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A fission yeast RCC1-related protein is required for the mitosis to interphase transition

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The isolation and characterization of the mutant *dcd*^{ts} (defect in chromatin decondensation) has led to the identification of two conserved proteins required for the re-establishment of the interphase state following the completion of mitosis. The gene that rescues the *dcd*^{ts} mutant encodes a protein similar to the human chromatin binding protein, RCC1. A suppressor of *dcd*^{ts} encodes a protein nearly identical to the human GTP-binding protein, RAN, encoded by the *TC4* gene. These results indicate that completion of mitosis is regulated at least in part by a GTPase molecular switch. The gene and suppressor of *dcd*^{ts} are identical to the previously described *Schizosaccharomyces pombe* genes *pim1* (premature initiation of mitosis) and *spi1* (suppressor of pim), but the *dcd*^{ts} mutant does not enter mitosis prematurely, a phenotype that has been reported for the *pim1*-46^{ts} mutant. Based on our studies we propose that the *pim1* gene product is required for regulating chromatin condensation with a primary role at the end of mitosis and pleiotropic effects on other aspects of cell behavior.

Introduction

Considerable progress has been made towards elucidating the molecular mechanisms that control entry into mitosis, when the replicated chromosomes are segregated into the two daughter cells. Central to this mechanism is a complex between the p34^{cdc2} protein kinase and cyclin, that becomes activated at the G_2-mitosis transition and brings about the phosphorylation of proteins involved in the process of mitosis (reviewed in Nurse, 1990). In contrast, rather less is known about the biochemical and structural changes associated with the re-establishment of the interphase state following mitosis. At a late stage in mitosis cyclin and other proteins are degraded, the p34^{cdc2} protein kinase is inactivated and cells exit from mitosis (Murray *et al.*, 1989; reviewed in Nurse, 1990; Holloway *et al.*, 1993). It is also likely that the proteins phosphorylated by the mitotic initiator p34^{cdc2} are dephosphorylated at the completion of mitosis, consistent with the fact that protein serine-threonine phosphatases are required for the completion of mitosis in *S.pombe*, *Aspergillus nidulans* and *Drosophila melanogaster* (Booher and Beach, 1989; Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990; Fernandez *et al.*, 1992).

Of the original collection of fission yeast cell cycle mutants none is defective at the mitosis-interphase transition (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981). This may be due to the fact that these temperature sensitive (ts) mutants were selected for their ability to elongate at the restrictive temperature, and cells do not elongate during the last quarter of the cell cycle during which mitosis takes place (Mitchison, 1970). However, visual screens for fission yeast mutants defective in various stages of mitosis have been successfully conducted (reviewed in Yanagida, 1989). In order to investigate specifically the mitosis-interphase transition we conducted a screen to identify fission yeast cell cycle mutants that have successfully completed mitosis but have not yet replicated their DNA. Temperature sensitive lethal mutants were isolated and screened for alterations in both DNA content and nuclear morphology. One of these mutants, called *dcd*^{ts} for defect in chromatin decondensation, is described here. This mutant is an allele of a previously described gene, *pim1*$^{+}$ (Matsumoto and Beach, 1991), that encodes an RCC1-related protein. A suppressor of *dcd*^{ts} was also isolated and found to be a GTPase encoded by the previously described *spi1*$^{+}$ gene (Matsumoto and Beach, 1991). Our results demonstrate that these two gene products function at the mitosis-interphase transition. This conclusion contrasts with an earlier suggestion that these genes are concerned with regulating the onset of mitosis and its coupling to completion of the previous S-phase (Matsumoto and Beach, 1991).

Results

Isolation and characterization of the dcd^{ts} *mutant*

In a screen designed to isolate fission yeast mutants defective in the mitosis-interphase transition, we characterized a bank of ts lethal mutants that grow normally at the permissive temperature of 25°C but do not form colonies at the restrictive temperature of 36°C. Each mutant was subjected to flow cytometric analysis to measure DNA content, and microscopic examination of DAPI-stained cells to determine alterations in nuclear morphology. The *dcd*^{ts} mutant isolated in this screen was of particular interest because upon incubation at 36°C it undergoes uniform cell cycle arrest with the chromosomes in a condensed post-anaphase state and a 1C DNA content per nucleus. Genetic analysis shows that the *dcd*^{ts} mutation is recessive, and that the ts phenotype is the result of this single mutation.

Cytological examination of the *dcd*^{ts} mutant indicates that it is unable to re-establish properly the interphase state following mitosis. *dcd*^{ts} cells in interphase have decondensed chromatin and nuclei that are uniformly stained with DAPI (Figure 1A, 1). At the restrictive temperature

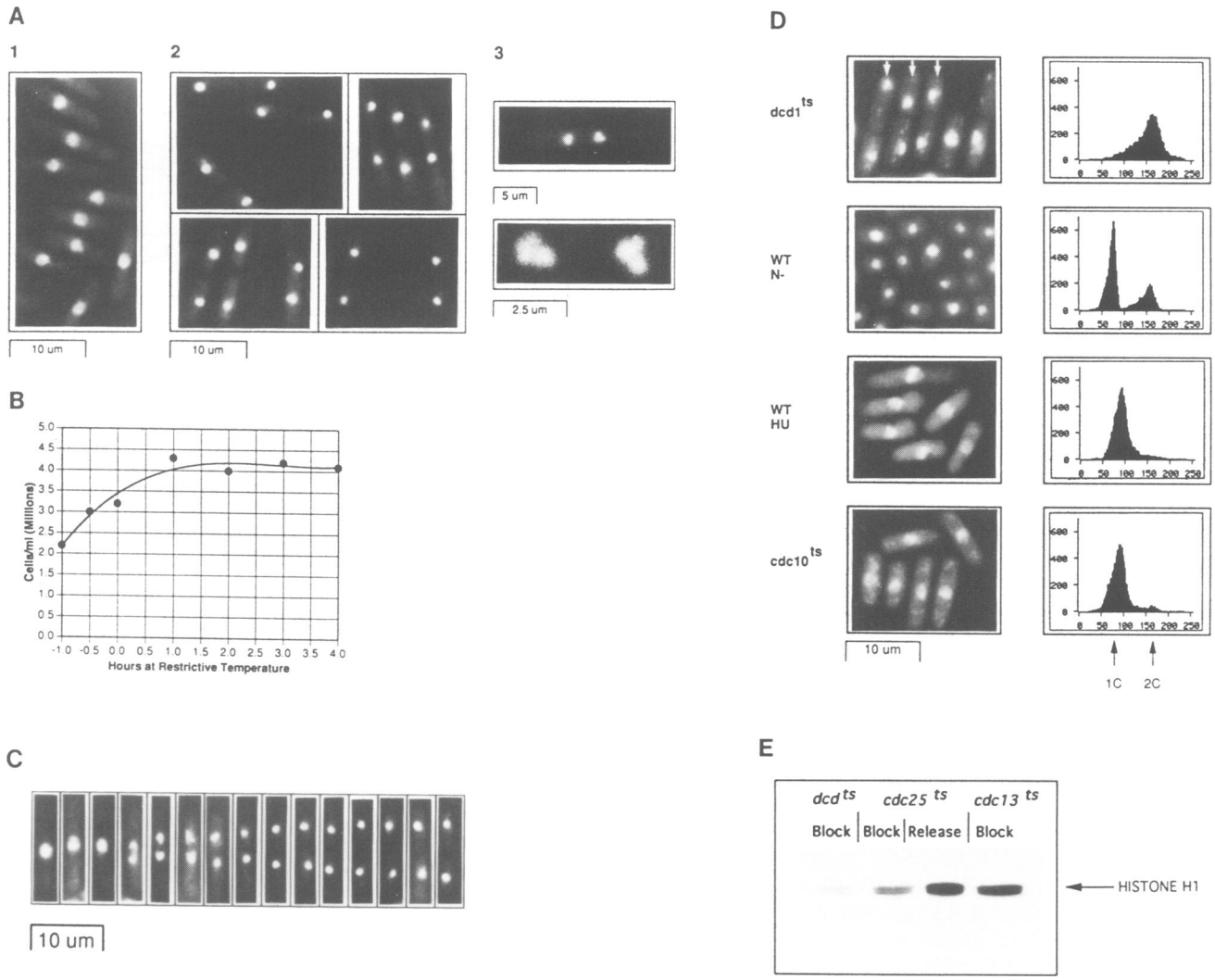

Fig. 1. *dcd*^{ts} cells arrest rapidly at the restrictive temperature with condensed mitotic chromatin. (**A**) Cells grown at 25°C were then incubated at 36°C and stained with DAPI. (1) *dcd*^{ts} cells showing interphase nuclei. (2) *dcd*^{ts} cells at 36°C showing condensed chromatin. (3) Two magnifications of *dcd*^{ts} cell with three nuclear lobes in each half of the cell. (**B**) Cell number was monitored in an asynchronous culture of *dcd*^{ts} cells grown at 25°C then incubated at 36°C for 4 h. (**C**) *dcd*^{ts} cells arrested at various stages of mitosis stained with DAPI and organized according to distance between the dividing nuclei. (**D**) Comparison of the nuclear morphology of DAPI-stained cells and the DNA content of *dcd*^{ts} arrested cells compared with those of nitrogen-starved wild type cells, HU arrested wild type cells and *cdc10*-129^{ts} arrested cells. Left panel: cells stained with DAPI. Right panel: DNA content measured by FACS. (**E**) Asynchronous cultures of *dcd*^{ts} (lane 1), *cdc25*-22^{ts} (lane 2; blocking at G_2-M) and *cdc13*-117^{ts} (lane 4; blocking in mitosis), were grown at 25°C and then shifted to 36°C for 4 h. Half of the *cdc25*-22^{ts} culture was then rapidly cooled to 25°C for 15 min to release the cells from the G_2 block and allow them to enter mitosis synchronously (lane 3). Protein kinase assays were performed on immunoprecipitated p34^{cdc2} using histone H1.

the cells arrest with two nuclei showing condensed chromatin and have a medial septum (Figure 1A, 2). Sometimes it is possible to see clearly three nuclear lobes in each cell half which may be the three condensed chromosomes (Figure 1A, 3).

The *dcd*^{ts} cell cycle arrest is established rapidly. When an asynchronous population of cells is shifted to 36°C, the cell number increases only ~1.2-fold at the restrictive temperature (Figure 1B). This indicates that the cell cycle block is imposed very rapidly. Because ~20% of the cells divide (those positioned between the *dcd*^{ts} execution point and cytokinesis) we estimate that the transition point is at 0.75 of a cell cycle, at the time of mitosis. This indicates that the primary defect of the mutant is at the end of the cell cycle.

The nuclear morphology of the *dcd*^{ts} mutant is indicative of cells arrested at the end of mitosis. When we examined mutant cells incubated at the restrictive temperature we found cells with condensed chromatin at all stages of mitosis, examples of which are shown in Figure 1C. From this we conclude that *dcd*^{ts} cells condense their chromosomes in mid-mitosis and that the chromosomes remain condensed from then on. Cells arrested at the *dcd*^{ts} block point do not undergo the subsequent S-phase. When an asynchronous culture is incubated at the restrictive temperature for 4 h, binucleated cells accumulate with a 2C DNA content per cell (Figure 1D) indicating that the DNA content per nucleus is 1C. This finding was confirmed by monitoring DNA content in a synchronous culture incubated for 8 h at the restrictive temperature (see Figure 4B).

To demonstrate that the small size of the nucleus in *dcd*^{ts} arrested cells is due to chromatin condensation and not to the reduction in DNA content from 2C to 1C per nucleus, we compared the morphology and DNA content of these cells

with those of three samples that also have a 1C DNA content per nucleus: wild type cells arrested with a 1C nuclear DNA content by nitrogen starvation, wild type cells arrested in G_1 with a 1C DNA content by hydroxyurea (HU), and the *cdc10*-129^{ts} mutant arrested in G_1 with a 1C DNA content at the restrictive temperature (Figure 1D). FACS analysis of the DNA content showed that the cells in all four samples have an identical (1C) DNA content per nucleus. The three arrowed cells in the *dcd*^{ts} panel show the terminal phenotype with condensed nuclei while the shorter cell, which has not yet reached the terminal phenotype, has a normal sized nucleus and is included for internal comparison. The DNA in the *dcd*^{ts} arrested cells is compacted relative to that in the HU and *cdc10*-129^{ts} arrested cells. These results show that the reduction in size of the DAPI-staining portion of the nuclei in the *dcd*^{ts} cells is due to an alteration in chromatin structure and not to a reduction in DNA content. The DNA in the nitrogen-starved cells is compacted more than that in the HU and *cdc10*-129^{ts} arrested cells and is similar to that in the *dcd*^{ts} cells. This is considered further later.

The *dcd*^{ts} cells have, however, undergone cytokinesis and have a medial septum (see Figure 1A, 2) indicating that cytoplasmic aspects of cell cycle progression continue although the nuclear cycle is blocked with the chromatin in a condensed state.

Because p34^{cdc2} protein kinase activity peaks at mitosis (Moreno *et al.*, 1989) it is a good marker of the mitotic state of a cell. We immunoprecipitated p34^{cdc2} from cell extracts followed by assay of H1 histone kinase activity from equal amounts of p34^{cdc2}. In a *dcd*^{ts} asynchronous culture incubated for 4 h at 36°C the p34^{cdc2} protein kinase activity is relatively low (Figure 1E, lane 1). This can be seen by comparison with the level of kinase activity found in mitotic cells generated either in the *cdc13*-117^{ts} mutant which blocks in mid-mitosis (Figure 1E, lane 4) or a *cdc25*-22^{ts} mutant first blocked in late G_2 (Figure 1E, lane 2) and then released to obtain a uniform population of mitotic cells (Figure 1E, lane 3). These data also indicate that the failure of the *dcd*^{ts} mutant to complete mitosis is not due to an inability to inactivate p34^{cdc2}.

Once cells have arrested at the *dcd*^{ts} block point they fail to progress into S-phase (see Figure 1D). However, we do not believe that *dcd*$^{+}$ is required for DNA replication because G_1 synchronized cells are capable of doubling their DNA content at the restrictive temperature. Haploid spores containing the *dcd*^{ts} allele are able to germinate and to undergo S-phase at the restrictive temperature (Figure 2A). DNA replication also occurs when *dcd*^{ts} cells arrested in G_1 by nitrogen starvation are released from this block at the restrictive temperature (Figure 2B).

These data indicate that *dcd*^{ts} cells are blocked at the exit from mitosis and have features both of the mitotic state, namely condensed chromatin, and of the G_1 interphase state, namely a septum and low p34^{cdc2} kinase activity.

dcd^{ts} *is an allele of pim1*$^{+}$

The gene complementing the *dcd*^{ts} mutation was cloned by transforming the mutant cells with a cDNA library expressed from the *nmt1* promoter suppressed to very low levels of transcription using thiamine (Maundrell, 1990). Integrative transformation followed by Southern blotting showed that one of the complementing cDNAs contained the authentic

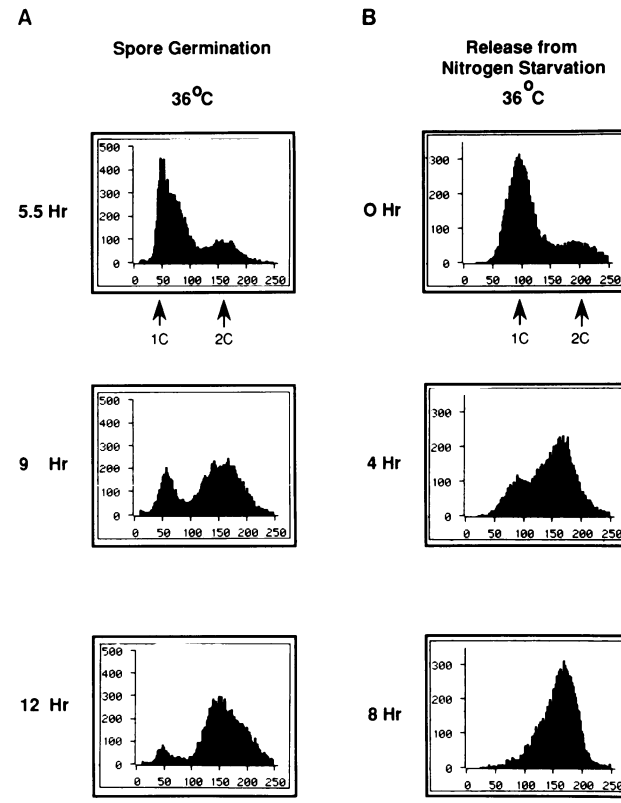

Fig. 2. *dcd*^{ts} cells synchronized in G_1 can undergo DNA replication at 36°C. (**A**) FACS analysis of the DNA content of *dcd*^{ts} germinated in yeast extract at 36°C. (**B**) FACS analysis of the DNA content of *dcd*^{ts} cells synchronized in G_1 by nitrogen starvation, and released to complete medium at 36°C.

gene and genetic mapping established that the locus is on the right arm of chromosome II between the *his2* and *cdc10* genes. The gene has been physically mapped using an ordered library of cosmid clones (Maier *et al.*, 1992; Hoheisel *et al.*, 1993) to a position ~265 kbp from *cdc10* and ~1150 kbp from the centromere. *dcd*^{ts} mutant cells transformed with this cDNA could grow at the restrictive temperature when expression was low but not when the level was high. Overproduction of the gene in wild type cells is also lethal.

While this work was in progress a paper was published describing the fission yeast mutant *pim1*-46 (premature initiation of mitosis) reported to advance cells into mitosis even in the absence of DNA replication (Matsumoto and Beach, 1991). The *dcd*^{ts} mutant is an allele of *pim1*$^{+}$, as demonstrated both by genetic mapping to the same chromosomal location as *pim1*$^{+}$ and by DNA sequence identity. We have therefore renamed the *dcd*^{ts} mutant *pim1*-d1^{ts}. A comparison of our cDNA sequence with the published genomic sequence of *pim1*$^{+}$ (Matsumoto and Beach, 1991) identifies a single 175 bp intron in the gene that extends from nucleotide 607 to 781 relative to the AUG codon at position 1, and a polyadenylation site 77 bp 3′ of the TAG stop codon.

A suppressor is allelic to spi1^{+}

The suppressor of *pim1*-d1^{ts} was isolated from a multicopy *S.pombe* genomic gene bank by its ability to rescue the ts lethality of *pim1*-d1^{ts} and genetic and Southern blot analyses showed that a plasmid integrated by homologous recomb-

ination was not linked to the *pim1*$^{+}$ locus, demonstrating that this gene encodes an extragenic suppressor of *pim1*-d1^{ts}. Sequencing showed that the suppressor, which we originally named *fyt1* (fission yeast TC4), was identical to *spi1*$^{+}$, an extragenic suppressor of *pim1*-46^{ts} (Matsumoto and Beach, 1991). The corresponding cDNA was isolated by hybridization and sequenced. Sequence comparison of this cDNA clone with the genomic sequence (Matsumoto and Beach, 1991) established that there are introns extending from nucleotide 126 to 307 and from 495 to 531 relative to the ATG codon at position 1. The predicted product of the *spi1*$^{+}$ gene is a small GTP-binding protein of the superfamily typified by p21^{ras} (Bourne *et al.*, 1991; Matsumoto and Beach, 1991). The spi1 protein is 80% identical and 92% similar to a human GTP-binding protein called RAN (Bishoff and Ponstingl, 1991a) encoded by the *TC4* gene (Drivas *et al.*, 1990). Bishoff and Ponstingl (1991b) have shown that human RCC1 acts *in vitro* as a nucleotide exchange factor for the RAN protein, facilitating its conversion to the GTP-bound form. We introduced mutations into the genomic clone of *spi1*, at positions 18 and 67 corresponding respectively to amino acids 12 and 61 in p21^{ras}, that stabilize p21^{ras} in its GTP-bound state (reviewed in Barbacid, 1987). When introduced into either wild type or *pim1*-d1^{ts} cells grown at the permissive temperature no transformants were obtained, consistent with the hypothesis that even in the presence of wild type spi1 protein overproduction of the mutant Arg18Arg67 or Val18 spi1 protein is lethal. This result is also consistent with results of a comparable experiment conducted in *S.cerevisiae* in which the activated form of the spi1 homolog, GSP1, had a transformation efficiency 1000-fold lower than that of the wild type gene (Belhumeur *et al.*, 1993). Introduction of the activated form of the human RAN protein into mammalian cells also causes a cessation of cell division (Ren *et al.*, 1993). We did not, however, observe this lethality when the Arg18 Arg67 mutations were introduced into the *spi1* cDNA transcribed to maximum levels from the *nmt1* promoter, a result that is probably due to inappropriate or reduced expression.

In *S.cerevisiae*, cells lacking the guanine nucleotide exchange factor, CDC25, can be rescued by expression of an activated form of RAS2 (Broek *et al.*, 1987; Robinson *et al.*, 1987). We tested the ability of the activated form of spi1 to rescue the pim1-d1^{ts} phenotype by plating *pim1*-d1^{ts} cells transformed with the Arg18 Arg67 or Val18 genomic *spi1* plasmids directly at the restrictive temperature. No transformants were obtained, suggesting that under the control of the *spi1* promoter this mutated gene could not rescue the pim1-d^{ts} phenotype.

pim1-d1^{ts} cells enter mitosis with normal kinetics at the restrictive temperature

Our analysis had shown that the pim1^{+} protein acts at the mitosis – interphase transition and produced no evidence that it is part of the control linking the initiation of mitosis to completion of S-phase. However, the sequence of *pim1*$^{+}$ is similar to that of RCC1, a protein thought to coordinate these two events in mammalian cells (Nishitani *et al.*, 1991), and Matsumoto and Beach (1991) have proposed that it plays a similar role in fission yeast. We therefore investigated whether the *pim1*-d1^{ts} mutant would enter mitosis prematurely in the absence of S-phase.

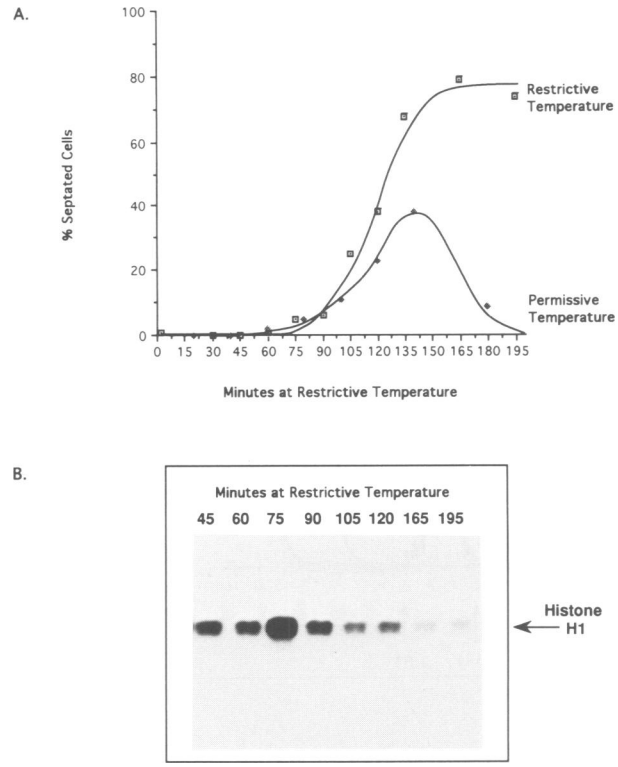

Fig. 3. *pim1*-d1^{ts} cells activate p34^{cdc2} histone H1 kinase with normal kinetics at the restrictive temperature. Small cells in early G_2 were selected by centrifugal elutriation of *pim1*-d1^{ts} at 25°C. The culture was then incubated either at 25°C (permissive temperature) or at 36°C (restrictive temperature). (**A**) The percentage of septated cells. (**B**) Protein kinase assays on immunoprecipitated p34^{cdc2} using histone H1.

First we determined whether the entry of *pim1*-d1^{ts} cells into mitosis was temporally accelerated. A population of G_2 cells was size selected from an asynchronous culture of *pim1*-d1^{ts} grown at 25°C using centrifugal elutriation. The synchronous culture was then incubated at the restrictive temperature of 36°C. We followed progression into mitosis by monitoring the percentage of cells with septa because the percentage of cells in mitosis, as monitored by the appearance of binucleated cells, exactly parallels the appearance of septated cells. We found no evidence of advancement into cell division. The percentage of septa in the culture incubated at the restrictive temperature increased at the expected time for normal entry into mitosis and septated cells with the typical pim1-d1^{ts} phenotype accumulated (Figure 3A). p34^{cdc2} protein kinase activation and inactivation also occurred on schedule (Moreno *et al.*, 1989). The kinase activity increased ~1 h prior to the peak of septation and dropped to a low level as the terminal phenotype appeared (Figure 3B). Therefore the timing of the entry into mitosis is not accelerated at 36°C.

pim1-d1^{ts} cells enter mitosis only after DNA replication has occurred

We also investigated the possibility that the *pim1*-d1^{ts} mutant could enter mitosis at the restrictive temperature if DNA replication is inhibited. Matsumoto and Beach (1991) concluded that at the restrictive temperature *pim*-46^{ts} prematurely initiates mitosis, based largely on the appearance of post-mitotic cells in the presence of the DNA synthesis inhibitor HU. Using an identical experimental protocol, in

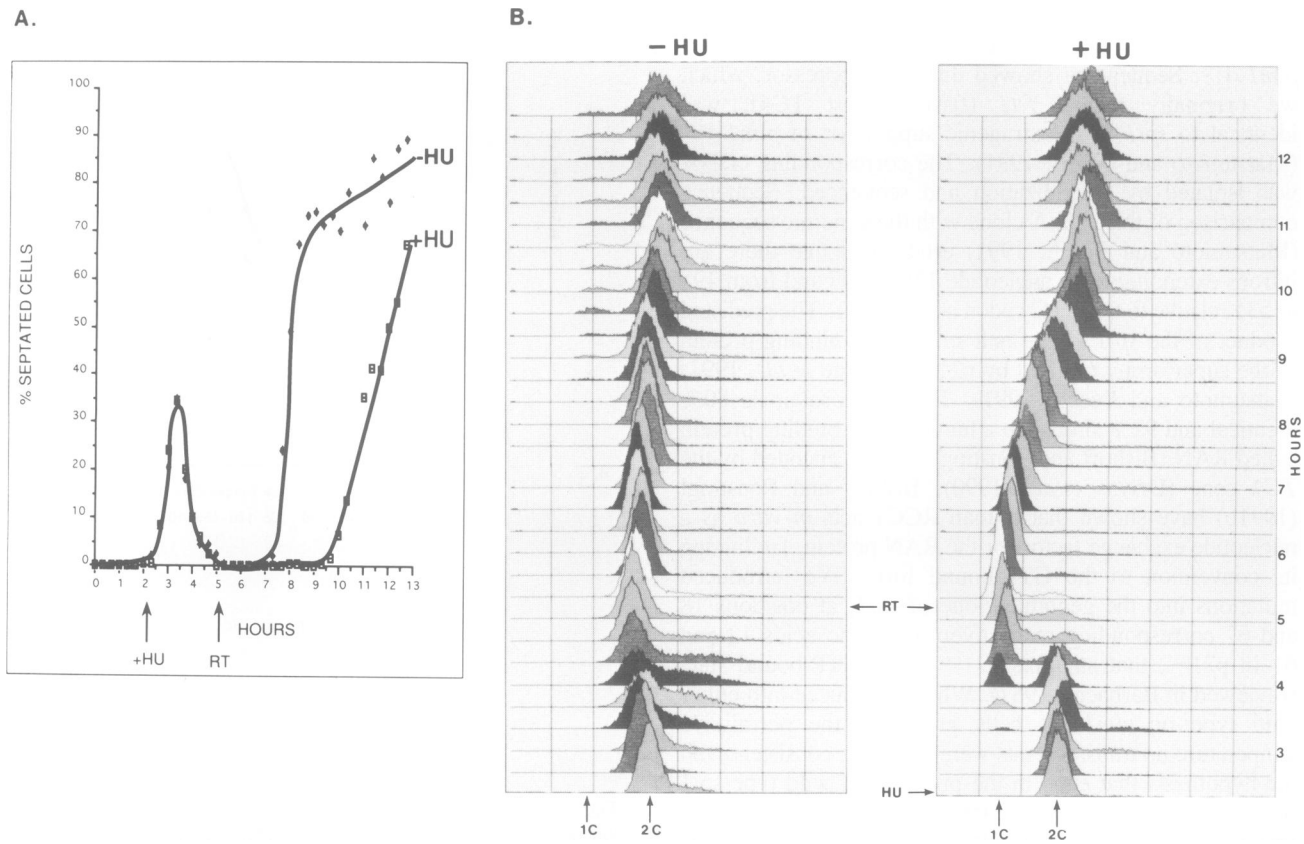

Fig. 4. *pim1*-d1^{ts} cells enter mitosis only after DNA replication has occurred. Small *pim1*-d1^{ts} cells in early G_2 were selected by centrifugal elutriation at 25°C. The culture was incubated at 25°C until just before the appearance of septated cells when HU was added to one-half of the cells to a final concentration of 12 mM (+HU). Following the first synchronous round of mitosis both cultures were shifted to 36°C (RT). (**A**) The percentage of septated cells. (**B**) DNA content of cells measured by FACS.

which cells are treated with HU for 8 h (4 h at the permissive temperature and 4 h at the restrictive temperature) we found that wild type, *pim1*-d1^{ts} and *pim1*-46^{ts} cells behave the same. They are only transiently inhibited by this HU block, double their DNA content and subsequently enter mitosis. To avoid possible complications due to population heterogeneity or long exposure of cells to the DNA synthesis inhibitor HU, we monitored cell cycle progression in a synchronous culture of *pim*-d1 treated with HU near the G_1-S boundary.

A synchronous culture of *pim1*-d1^{ts} was prepared by centrifugal elutriation and inoculated at 25°C. Because DNA replication occurs immediately after septation, HU was added just before the appearance of septated cells in order to block the next S-phase (Figure 4A). The timing of HU addition minimized the exposure of cells to HU and effectively blocked replication (Figure 4B). Cells were then shifted to the restrictive temperature before initiation of the next mitosis. Control cells to which no HU had been added underwent septation on schedule, and accumulated septated binucleate cells typical of the pim1-d1^{ts} phenotype. In contrast, no septation was observed at this time in the culture to which HU had been added. Septated cells eventually accumulate in the HU-treated culture but only 3 h after septation had been observed in the control cells. Microscopic observation of fixed cells stained with the fluorescent DNA-binding dye DAPI confirmed the timing of cell cycle events described above, and was used to determine that at the restrictive temperature the appearance of cells with the pim1-d1^{ts} phenotype exactly parallels the appearance of septated cells both in the absence and presence of HU. To determine if these cells had entered mitosis prior to the completion of S-phase we monitored the DNA content of cells using flow cytometry (Figure 4B). The untreated control culture maintains a 2C DNA content throughout the course of the experiment. In the treated cells, DNA synthesis is completely blocked for 2 h and replication occurs gradually during the ensuing 2 h. It is only after this increase in DNA content that the *pim1*-d1^{ts} cells enter mitosis. Thus *pim1*-d1^{ts} cells do not enter mitosis prematurely when DNA synthesis is blocked by HU. The presence of HU in fact delays the onset of mitosis by several hours.

DNA replication can also be inhibited by arresting cells in G_1 at start using the ts *cdc10*-129 mutant. A double mutant, *pim1*-d1^{ts} *cdc10*-129^{ts}, was constructed and starved of nitrogen to synchronize the cells in G_1. This synchronization step ensures that upon release into complete medium the cells will reach the *cdc10*-129^{ts} G_1 block point before that imposed by *pim1*-d1^{ts}. As previously discussed (Figure 1D) nitrogen-starved fission yeast cells have condensed chromatin. After starvation, cells were transferred to complete medium at the restrictive temperature and chromatin condensation was monitored. After 2–4 h at the restrictive temperature in complete medium, wild type and *cdc10*-129^{ts} cells decondense their chromatin (Figure 5A). The *pim1*-d1^{ts} mutant is somewhat delayed in decondensing

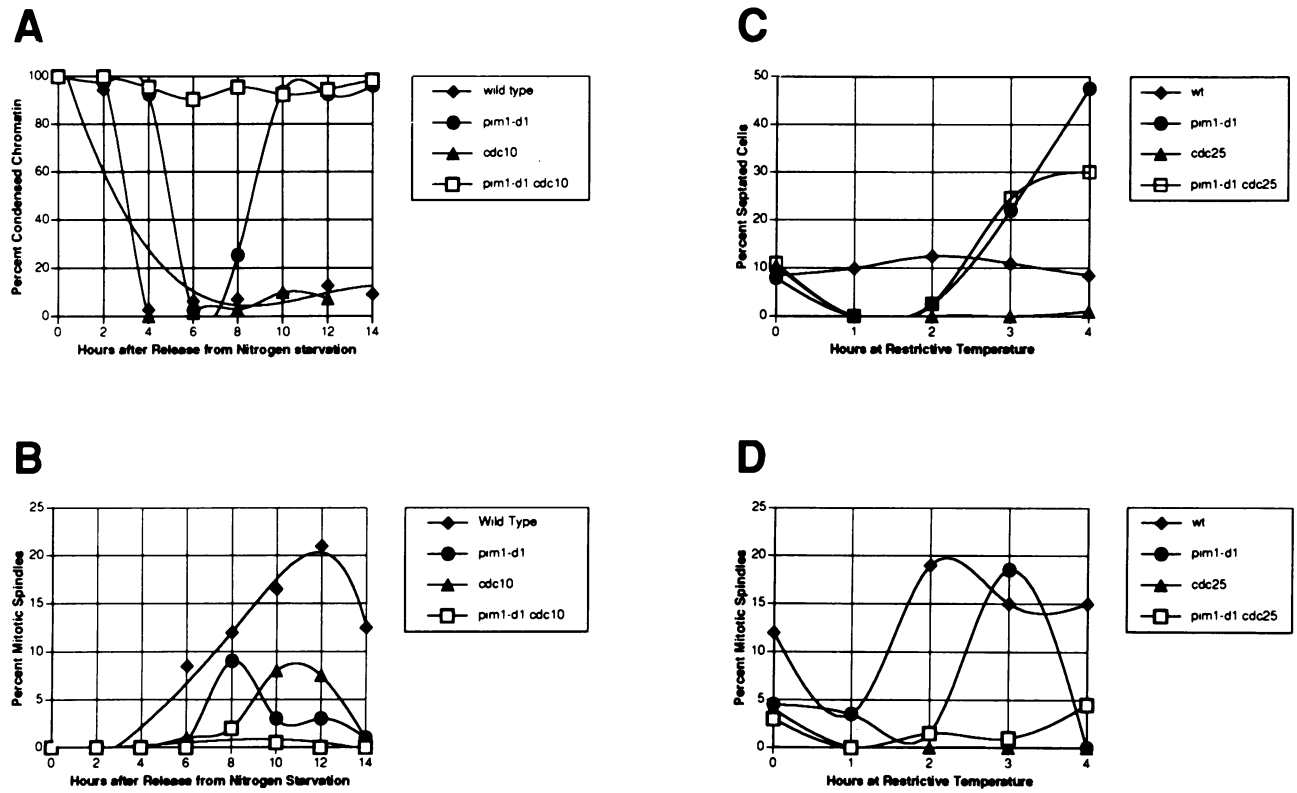

Fig. 5. *pim1*-d1^{ts} cells do not enter mitosis from a G_1 or a G_2 cell cycle block. (**A** and **B**) Wild type cells, the *pim1*-d1^{ts} and *cdc10*-129^{ts} single and the *pim1*-d1^{ts} *cdc10*-129^{ts} double mutant were grown at 25°C and starved of nitrogen for 24 h to arrest cells in G_1 prior to the *cdc10*-129^{ts} block point. Cells were then shifted to complete medium at 36°C. Cells were stained with DAPI and the anti-tubulin antibody TAT-1. (A) The percentage of cells with condensed chromatin. (B) The percentage of cells with mitotic spindles. (**C** and **D**) Wild type cells, the *pim1*-d1^{ts} and *cdc25*-22^{ts} single and the *pim1*-d1^{ts} *cdc25*-22^{ts} double mutants were grown at 25°C and shifted to 36°C for 4 h. Cells were stained with the TAT-1 anti-tubulin antibody. (C) The percentage of cells that are septated. (D) The percentage of cells with mitotic spindles.

its chromatin, taking 4–6 h, roughly coincident with the doubling in DNA content (Figure 2B). At 8–10 h the chromosomes condense as the cells undergo mitosis and express the pim1-d1^{ts} terminal phenotype (Figure 5A and B). In contrast we found that the *pim1*-d1^{ts} *cdc10*-129^{ts} double mutant never decondenses its chromosomes (Figure 5A), suggesting that it cannot recover properly from the condensed chromosome state brought about by nitrogen starvation. The terminal phenotype of the *pim1*-d1^{ts} *cdc10*-129^{ts} double mutant is a single condensed nucleus with no septum. This is identical to the phenotype of the *pim1*-46^{ts} *cdc10*-129^{ts} double mutant (Matsumoto and Beach, 1991) and differs from the terminal phenotype of *pim1*-d1^{ts} and *pim1*-46^{ts} cells with two condensed nuclei and a septum. It appears that the *cdc10*-129^{ts} mutation, which blocks cells in G_1 before S-phase, prevents expression of the normal late mitotic pim1-d1^{ts} mutant phenotype. This conclusion was confirmed by monitoring the percentage of mitotic spindles during this experiment (Figure 5B). The wild type, *cdc10*-129^{ts} and *pim1*-d1^{ts} cells generated mitotic spindles as they underwent mitosis. Consistent with our conclusion that the double mutant does not enter mitosis prematurely is the observation that in this strain almost no mitotic spindles were observed.

Behavior of pim1-d1^{ts} cdc25-22^{ts} cells

We also examined the effect of blocking cells later in the cell cycle on the ability of the *pim1*-d1^{ts} mutant to enter mitosis. The *cdc25*-22^{ts} mutant blocks cells in late G_2 before initiation of mitosis (Fantes, 1979). The double mutant with *pim1*-d1^{ts} gives an unusual phenotype similar to that reported for *pim1*-46^{ts} *cdc25*-22^{ts} cells. A proportion of cells undergo septation with a single nucleus located in one of the two daughter cells (Figure 5C; Matsumoto and Beach, 1991) but there is no evidence of chromosome segregation and no mitotic spindles were constructed during generation of the pim1-d1^{ts} cdc25-22^{ts} phenotype (Figure 5D). Thus these cells arrested in late G_2 do not appear to undergo a normal mitosis although they do become septated which is characteristic of an event occurring at the end of mitosis. This may be related to the role of *pim1*$^{+}$ at the end of mitosis.

Discussion

pim1-d1^{ts} cells fail to re-establish properly the interphase state following mitosis

The proper completion of mitosis involves structural and biochemical changes necessary for re-establishment of the interphase state and preparation for the next cell division. Prominent among these changes is the conversion of the condensed mitotic chromosomes into a decondensed interphase state. We have isolated a ts fission yeast cell cycle mutant defective in a gene we originally named *dcd*^{ts} for defect in chromatin decondensation. Because we now know that *dcd*^{ts} is an allele of *pim1*$^{+}$ we have renamed it *pim1*-d1^{ts}. A G_1 population of *pim1*-d1^{ts} cells incubated at the restrictive temperature undergoes DNA synthesis and mitosis as do wild type cells, but following mitosis the chromatin fails to decondense and the cells do not initiate

the subsequent round of DNA synthesis. Other changes associated with progression into interphase, such as septation, and a decrease in p34^{cdc2} protein kinase activity take place normally. At the restrictive temperature the kinetics of entry into mitosis and the activation of p34^{cdc2} in *pim1*-d1^{ts} cells grown are similar to those of wild type cells.

pim1-d1^{ts} arrested cells display certain characteristics of an interphase state in the presence of condensed chromatin but fail to progress into S-phase. The fact that G_1-synchronized *pim1*-d1^{ts} cells can replicate their DNA when incubated at the restrictive temperature argues that the pim1$^+$ protein is not required for DNA replication *per se*. The failure of *pim1*-d1^{ts} arrested cells to enter S-phase may therefore be due to a more general effect such as the inability of this hyper-condensed chromatin to be replicated and/or transcribed.

pim1$^+$ was initially reported to prevent mitotic entry in the absence of DNA synthesis

While our work was in progress, a mutant, *pim1*-46^{ts} (premature initiation of mitosis), with a phenotype similar to that of *pim1*-d1^{ts}, was isolated (Matsumoto and Beach, 1991). *pim1*-d1^{ts} and *pim1*-46^{ts} are alleles of the same gene and are suppressed by the same *TC4/RAN*-like gene. Furthermore, the cytological phenotypes of asynchronous populations shifted to the restrictive temperature and of double mutants constructed with *cdc10*-129^{ts} and *cdc25*-22^{ts} are identical. We have shown that the *pim1*-d1^{ts} mutant is defective in the mitosis−interphase transition and only enters mitosis after replicating its DNA. In contrast, Matsumoto and Beach (1991) conclude that the appearance of mitotic cells in a culture of *pim1*-46^{ts} cells treated for 8 h in HU was evidence of premature initiation of mitosis. We found that wild type cells also undergo mitosis after an 8 h treatment with HU suggesting that this phenotype is not the result of a mutation in the *pim1* gene. We have also found that in both cases the cells enter mitosis only after recovery from the transient inhibition of DNA synthesis by HU. The premature activation of p34^{cdc2} reported when *pim1*-46^{ts} cells are shifted to the restrictive temperature is <2-fold, does not correlate with tyrosine dephosphorylation of p34^{cdc2} and occurs only 3 h after a shift to the restrictive temperature (Matsumoto and Beach, 1991). We obtained no evidence of premature increase in kinase activity in either asynchronous or synchronous cultures of *pim1*-d1^{ts}.

Phenotypic analysis of double mutants constructed with *cdc10*-129^{ts}, which blocks cells in G_1, and with *cdc25*-22^{ts}, which blocks cells in late G_2, led to the conclusion that *pim1*-46^{ts} cells can enter mitosis prematurely from either G_1 or G_2 (Matsumoto and Beach, 1991). The appearance of *pim1*-46^{ts} *cdc10*-129^{ts} cells with condensed chromatin after 10 h of recovery from nutrient starvation at the restrictive temperature was interpreted as evidence of entry into mitosis from G_1. *pim1*-d1^{ts} *cdc10*-129^{ts} double mutant cells also have a single nucleus containing condensed chromatin but this is due not to premature chromosome condensation but to the fact that the chromatin does not decondense after nitrogen starvation. Probably the same explanation holds for the reported pim1-46^{ts} cdc10-129^{ts} phenotype. This result implies that *pim1$^+$* may have some general role in maintaining chromatin structure, and that prolonged arrest in G_1 prevents the chromatin in a *pim1*-d1^{ts} mutant from becoming decondensed. This is consistent with the finding that the longer tsBN2 cells are arrested in G_1 by serum starvation the less capable they are of re-entering the cell cycle (Nishimoto *et al.*, 1978).

The conclusion that *pim1*-46^{ts} cells can prematurely enter mitosis from G_2 is based on an analysis of the *pim1*-46^{ts} *cdc25*-22^{ts} mutant which at the restrictive temperature is septated and has a single asymmetrically positioned nucleus (Matsumoto and Beach, 1991). A similar phenotype is seen for *pim1*-d1^{ts} *cdc25*-22^{ts} mutant cells. However, it does not appear to be the result of premature advancement into a normal mitosis because these cells neither segregate their chromosomes nor assemble a mitotic spindle at the restrictive temperature. One possible interpretation of the phenotype of *pim1*-d1^{ts} *cdc25*-22^{ts} cells is that pim1$^+$ plays a role in influencing the timing of cytoskeletal reorganization, nuclear migration and septation. In certain circumstances in the absence of functional pim1$^+$ protein these cytoplasmic processes can take place even in the absence of nuclear mitotic events such as spindle formation and chromosome segregation. This results in the formation of a septum with a single nucleus positioned in one of the two daughter cells.

The pim1$^+$ protein is related to the RCC1 family of chromatin-associated proteins

The *pim1* gene encodes a protein with sequence similarity to mammalian RCC1 believed to be involved in monitoring the completion of S-phase in order to prevent cells from condensing their chromosomes and entering mitosis prematurely (Nishitani *et al.*, 1991). Genes encoding RCC1-related proteins have been cloned and characterized in a number of organisms. In addition to affecting chromatin structure, mutations in these proteins result in a variety of phenotypes including defects in transcription initiation, RNA processing and RNA transport (reviewed in Dasso, 1993). The *Drosophila* BJ1 protein, like human and *Xenopus* RCC1, is uniformly distributed along the chromatin in interphase nuclei but is found throughout the cytoplasm at mitosis. This is consistent with a direct role for these proteins in the maintenance of chromosomes in their decondensed interphase state. The functional homology of these proteins has been demonstrated by complementation analysis (reviewed in Dasso, 1993). If RCC1-like proteins are involved in the maintenance of chromatin structure, then cells lacking this function may be expected to exhibit a variety of pleiotropic effects. The precise terminal phenotype observed in different cell types might depend on the regulatory systems that are operative and what process is most sensitive to depletion of RCC1 activity. Another possibility is that the RCC1-like proteins are structurally related and normally perform different functions, but that when they are highly expressed they can compensate for one another.

One clear difference probably indicative of functional diversity between the *pim1*-d1^{ts} mutant and the tsBN2 mutant is that at the restrictive temperature the *S.pombe* mutant does not enter mitosis prior to the completion of S-phase either in the presence or absence of the DNA synthesis inhibitor HU. Another striking difference in the apparent role of RCC1 is that in *Xenopus* egg extracts the depletion of RCC1 leads to an inhibition of DNA synthesis (Dasso *et al.*, 1992) whereas we have confirmed the results of Matsumoto and Beach (1991) showing that *S.pombe* can replicate its DNA in the absence of functional pim1$^+$ protein. Because demembranated sperm chromatin does not contain the normal complement of chromosomal proteins

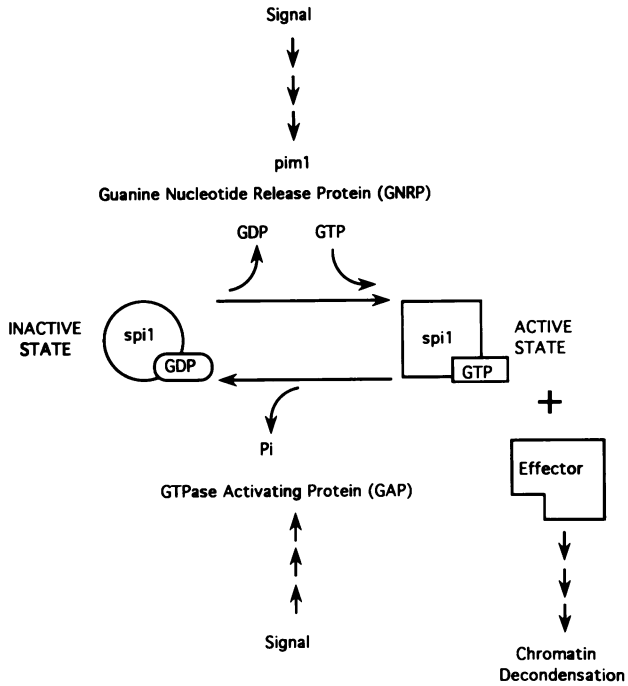

Fig. 6. Model of the pim1 switch at mitotic exit. spi1 is a GTPase that can exist in either an inactive GDP-bound form or an active GTP-bound form. It is only in the GTP-bound form that spi1 can interact with its cellular effectors and bring about chromatin decondensation. The abundance of active spi1 is governed by the relative activities of pim1, the guanine nucleotide exchange protein (GNRP) and the yet to be identified GTPase activating protein (GAP).

(Poccia, 1986) the ability of RCC1-depleted *Xenopus* extracts to decondense this chromatin (Dasso *et al.*, 1992) may not be analogous to the role of RCC1 in post-mitotic chromosome decondensation.

While we cannot rule out the possibility that as yet unidentified mutations in the *pim1* gene might result in a phenotype more similar to the mammalian *RCC1* mutant, this seems unlikely given that a null allele of this gene (Matsumoto and Beach, 1991) has a terminal phenotype that is cytologically identical to that of *pim1*-d1^{ts} and *pim1*-46^{ts} cells. That the pim1 pathway may be regulated at least in part by protein phosphorylation is suggested by the finding that a cold sensitive suppressor of both the pim1-d1^{ts} and pim1-46^{ts} phenotype (Matsumoto and Beach, 1993; X.He and S.Sazer, unpublished results) encodes ppe1, a type 2A protein phosphatase (Shimanuki *et al.*, 1993).

The pim1-d1^{ts} mutation is suppressed by the spi1 GTPase

The *pim1*-d1^{ts} mutation is suppressed by the *spi1* gene, which encodes a low molecular weight GTPase nearly identical to the vertebrate RAN/TC4 protein. GTPases are essentially molecular switches (Bourne *et al.*, 1990) and thus it is possible that the spi1 protein is involved in signalling the completion of mitosis, perhaps by reversing some of the changes brought about by the p34^{cdc2} protein kinase at mitotic entry. Because RCC1 is a highly abundant protein (Bishoff and Ponstingl, 1991a; Frasch, 1991; Dasso *et al.*, 1992) it is also possible that the pim1/spi1 switch is acting not in a signalling pathway but in a manner analogous to small GTPases that target interactions between intracellular vesicles (reviewed in Pfeffer, 1992). In this case spi1 may play a role in facilitating the interaction between interphase chromosomes and structural elements of the nucleus. A candidate for this interaction is NuMA, a component of the nuclear matrix required at the completion of mitosis in mammalian cells (Compton and Cleveland, 1993). Expression of a truncated form of NuMA causes a phenotype identical to that of tsBN2 at the restrictive temperature and overproduction of full-length NuMA rescues certain of the tsBN2 morphological defects at the end of mitosis (Compton and Cleveland, 1993). A further role for spi1 is suggested by the recent demonstration that the vertebrate homolog, RAN/TC4, is a component of the nuclear protein import system (Moore and Blobel, 1993).

Model of the pim1/spi1 switch at mitotic exit

Based on the biochemical characterization of the RCC1/RAN molecular switch in mammalian cells (Bishoff and Ponstingl, 1991a,b), it is likely that pim1 is the guanine nucleotide release protein (GNRP) for the spi1 GTPase (Figure 6) and that their interactions are similar to those seen for other GTPases in the ras superfamily (Bourne *et al.*, 1990). The constitutive activation of this GTPase pathway is lethal: wild type cells expressing either an activated form of spi1 comparable to activated p21^{ras} mutants stabilized in their GTP-bound state or overexpressing the pim1^{+} protein are inviable. These findings are consistent with the model in which pim1^{+} facilitates the conversion of spi1^{+} to its active GTP-bound form. Still to be identified are the signals to which this molecular switch is responding and the effector(s) that return the nucleus to its interphase state following mitosis.

Materials and methods

Cells and cell culture

Strains and media. Wild type *S.pombe* cells, strain 972 h^{-} (Gutz *et al.*, 1974), ts mutants *cdc25*-22 (Fantes, 1979), *cdc10*-129 (Nurse *et al.*, 1976), *pim1*-46^{ts} (Matsumoto and Beach, 1991), *pim1*-d1^{ts} and double mutant strains *pim1*-d1^{ts} *cdc25*-22^{ts} and *pim1*-d1^{ts} *cdc10*-129^{ts} were cultured in minimal medium as previously described (Nurse, 1975) unless otherwise noted. HU was added when stated to a final concentration of 12 mM and thiamine was added when stated to a final concentration of 5 μg/ml. Genetical manipulations were performed using standard techniques (Gutz *et al.*, 1974; Moreno *et al.*, 1991).

ts arrest. In experiments involving a temperature shift cells were grown to a density of 4 × 10^{6} cells/ml at 25°C before being shifted to 36°C. Cell number was determined using a Coulter counter.

Nitrogen starvation. Cells arrested in G_1 by nitrogen starvation were grown at 25°C to a density of ~2 × 10^{6} cells/ml in minimal medium, washed three times in minimal medium from which the nitrogen source had been omitted, resuspended in nitrogen-free medium and incubated at 25°C for 24 h. Cells were pelleted, resuspended in complete minimal medium and incubated at 36°C for 12 h. Arrest in G_1 was monitored by FACS and by microscopic examination of DAPI-stained cells.

Spore germination. Sporulation of a *pim1*-d1^{ts} homozygous diploid strain and germination of the spores was carried out using standard methods (Moreno *et al.*, 1989).

Synchronous culture. Four liters of cells were grown to a density of ~5 × 10^{6} cells/ml in minimal medium at 25°C. Small G_2 cells were isolated using a Beckman JE-5.0 Elutriation Rotor and incubated at 25°C or 36°C as indicated.

Mutagenesis. Yeast cells were mutagenized to 50% survival using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described in Moreno *et al.* (1991). Cells were grown at 25°C and then replica plated to 36°C for 2 days on plates containing phyloxine B (Moreno *et al.*, 1991).

Flow cytometry

Cells were pelleted, washed once and resuspended in sterile water at 4°C, then vortexed as ethanol at −20°C was added to 70%. Fixed cells were stored at 4°C. Cellular DNA content was measured in fixed cells stained with the DNA fluorochrome propidium iodide and analyzed using a Becton Dickinson FACScan or a Coulter Epics 753 flow cytometer as previously described (Sazer and Sherwood, 1990).

Cytology

DNA. Cells were fixed in ethanol as described above. For staining cells were pelleted, resuspended in PBS and air dried on coverslips coated with 1 mg/ml poly-L-lysine. The coverslips were mounted on slides with 50% glycerol containing 1 μM fluorescent DNA-binding dye, 4′,6-diamidino-2-phenylindole (DAPI), and 1 mg/ml paraphenylamine diamine, an anti-fading agent.

Microtubules. Cells were fixed using the aldehyde fixation method developed by Hagan and Hyams (1988). They were stained using a primary anti-α-tubulin monoclonal antibody, TAT-1 (Woods *et al.*, 1989; a generous gift of K.Gull, University of Manchester), and FITC-conjugated rabbit anti-mouse secondary antibodies (Cedar Lane Laboratories). The DNA was stained with DAPI as described above.

Light microscopy. Cells were observed with a Zeiss Axioskop photomicroscope using a 100× objective and photographed using T-Max 400 film.

Protein kinase assays

p34^{cdc2} was immunoprecipitated with the Ab 4711 antiserum (Gould and Nurse, 1989; a generous gift of K.Gould, Vanderbilt University), from nondenatured lysates. Half was assayed for histone H1 kinase activity and the other half was Western immunoblotted with Ab 4711 (Moreno *et al.*, 1989; Fleig *et al.*, 1992).

Cloning the pim1$^+$ gene

pim1-d1^{ts} *leu1*-32 *ura4*-D18 h$^-$ cells were transformed using the protoplast method (Moreno *et al.*, 1991) with the *S.pombe* cDNA library pREPsp [generous gift of B.Edgar (Fred Hutchinson Cancer Research Center) and C.Norbury (University of Oxford)]. The level of expression is controlled by the *nmt1* promoter (Maundrell, 1990) and can be regulated by the presence and absence of thiamine. Because we suspected that overexpression of *pim1*$^+$ was lethal to cells we selected transformants that grew at 36°C in the presence of 5 μg/ml thiamine when expression was low, but died in the absence of thiamine when expression was high. The plasmid was recovered from yeast and transformed into bacterial strain JA226 (Hagan *et al.*, 1988). The 1.9 kb insert was subcloned into pTZ19R (Pharmacia) for sequencing using Sequenase (United States Biochemical). Southern blot analysis was done using Gene-Screen Plus membrane (New England Nuclear).

Physical mapping of the pim1$^+$ gene

A high density gridded *S.pombe* genomic cosmid library filter (Maier *et al.*, 1992; Hoheisel *et al.*, 1993) was hybridized to the 1 kb *Hpa*II fragment of the *pim1* cDNA using standard techniques. Data analysis was kindly performed by J.Hoheisel as described.

Cloning the spi1 gene

pim1-d1^{ts} *leu1*-32 *ura4*-D18 h$^-$ cells were transformed using the protoplast method (Moreno *et al.*, 1991) with the *S.pombe* genomic library pURSP2 (Barbet *et al.*, 1992; generous gift of T.Carr, University of Sussex) and colonies growing at 36°C were isolated. Plasmid was isolated as described above and the 2 kb insert from the smallest of the rescuing plasmids was subcloned into plasmid PTZ19R for sequencing. The genomic clone was used as a hybridization probe to isolate the encoded cDNA from an *S.pombe* library (Fikes *et al.*, 1990; generous gift of J.Fikes and L.Guarente, MIT) using standard methods (Maniatis *et al.*, 1982), and was then subcloned and sequenced as described above. Mutagenesis of the *spi1* gene was carried out using the Amersham oligonucleotide-directed *in vitro* mutagenesis system. The mutations changed the Gly18 GGT codon, equivalent in position to Gly12 in p21^{ras} to either CGT (encoding Arg) or CTT (encoding Val) and the Glu68 CAA codon, equivalent in position to Glu61 in p21^{ras} to CGA (encoding Arg).

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