C in EMM, then shifted to 36°C for 4 h. Live cells were observed microscopically either to determine the steady state localization of the GFP-SV40-NLS-LacZ protein, or to monitor the localization of the nucleocytoplasmic shuttling protein, GFP-Pap1p, before hydrogen peroxide addition (to test for nuclear protein export) or 15 min after addition of 0.8 mM hydrogen peroxide (to test for nuclear protein import).

GTP Binding Assays

Three E. coli BL21 cell-produced His(6)-tagged substrates His-Spi1p in pTrchHisB (Matynia et al., 1996), His-Spi1p-25 in pTrchHisB, and S. cerev.
Table 1. Strains Used in This Study

| Strain   | Genotype                                      | Source          |
|----------|-----------------------------------------------|-----------------|
| YP10.22  | h+ leu1-32 ade6-M210 ura4-D6 Ch[+ade6-M216]   | U. Fleig        |
| YP10.22a | h+ ade6-M210 ura4-D6 Ch[+ade6-M216]           | U. Fleig        |
| UFY135   | h+ mal3Δ::his3+ leu1-32 ade6-M210 ura4-D18 his3Δ | U. Fleig        |
| UFY250   | h+ spi1-25 leu1-32 ade6-M210 ura4-D6 Ch[+ade6-M216] | U. Fleig        |
| UFY250R  | h+ spi1-25 leu1-32 ade6-M210 ura4-D6          | U. Fleig        |
| UFY25CX  | h+ spi1-25 leu1-32 ade6-M210 ura4-D6 his3Δ    | U. Fleig        |
| UFY156   | h+ spi1Δ::spil-25cDNA/his3+ leu1-32 ade6-M210 ura4-D18 his3Δ | U. Fleig        |
| UFY192   | h+ spi1Δ::spil-25cDNA/his3+ leu1-32 ade6-M210 ura4-D18 his3Δ | U. Fleig        |
| UFY153   | h+ spi1-25 mad2Δ::ura4+ ade6-M210 leu1-23 ura4-D18 | U. Fleig        |
| UFY154   | h+ spi1-25 mphp1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 | U. Fleig        |
| UFY193   | h+ spi1-25 mal3Δ::his3+ ade6-M210 leu1-32 ura4-D18 his3Δ | U. Fleig        |
| YPK2G246 | h+ leu1-32 ade6-M210 ura4-D18 his3Δ           | K. Gould        |
| 80       | h+ cut11-2 leu1-32 ura4-D18                   | J.R. McIntosh   |
| 83       | h+ cut11-3 leu1-32 ura4-D18                   | J.R. McIntosh   |
| 91       | h+ cut11-7 leu1-32 ura4-D18                   | J.R. McIntosh   |
| 317      | h+ cut11::GFP::ura4+::leu1-32 ura4-D18        | J.R. McIntosh   |
| SS446    | h+ leu1-32 ade6-M210 ura4-D18                 | S. Sazer        |
| SS767    | h+ int::GFP-pap1/pRep41::leu1-32 ura4-D18 ade6-M210 | S. Sazer        |
| SS893    | h+ int::GFP-pap1/pRep41::leu1-32 ura4-D18 ade6-M210 | S. Sazer        |
| SS131    | h+ pim1-1-d1 leu1-32 ade6-704                 | S. Sazer        |
| SS560    | h+ mphp1Δ::ura4+ leu1-32 ade6-M216 ura4-D18   | S. Sazer        |
| SS638    | h+ mad2Δ::ura4+ leu1-32 ade6-M210 ura4-D18    | S. Sazer        |
| SS482    | h+ int::GFP-NLS-LacZ/pREP4X::ura4+           | S. Sazer        |
| SS890    | h+ spi1-25 int::GFP-NLS-LacZ/pREP4X::ura4+   | S. Sazer        |

Visiae His-UBC4 in PET (Novagen; construct a gift of J.W. Harper, Baylor College of Medicine, Houston, TX) were obtained using standard methods and purified on a nickel-nitrilotriacetic acid resin (Qiagen) in sonication buffer (50 mM NaPO4, pH 8.0, 300 mM NaCl) plus 10 mM imidazole, 0.1 mM PMSF, and 10 μg/ml leupeptin. Protein was determined by quantifying the intensity of Coomassie blue staining of the protein gel band using a DC-129 digital camera and Digital Science Electrophoresis Documentation and Analysis System 120 software (both from Eastman Kodak Co.). 10 μl of nickel resin with 3–5 μg His-tagged substrate was aliquoted into SpinX² (Costar) columns containing 180 μl GTP Binding Buffer (50 mM NaPO4, pH 7.0, 5 mM MgCl2) plus 10 μM imidazole, 0.1 mM PMSF, and 10 μg/ml leupeptin. The binding reactions were initiated by the addition of 10 μCi of α-32P[GTP and stopped by spinning for 30 s and washing three times. Samples were added to Scinti-safe Gel scintillant (Fisher Scientific) and bound radioactive nucleotide was quantified using a Beckman LS-3801 scintillation counter.

Figure 1. Phenotypic characterization of the mald25-1 strain. (A) Sectoring phenotypes of wild-type (wt) and mald25-1 strains grown on indicator plates (Fleig et al., 1996) for 6 d at 24°C. (B) Serial dilution patch test for sensitivity to the microtubule-destabilizing drug TBZ. Dilutions shown were 10-fold. Although the isogenic wild-type strain (b and d) shows slightly reduced growth at 24°C, the mald25-1 strain (a and c) is unable to grow. (C) The mald25-1 strain shows growth defects at higher temperatures. Serial dilution patch tests of wild-type strain (a, c, and e) and mald25-1 strain (b, d, and f) grown at 24°C, 30°C, and 36°C, respectively) are shown. (D) Photomicrographs of wild-type (insert) and mald25-1 strain show an abnormal, elongated cell form of mutant strain. At 30°C, wild-type cells had an average length of 13.2 ± 1.2 μm at septa formation, whereas mald25-1 cells were 19.0 ± 3 μm in length. Arrows indicate septum. Bar, 10 μm.
Results

Characterization of mal25-1 Mutant and Establishment of Allelism between mal25-1 and the Gene Encoding the SpRan GTPase Spi1p

From a previously described screen (Fleig et al., 1996), we isolated a mutation named mal25-1 that strongly decreased the transmission fidelity of a nonessential minichromosome that was identified as an increase in the number of red sectors in a white colony (Fig. 1A) using an ade6-based colony color assay. Based on prior analysis of other mal mutants we estimate that presence of mal25-1 leads to an \( \geq 400 \)-fold increase in minichromosome loss (Fleig et al., 1996; Beinhauer et al., 1997). In addition, the mal25-1 strain was hypersensitive to the microtubule-destabilizing drug TBZ (Fig. 1B), had reduced growth at \( \geq 30^\circ \text{C} \) (Fig. 1C), and accumulated elongated, abnormally shaped cells (Fig. 1D).

Multicopy plasmid-borne suppression of TBZ hypersensitivity of the mal25-1 strain by transformation with an \( S. \text{pombe} \) genomic library identified repeatedly the spi1 and mal3 ORFs (Fig. 2). spi1 codes for the fission yeast Ran GTPase, whereas mal3 encodes an MAP (Matsumoto and Beach, 1991; Sazer and Nurse, 1994; Beinhauer et al., 1997). The mal3 gene only suppressed the TBZ hypersensitivity, whereas spi1 could suppress all phenotypes associated with the mal25-1 mutation (data not shown). To determine via linkage analysis if mal25-1 and spi1 were allelic, the mal25-1 strain was crossed to a strain carrying a wild-type spi1 allele tagged with the kanR marker. Among 500 resulting mal25-1 spores tested by random spore analysis, none carried the kanR marker, indicating that mal25-1 and spi1 were linked. Sequence analysis of the spi1 gene in the mal25-1 strain identified a single base pair change from G to A at position 315 of the ORF, resulting in a change from valine to isoleucine at position 44 of Spi1p that corresponds to V45 in mammalian Ran. This amino acid is in the Switch I region (Chook and Blobel, 1999; Vetter et al., 1999) of Ran, which undergoes a substantial conformational change depending on whether the protein is bound to GDP or GTP and is important for the binding of factors of the importin \( \beta \) family and other effectors to Ran-GTP (Chook and Blobel, 1999). To confirm that all phenotypes observed in the spi1-25 strain were caused by the single amino acid change in Spi1p, we re-made the mutation in vitro in the spi1 cDNA, tagged it with the S. pombe his3 marker, and replaced the genomic copy of a wild-type S. pombe strain with this mutated ver-

Table II. spi1-25 Interactions

| Gene | Function | Interaction |
|------|----------|-------------|
| nda2 | \( \alpha \)-Tubulin | No |
| sbp1 | Ran-GTP BP | Yes |

Genetic interactions

| Gene | Function | Phenotype |
|------|----------|-----------|
| pim1d | Ran GEF | Synthetic lethal |
| cut1-2 | SPB component | Synthetic lethal |
| cut1-3 | SPB component | Synthetic lethal |
| cut1-7 | SPB component | Poor growth at 24°C, dead at 30°C |
| mph1 \( \Delta \) | Spindle checkpoint component | Extremely poor growth at 24°C; increase in chromosome missegregation |
| mad2 \( \Delta \) | Spindle checkpoint component | None |
| mal3 \( \Delta \) | MAP | Very poor growth at 24°C; dead on sublethal doses of TBZ; increase in abnormal cell form; increase in chromosome missegregation |

Multicopy suppression

| Overexpression | Function | Allele to be rescued | Phenotype |
|----------------|----------|----------------------|-----------|
| spi1+ | Ran-GTP BP | spi1-25 | Lethal as for spi1+ |
| rna1+ | Ran-GAP | spi1-25 | Lethal as for spi1+ |
| spi1+ | Ran | pim1d | Good rescue |
| spi1-25 | Ran | pim1d | Moderate rescue |

Figure 2. The TBZ hypersensitivity of the mal25-1 strain is rescued by plasmid-borne copies of mal3\(^+\) and spi1\(^+\). Left and right panels show serial dilution patch tests (10\(^4\) to 10\(^2\) cells) of mal25-1 transformants grown on selective minimal medium without TBZ or with 7 \( \mu \)g/ml TBZ, respectively. Vector control indicates plasmid without insert.
served.

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...or by deletion or overexpression of its regulators (Sazer and Nurse, 1994; Matynia et al., 1996; He et al., 1998a) or by mutation in the ORF caused all phenotypes observed. *mal25-I* was thus renamed *spi1-25*.

**Interaction of Mutant Spi1p with Components of the SpRan GTPase System**

Misregulation of the Ran system in fission yeast by mutation or overexpression of its regulators (Sazer and Nurse, 1994; Matynia et al., 1996; He et al., 1998a) or by deletion of *spi1* (data not shown) results in a characteristic terminal phenotype. Cells arrest with normal size and shape, condensed postmitotic chromosomes, fragmented nuclear envelopes, and a wide medial septum.

The terminal phenotype of the *spi1-25* strain is clearly different from the *spi1* null strain, but the Spi1p protein levels are similar (data not shown), suggesting that Spi1-25p might be a separation of function mutant that retains normal interactions with some but not all binding partners. Alternatively, Spi1-25p could be a partial loss of function mutant in which the activity of the protein is lower than normal. To distinguish between these possibilities, we first asked whether the Spi1-25p mutant protein is able to interact with known regulatory and effector binding partners.

Spi1p interacts with proteins that modulate its nucleotide bound state such as the guanine nucleotide exchange factor Pim1p, the GTPaseactivating protein Rna1p, and the Ran binding proteins including Sbp1p (Matsumoto and Beach, 1991; Melchior et al., 1993; Sazer and Nurse, 1994; He et al., 1998a). Both Spi1p and Spi1p interacted with Sbp1p in the two-hybrid assay (Table II, data not shown), indicating that association of this Ran-GTP binding protein with Spi-25p was possible. Furthermore, as has been shown for *spi1* wild-type strains (Matynia et al., 1996; He et al., 1998a), overexpression of *sbp1* or *rna1* from the regulatable *nmt1* promoter in a *spi1-25* strain background was lethal (Table II), confirming the ability of the mutant protein to interact normally in vivo.

The nongrowth phenotype of strains carrying the *pim1*-*dlts* temperature-sensitive mutation can be rescued by *spi1* cDNA under the control of the full strength *nmt1* promoter at both low and high levels of expression (Sazer and Nurse, 1994). We found that the *spi1-25* cDNA could rescue the nongrowth phenotype of a *pim1-dlts* strain only with high level expression (data not shown), indicating either that the mutant protein has reduced activity or that only a portion of the protein is functional.

*spi1-25* and *pim1-dlts* showed a strong genetic interaction as spores from double mutant strains grown at 25°C could germinate but then ceased growth (data not shown).

**Spi1p Is Deficient in Nucleotide Binding**

To further characterize the consequences of the V44I mutation, we asked whether the Spi1p protein is properly folded by monitoring its ability to bind nucleotide. Histidine-tagged wild-type Spi1p and Spi1p were incubated with radiolabeled GTP. By comparing the maximum amount of bound nucleotide per microgram of protein, we found that Spi1p is only 30% as efficient in binding as wild-type Spi1p. Histidine-tagged wild-type Spi1p and Spi1p were incubated with radiolabeled GTP and the amount of bound nucleotide was quantitated. The initial binding kinetics of wild-type and mutant Spi1p are similar, but only 30% of Spi1p is competent to bind nucleotide. Data for each experiment were normalized for protein amount and calculated as percentage of maximal wild-type binding. Values from four independent experiments were averaged and graphed as percentage of maximal wild-type binding. Values from four independent experiments were averaged and graphed as percentage of maximal wild-type binding. Values from four independent experiments were averaged and graphed as percentage of maximal wild-type binding. Values from four independent experiments were averaged and graphed as percentage of maximal wild-type binding.

**Figure 3.** Comparison of the kinetics and efficiency of GTP binding between wild-type (wt) Spi1p and mutant Spi1-25p. Histagged Spi1p, his-tagged Spi1-25p, or UBC4 as a negative control were incubated with [γ-32P]GTP as described in Materials and Methods and the amount of bound nucleotide was quantitated. The initial binding kinetics of wild-type and mutant Spi1p are similar, but only 30% of Spi1p is competent to bind nucleotide. Data for each experiment were normalized for protein amount and calculated as percentage of maximal wild-type binding. Values from four independent experiments were averaged and graphed as percentage of maximal wild-type binding. Values from four independent experiments were averaged and graphed as percentage of maximal wild-type binding. Values from four independent experiments were averaged and graphed as percentage of maximal wild-type binding.

**Figure 4.** Nucleocytoplasmic transport is normal in *spi1-25* cells. Wild-type or *spi1-25* cells with either integrated GFP-SV40, NLS-LacZ, or GFP-pap1 were grown to midlog phase at 25°C then shifted to 36°C for 4 h. The GFP-SV40 NLS-Galactosidase reporter protein is exclusively nuclear localized in wild-type (A) and *spi1-25* cells (B). Without hydrogen peroxide treatment, GFP-Pap1p is exported from the nucleus in both wild-type (C) and *spi1-25* cells (D). 15 min after the addition of 0.8 mM hydrogen peroxide, the fusion protein is imported into the nucleus in wild-type cells (E) and *spi1-25* cells (F). Bar, 10 μm.
GTP binding compared with Spi1p, but that the initial kinetics of binding appears similar to those of the wild-type protein (Fig. 3). These data demonstrate that although the kinetics of nucleotide binding is normal, only a portion of Spi1-25p is competent to bind nucleotide, perhaps due to misfolding.

**Mutant Spi1p Protein Does Not Cause Nucleocytoplasmic Transport Defects**

To test if nucleocytoplasmic transport was affected in *spi1-25* cells, we first monitored the localization of a GFP-β-galactosidase reporter protein constitutively targeted to the nucleus by the SV-40 NLS. In both *spi1-25* and *spi1*+ cells, this reporter protein was predominantly localized to the nucleus (Fig. 4, A and B). To more precisely monitor nucleocytoplasmic transport, we followed localization of a GFP-Pap1p fusion protein. Pap1p is an AP1-like transcription factor with a bipartite type1 nuclear localization sequence (Ding et al., 2000) and a nuclear export sequence (Kudo et al., 1999) that continuously shuttles between the nucleus and the cytoplasm. At steady state it is actively exported from the nucleus and appears predominantly cytoplasmic; under oxidative stress conditions, such as the presence of hydrogen peroxide, it accumulates in the nucleus (Toone et al., 1998).

In both untreated *spi1*+ or *spi1-25* strains, the GFP-Pap1p fusion protein is actively exported to the cytoplasm and relocizes to the nucleus upon oxidative stress (Fig. 4, E and F), indicating that the mutant is competent for both nuclear protein export and import. The ratio of nuclear to cytoplasmic GFP-Pap1p fluorescence (see Materials and Methods) in *spi1-25* cells was similar to that of wild-type cells: before hydrogen peroxide treatment, the ratios were 0.8 ± 0.07 for both strains; 15 min after hydrogen peroxide addition, the ratios were 3.8 ± 0.7 and 3.1 ± 0.8 for wild-type and *spi1-25* cells, respectively.

**The *spi1-25* Mutant Strain Shows Altered Interphase Microtubule Arrays**

At 24°C, ~10% of *spi1-25* cells did not have wild-type cylindrical shape but were curved, bent, or branched (see Figs. 1 D and 8 B). At temperatures ≥30°C, this number increased to 21%. Because an abnormal microtubule cytoskeleton is known to cause changes in cell shape (Hiraoka et al., 1984; Verde et al., 1995; Beinhauer et al., 1997; Sawin and Nurse, 1998), we analyzed microtubules in *spi1-25* cells by indirect immunofluorescence. In wild-type cells, interphase microtubules are aligned along the long axis of the cell reaching the cell tips (Fig. 5 e). In contrast, the cytoplasmic microtubules in most branched and 10% of normal cylindrically shaped *spi1-25* cells failed to reach the cell tips and in some cases were shorter than normal (Fig. 5, a–c). Cell elongation alone cannot explain the inability of the microtubules to reach the cell tips of *spi1-25* cells because elongated *cdc25* null mutant cells have interphase microtubules that are much longer than those of wild-type cells and do reach the cell tips (Hagan and Hyams, 1988).

Curved *spi1-25* cells (Fig. 5 d) usually displayed a single microtubule bundle on the convex side of the cell as has been described for other curved *S. pombe* mutants (Verde et al., 1995). Therefore, a significant proportion of cells carrying the defective *spi1-25* allele display defects of the cytoplasmic microtubule cytoskeleton.

**The *spi1-25* Mutation Affects Formation and Function of the Mitotic Spindle**

At 25°C, the *spi1-25* strain showed increased loss of a nonessential chromosome (see Fig. 1 A) and ~2% of cells in a population showed abnormal mitosis as seen by staining of the chromatin with DAPI (data not shown). At temperatures ≥30°C *spi1-25* cells showed reduced growth (see Fig. 1 C) and increased mis segregation of chromosomes. We identified three phenotypic classes: (a) cytokinesis without...
prior completion of mitosis, resulting in a displaced nucleus and an anucleate daughter cell; (b) chromosomes segregated asymmetrically to the two ends of a cell; and (c) highly condensed chromatin in the middle fifth of the cell.

To determine the cause of the chromosome missegregation phenotype, the mitotic spindle in spi1-25 cells was analyzed. The spi1-25 allele is not a temperature-sensitive lethal mutation but shows significantly reduced growth and only 61% viability at 36°C. spi1-25 cells were incubated at 36°C to determine spindle structure, chromatin structure, and SPB localization. We found two main phenotypic aberrations in the formation of the mitotic spindle. One was a star- or fan-shaped tubulin staining pattern indicating multiple microtubule bundles that originated from a single focal point giving rise to a monopolar spindle (Fig. 6, c and g). DAPI staining showed hypercondensed chromatin (Fig. 6, b and f) and a tiny pre-metaphase spindle (c, top cell; k) between duplicated and separated spindle pole bodies (d, top cell; l) and not visibly condensed chromatin (b, top cell; j). Tubulin staining of the nucleus in k shows nuclear import of tubulin. Bars, 10 μm.

The aberrant mitotic spindle phenotype seen in spi1-25 cells indicated that SpRan was required for an early stage in spindle formation. We used the SPB localization of the Cut11p protein in spi1-25 cells grown at 36°C to define this stage more precisely. In prophase, cells have a single bright Cut11-GFPp spot, whereas at later mitotic stages two spots are observed (West et al., 1998). 7.3 ± 2.1% spi1-25 cells grown for 1 h at 36°C showed a single bright spot compared with 0.8 ± 0.4% for wild-type cells, indicating that there is an approximately eightfold increase in prophase cells, whereas the percentages of mutant and wild-type

Figure 6. spi1-25 cells show mitotic spindle defects. Early mitotic spindle defects in an asynchronous culture of the spi1-25 mutant held at 36°C for 3 h when most aberrant phenotypes were scored. Each panel shows four different images of the same cell: the first DIC with DAPI staining (a, e, and i) shows the cell outline and position of the chromatin; the second shows chromatin staining by DAPI (b, f, and j); the third and fourth show immunofluorescence of tubulin (c, g, and k) and the spindle component Sad1p (d, h, and l), respectively. The two predominant spindle defects are shown: star-shaped monopolar spindles and condensed chromatin (c and b, bottom cell; g and f) and a tiny pre-metaphase spindle (c, top cell; k) between duplicated and separated spindle pole bodies (d, top cell; l) and not visibly condensed chromatin (b, top cell; j). Tubulin staining of the nucleus in k shows nuclear import of tubulin. Bars, 10 μm.
 Spi1-25 is Synthetically Lethal with cut11ts Alleles

Because the spi1-25 strain expressing cut11-GFP grew slower than the isogenic spi1-25 strain, possibly due to a subtle change in the properties of the Cut11p protein that was amplified in the spi1-25 strain, we tested whether cut11 and spi1 interact genetically by constructing spi1-25 cut11ts double mutants. Three different temperature-sensitive cut11ts alleles were used (see Table I) (West et al., 1998). The resultant double mutants showed allele specific synthetic lethal interactions (see Table II). At 25°C, both single mutants grow, but the cut11-2 spi1-25 and cut11-3 spi1-25 strains arrested after three to four cell divisions with highly elongated cells. The synthetic lethality between cut11-7 and spi1-25 was seen only at 30°C. These data indicate that spi1-25 and cut11ts encoding an SPB component interact genetically.

Spi1-25 Is Synthetically Lethal with the Spindle Checkpoint Mutant mph1Δ

As the spi1-25 strain showed a variety of abnormal spindle phenotypes, we asked if the viability of the strain was dependent on the spindle checkpoint, which monitors the correct alignment of chromosomes on the spindle (for review see Gardner and Burke, 2000), was activated by constructing double mutants of spi1-25 with mutant components of the spindle checkpoint pathway. We used deletion variants of mad2+ and mph1+ (He et al., 1997, 1998b), which are evolutionarily conserved components of this pathway. We found that the spi1-25 mad2Δ strains showed no apparent difference in growth or TBZ sensitivity compared with the single spi1-25 mutant (Table II, data not shown). However, spi1-25 mph1Δ double mutant strains were severely affected. At 25°C, both single mutants grew, whereas the double mutant barely grew (Fig. 7 A). At temperatures >30°C or in the presence of sublethal doses of TBZ (6 μg/ml), the spi1-25 mph1Δ strain barely grew (data not shown). The severely reduced growth phenotype is most likely a consequence of increased aberrant mitosis in the double mutant strains. As shown diagrammatically in Fig. 7 B, absence of the mph1+ gene product gave rise to a significant increase of abnormal mitoses from 18% for the single spi1-25 mutant to 39% for the double mutant. These data indicate that the missegregation of chromosomes is reduced in spi1-25 cells due to the spindle checkpoint system.

Mal3p and Spi1p Probably Act in Parallel Pathways

Mal3p is an evolutionary conserved microtubule-interacting protein that has been shown to associate with cytoplasmic and spindle microtubules (Beinhauer et al., 1997). Extra copies of mal3+ were able to rescue the TBZ hypersensitivity of the spi1-25 strain (see Fig. 2) but not other phenotypes associated with spi1-25, indicating that extra Mal3p can partially complement spi1-25 malfunction. However, we were unable to communoprecipitate Mal3p and Spi1p (data not shown), indicating that Mal3p and Spi1p probably do not interact physically.

We also found that nuclear transport of Mal3p was normal in the spi1-25 mutant by monitoring the in vivo localization of the Mal3p-GFP fusion protein (Beinhauer et al., 1997; data not shown).
Finally, genetic interactions between *spi1*<sup>+</sup> and *mal3*<sup>+</sup> were analyzed by constructing a *spi1*-25 *mal3*Δ double mutant. We found that the double mutant showed significantly reduced growth at 25°C, a temperature where *mal3*Δ single mutants grow normally, and *spi1*-25 mutants are affected only slightly (Fig. 8A, left). Other phenotypes were also additive: the double mutant population had 53% cells with abnormal cell form, whereas the single mutants *mal3*Δ and *spi1*-25 showed 26.5 and 9.7% aberrant cells, respectively (Fig. 8B). The *mal3*Δ *spi1*-25 strain was unable to grow on medium containing TBZ, whereas the single mutant strains showed reduced growth only (Fig. 8A). The finding that *spi1*-25 and *mal3*Δ mutations are additive in terms of phenotypes (Table II) indicates that their gene products probably act in parallel pathways.

**Discussion**

**Spi1-25 Has a Mutation in the Switch I Region of Fission Yeast SpRan**

The V44I mutation in Spi1-25p (V45 in mammalian Ran) is located in the Switch I effector binding region, which adopts a dramatically different structure depending on whether the protein is bound to GDP or GTP (Chook and Blobel, 1999; Vetter et al., 1999). A mutation in the T42 residue within the Switch I region of the mammalian Ran protein is able to bind to some, but not all, of its known binding partners. Because the *spi1*-25 mutation is within the Switch I region, and because the phenotype of *spi1*-25 mutant cells is different from that of *spi1* null cells, we first tested the possibility that, like T42A of human Ran, the V44I mutation in SpRan is a separation of function mutant. We found, however, that Spi1-25p is capable of interacting with several of its known binding partners in vivo and/or in vitro. These results, and the fact that *spi1*-25 mutant cells are viable, indicated that *spi1*-25 might, in fact, be a partial loss of function mutant. Consistent with this possibility, we found that the level of SpI1p was similar in wild-type and mutant cells but that only 30% of the *Spi1*-25 protein was capable of binding GTP in vitro. We presume that the remaining 70% of protein is misfolded. The mutant protein is also less efficient than wild-type in its ability to rescue the temperature-sensitive lethality of the *pim1*-d<sup>1ts</sup> SpRan-GEF mutant when overexpressed.

Even with a reduced level of active protein, *spi1*-25 cells are competent for nucleocyttoplasmic transport of both the endogenous Pap1p protein and a fusion protein targeted to the nucleus by the classical SV-40 NLS. We cannot exclude the possibility that the mutant has subtle defects in transport not detected in our assays or that it is defective in the transport of only a small subset of proteins that affect microtubule function. However, we did rule out the possibility that the microtubule defect in *spi1*-25 was the result of an inability to import the MAP Mal3p, which rescues this defect at elevated levels of expression.

When the pool of functional SpRan protein is reduced by the *spi1*-25 mutation cells have a specific defect in microtubule integrity. This is in contrast to the observations that temperature-sensitive mutations in the Ran-GEF in both fission yeast and mammalian cells have no obvious effect on spindle formation. Overexpression of Ran and RanBP1 cause chromosome missegregation and TBZ sensitivity in budding yeast by an unknown mechanism (for review see Sazer and Dasso, 2000).

Our results suggest the possibility that fission yeast SpRan has multiple independent functions which are differentially sensitive to loss of function of the GTPase system. The following data are consistent with this possibility: (a) our finding that imp2, a gene that encodes a protein that destabilizes the actin ring during septation is a high copy suppressor of the lethality of the RanGEF mutant *pim1*-d<sup>1ts</sup> at its semipermissive temperature of 34°C but not its restrictive temperature of 36°C (Demeter and Sazer, 1998); and (b) the observation that diploid fission yeast cells with a single copy of the wild-type *spi1*<sup>+</sup> gene lose chromosomes and haploidize (Matsumoto and Beach, 1991). We are currently testing this model by monitoring the phenotypes of cells with different levels of functional SpRan.

**SpRan Is Involved in the Integrity of Interphase Microtubules**

*spi1*-25 cells showed a variety of abnormal cell morphologies instead of the normal linear rod shape (see Fig. 1D) that were apparent at all temperatures but became more prominent with increasing temperature. Cytoplasmic microtubules play an important role in fission yeast cell morphogenesis (Verde et al., 1995; Mata and Nurse, 1997; Sawin and Nurse, 1998), and mutations causing altered interphase microtubule arrays lead to misshapen cells. Tubulin mutants (Toda et al., 1984), microtubule destabilizing drugs (Walker, 1982; Sawin and Nurse, 1998), or mutations in genes such as *tea2*<sup>+</sup> and *mal3*<sup>+</sup> (Verde et al., 1995; Beinhauer et al., 1997) that give rise to abnormally short interphase microtubules all lead to abnormally shaped cells. In wild-type cells, interphase microtubules extend along the long axis of the cell, reaching the cell tips (Hagan and Hyams, 1988). In contrast, the cytoplasmic microtubules in *spi1*-25 cells were often abnormally short or were positioned aberrantly (see Fig. 5). These data imply that SpRan is involved in the integrity of the cytoplasmic microtubule cytoskeleton.

**SpRan Affects the Formation and Function of the Mitotic Spindle**

We found two main aberrations in the formation of the mitotic spindle in *spi1*-25 cells. One phenotype was a star- or fan-shaped tubulin staining pattern, indicating that multiple microtubule bundles originate from a single focal point. Such staining patterns are typical of mutants with a defective SPB component (Hagan and Yanagida, 1995; Bridge et al., 1998; West et al., 1998) or mitotic motor protein (Hagan and Yanagida, 1992). Condensed chromatin was found in close proximity to the tubulin staining. The second phenotype was a tiny bipolar premetaphase spindle between barely separated SPBs. The chromatin in these cells still had the hemispherical appearance of an interphase nucleus, indicating a delay and/or arrest in very early spindle formation. We attempted to clarify these spindle phenotypes by various methods of cell synchronization but were unsuccessful due to the heterogeneous cell shapes of *spi1*-25 cells and their tendency to clump. Using
the mitotic stage–specific localization pattern of Cut11-GFPp (West et al., 1998), we found that spil-25 cells have an eightfold increase in prophase cells. In addition, spil-25 was synthetically lethal, with mutant alleles of cut11 encoding an SPB component required for bipolar spindle formation (West et al., 1998). Cells expressing this particular mutant SpRan protein are thus able to nucleate spindle microtubules but have problems with the establishment of a bipolar spindle or show a delay and/or arrest in the transition from a premetaphase to metaphase spindle.

Our phenotypic in vivo data are in accordance with recently obtained in vitro results demonstrating that Ran regulates spindle assembly in M phase Xenopus egg extracts by a yet unknown mechanism independent of nucleocyttoplasmic transport (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). When Ran-GTP levels were lowered in these extracts, spindle assembly was blocked, whereas high Ran-GTP levels promoted formation of spindle structures. We do not know whether the balance between Ran-GDP and Ran-GTP is altered in spil-25 cells, but there is a strong genetic interaction between spil-25 and the piml-d1Δ Ran-GEF mutant, which is predicted to have low Ran-GTP levels.

**spil-25 Interacts Genetically with the Spindle Checkpoint Pathway**

The spindle checkpoint pathway arrests cells at the metaphase to anaphase transition when chromosomes are not attached properly to the mitotic spindle (for review see Gardner and Burke, 2000). Although several mitotic spindles in spil-25 cells appear morphologically normal, the increase in chromosome loss and missegregation indicates that these spindles are functionally defective. We confirmed this by showing that loss of the spindle checkpoint gene mphl (He et al., 1998b) exacerbates the growth and chromosome missegregation defects of spil-25. Because Mph1p is required for checkpoint activation, but unlike its *S. cerevisiae* homologue Mps1p is not essential for viability or spindle pole body duplication (He et al., 1998b), our results suggest that the spindle defects in spil-25 are monitored by the spindle checkpoint pathway which transiently arrests cells in metaphase until the defects are corrected.

Although we cannot rule out the possibility that Mad2p is not required for this cell cycle delay, the lack of interaction between mad2Δ (He et al., 1997) and spil-25 most likely reflects these facts: (a) mad2Δ is less sensitive to microtubule destabilizing drugs than mphlΔ (Kadura, S., and S. Sazer, unpublished results); (b) Mph1p acts upstream of two branches of the checkpoint pathway, only one of which includes Mad2p (for review see Taylor, 1999); and (c) the interactions among components of the checkpoint pathway that have been placed in a linear genetic pathway are complex and dynamic (Brady and Hardwick, 2000).

**Mal3p Can Suppress the TBZ Hypersensitivity of spil-25 Cells**

Although the exact role of the SpRan GTPase in spindle formation is not yet known, the finding that extra copies of mal3Δ, encoding a MAP, were able to partially complement spil-25 malfunction points to a role of Spilp in microtubule integrity. Mal3p may act by stabilizing microtubules since the lethal overexpression phenotype of mal3Δ can be rescued by decreasing microtubule stability by various means (Beinhauer et al., 1997), and the absence of Mal3p in vivo affects microtubule dynamics by leading to a reduction in the microtubule growth rate (Ding, D.-Q., and Y. Hiraoka, personal communication).

spil-25 and mal3Δ mutations cause different spindle defects and are additive in all phenotypes analyzed, suggesting that these two gene products act in parallel pathways. The exact role of Mal3p on mitotic spindle formation and/or function is at present unclear. Absence of mal3Δ leads to greatly reduced spindle staining and an increased chromosome condensation index (Beinhauer et al., 1997). The *mal3+* homologue *BMI1/YEB1* in *S. cerevisiae* also influences formation and function of the mitotic spindle (Schwartz et al., 1997; Mu-hua et al., 1998; Tirnauer et al., 1999). Bim1 was isolated in a budding yeast SPB preparation (Wigge et al., 1998), indicating an affinity of this protein family for the SPB. In this context, it is interesting to note that we have identified a novel, evolutionary conserved SPB component in a suppressor screen of *mal3-1* mutant phenotypes (Decker, S., and U. Fleig, unpublished data).

Given the observation that Mal3p can partially suppress Spil-25p malfunction together with the mitotic spindle defects seen in *spil-25* cells, we propose that the SpRan GTPase is required for the very early stages of spindle formation by possibly exerting a microtubule stabilizing function. We do not propose that SpRan is involved directly in microtubule integrity, as purified Ran has no effect on microtubule polymerization (Wilde and Zheng, 1999), and Spilp does not interact with tubulin (see Table II), but suggest that it regulates a component(s) required for microtubule dynamics. Analysis of our remaining *spil-25* multicopy suppressors might help in the identification of such a component.

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