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Interaction of the pim1/spi1 Mitotic Checkpoint with a Protein Phosphatase

Tomohiro Matsumoto and David Beach

Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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Loss of $p58^{pim1}$, a homolog of human RCC1, results in uncoupling of mitosis from the completion of DNA replication in fission yeast. An extragenic suppressor of a mutant allele of *pim1*, *esp1*, has been isolated and characterized. *esp1* encodes a predicted product of 305 amino acid residues, which shares 71% identity with budding yeast SIT4, a type2A related protein phosphatase. $p58^{pim1}$ binds $p25^{spi1}$, a 25-kd ras-related GTPase previously isolated as a high dosage suppressor of *pim1*. The complex dissociates in the presence of guanine nucleotides and Mg^{2+}. The mutant $p58^{pim1}$ is defective in its ability to bind $p25^{spi1}$, suggesting that the physical interaction is essential for the maintenance of the interdependency of cell cycle event. In the *esp1 pim1* double mutant, the mutant $p58^{pim1}$ protein is still defective in its ability to bind to $p25^{spi1}$. However, *pim1* induced premature mitosis is completely suppressed, suggesting that *esp1* may act downstream of the $p58^{pim1}/p25^{spi1}$ physical interaction but upstream of the activation of the M-phase specific histone H1 kinase.

INTRODUCTION

Entry into mitosis is triggered by activation of the M-phase specific histone H1 kinase, $p34^{cdc2}$ (Arion *et al.*, 1988; Dunphy *et al.*, 1988; Booher *et al.*, 1989; Draetta *et al.*, 1989; Gautier *et al.*, 1989; Meijer *et al.*, 1989). Premature activation of the M-phase specific kinase would result in daughter cells, which lack a complete set of cellular components and genetic information.

To address the question of how mitosis is coupled to the completion of DNA replication, we have undertaken a genetic approach to dissect this pathway in fission yeast, *Schizosaccharomyces pombe*. Activation of M-phase specific kinase is regulated by at least two independent parallel pathways. Negative regulation is achieved through the activity of the mik1/wee1 kinases (Lundgren *et al.*, 1991) and a positive regulation through the cdc25 tyrosine phosphatase (Russell and Nurse, 1986). These pathways regulate the activation of M-phase specific kinase through the phosphorylation state of a tyrosine residue on the catalytic subunit of the kinase. Either loss of function of the mik1/wee1 kinases, or high dosage expression of cdc25 tyrosine phosphatase causes premature entry into mitosis (Enoch and Nurse, 1990; Lundgren *et al.*, 1991), suggesting that timing of the activation of $p34^{cdc2}$ is regulated by the balance between these pathways. To investigate events upstream of this activation, which insure that DNA synthesis is complete before mitosis is initiated, we have characterized a mutant (*pim1*) in which the onset of mitosis is uncoupled from the completion of DNA replication (Matsumoto and Beach, 1991). The *pim1* mutant at the restrictive temperature can undergo mitotic chromosome condensation and mitotic spindle formation without the completion of S-phase and without the activity of the cdc25 mitotic inducer. The M-phase specific kinase activity is required for pim1-induced mitosis and becomes activated upon temperature shift. $pim1^{+}$ encodes a homolog of the human nuclear protein RCC1 (Ohtsubo *et al.*, 1987). Loss of RCC1 function in baby hamster kidney (BHK) cells causes premature initiation of mitosis (Nishimoto *et al.*, 1978). $p25^{spi1}$, a high dosage suppressor of *pim1*, encodes a 25-kd ras-related GTPase (Matsumoto and Beach, 1991). Genetic analysis demonstrated that $p25^{spi1}$ does not bypass the function of $p58^{pim1}$, suggesting a possible physical interaction between the two gene products.

It has been shown that RCC1 acts as a nucleotide exchanger on a human homolog of the spi1 guanosine 5′-triphosphatase (GTPase) (Bischoff and Ponstingl, 1991). The structural and functional conservation of these proteins through evolution suggests that the

pim1/spi1 pathway plays an important role in the establishment of cell cycle interdependency.

Here, we report the identification of a novel protein phosphatase, *esp1*, interacting with the pim1/spi1 cell cycle checkpoint. In the background of *esp1* mutant, *pim1* induced premature initiation of mitosis is suppressed, resulting in normal cell growth at the restrictive temperature for *pim1* mutant. We also show that fission yeast $p58^{pim1}$ and $p25^{spi1}$ form a complex and that $p58^{pim1}$ mutant protein is defective in its binding to the $p25^{spi1}$.

MATERIALS AND METHODS

Strains and Media

All strains were derived from wild-type strains originally described by Leupold (1970). *S. pombe* was grown in standard yeast extract medium (YEA), yeast extract medium plus adenine (YE), pombe minimal medium (PM), and pombe minimal medium plus adenine (PMA) media (Beach *et al.*, 1985) containing additional amino acids as described at 75 μg/ml.

Isolation and Cloning of esp1

To isolate an extragenic suppressor of *pim1*, SP1027 (*h⁻ leu1-32 pim1-46*), was mutagenized by NTG (nitrosoguanidine) as described (Uemura and Yanagida, 1984). From 1×10^7 survivors, 55 were isolated as revertants, which were no longer temperature sensitive. These revertants were crossed to wild-type strain SP257 (*h⁺ ade6-210*) to test whether they were intragenic or extragenic. Genetic analysis indicated that one revertant carried a mutation (designated as *esp1-68*), which suppressed the *pim1-46* mutation. SP1122 (*h⁻ leu1-32 pim1-46 ade6-210 esp1-68*) was crossed to SP49 (*h⁺ lys1*), and tetrads were dissected to confirm that *esp1-68* suppressed *pim1-46*. The *esp1* mutation itself displays a cold sensitive phenotype and is defective in conjugation. All crosses with *esp1-68* mutants were carried out by constructing diploids before sporulation.

To clone the esp1 gene, SP1123 (*h⁻ leu1-32 esp1-68 ade6-210*) was transformed with an *S. pombe* genomic DNA cosmid library (Nakaseko *et al.*, 1986) and 37 transformant, which were no longer cold sensitive, were isolated from 5300 Leu^+ transformants. Cosmid DNAs were recovered from these transformants, and introduced into *Escherichia coli*. Restriction mapping revealed that four different cosmid clones were recovered, which share about a 10 kb overlapping region. After several steps of subcloning, a 1.3 kb *Xba* I-*Mlu* I fragment was found as an active fragment. Integration mapping using the pYC10 vector (Chikashige *et al.*, 1989) indicated that the fragment is derived from the *esp1* locus. Nucleotide sequencing analysis was done by a semiautomatic sequencer (ABI 373A DNA sequencer).

Antibodies

anti-pim1 Antibody. To obtain a cDNA corresponding to the *pim1*, polymerase chain reaction (PCR) was carried out with the use of an *S. pombe* cDNA library as a template. A pair of primers (GGGGCATATGAAAAATGGCAAAAAGCCGGTTAAACGT and GGGGCATATGCTAAGCAGTGGTGGAGCTGGGTTCGAGAACA) were synthesized, and added to the reaction after phosphorylation by T4 nucleotide kinase to obtain the fragment containing the gene franked by *Nde* I sites. A 1.6 kb PCR product was inserted into the *Sma* I site of pUC119 (designated as pUC119-pim1-2) after the product was filled in by the use of Klenow fragment. Nucleotide sequence analysis confirmed that a putative intron was correctly removed in the cDNA and cDNA encodes $p58^{pim1}$ gene product. An *Nde* I fragment was recovered from pUC119-pim1-2 and cloned into a yeast expression vector, pART3 (designated as pART3-pim1-2) or a bacterial expression vector pRK171A (designated as pRK171-pim1-2). pART3-pim1-2 could rescue the *pim1* mutant indicating the product is biologically active. pRK171-pim1-2 allows expression of a 507 amino acid polypeptide after induction with isopropyl-β-D-thiogalactopyranosid (IPTG) in the *E. coli* strain BL21(DE3). BL21(DE3) carrying pRK171-pim1-2 was cultured in Luria-Bertani medium (LB) overnight and diluted into fresh LB. After 1 h at 37°C, IPTG was added to a final concentration of 0.4 mM. After 3 or 4 h, cells from 1 l of culture were harvested and lysed in 50 ml of buffer A (50 mM tris(hydroxymethyl)aminomethane [Tris]-Cl pH 7.0, 5 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol [DTT], 0.1% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]) by sonication. After centrifugation for 20 min at 4°C (18 000 × *g*), the supernatant was applied to a DE-52 column, and the unbound fraction was applied to S-Sepharose or FPLC (Mono-S). The polypeptides were eluted at a salt concentration of 0.35–0.4 M. To immunize rabbits, some fractions were further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then the polypeptides were eluted into phosphate-buffered saline (PBS) from the gel. Immunization was carried out by Pocono Rabbit Farm (Canadensis, PA). For the affinity purification of anti-$p58^{pim1}$ antibody, the polypeptides eluted from S-Sepharose were coupled to activated CH Sepharose 4B (Pharmacia LKB, Piscataway, NJ) by the method recommended by the manufacturer. The beads were incubated with crude serum in PBS containing 3% bovine serum albumin (BSA) at 4°C for overnight. After three washes with PBS containing 0.1% Tween-20, the antibody was eluted by the addition of 2 ml of 100 mM glycine pH 2.5, and 400 μl of 2 M Tris-Cl pH 8.0 was added.

anti-spi1 Antibody. A similar procedure was followed to obtain a cDNA corresponding to the *spi1*. A pair of primers (GGGGCATATGGCTCAACCACAAAACGTTCCTACCTTT and GGGGCATATGTTACAAATCAGCGTCATCCTCATCAGG) were used to obtain the cDNA by PCR. Nucleotide sequence analysis revealed that putative two introns were removed. A 0.65 kb PCR product flanked by *Nde* I sites was cloned into pRK171A (designated as pRK171-spi1) or yeast expression vector (designated as pART3-spi1). pART3-spi1 could rescue the pim1 mutant. Expression of polypeptides was induced in BL21(DE3) carrying pRK171-spi1. The cell pellet was lysed in buffer B (20 mM Tris-Cl pH 8.0, 5 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 10% glycerol, 20 mM $MgCl_2$). After centrifugation for 20 min at 4°C (18 000 × *g*), the pellet was resuspended in buffer B containing 4 M urea. After centrifugation for 20 min at 4°C, the pellet was resuspended in buffer B containing 6 M urea. The soluble fraction containing the polypeptide was purified by SDS-PAGE, and the polypeptides was eluted from the gel. Immunization was done by Pocono Rabbit Farm. For the affinity purification of the antibody, the method described by Jessus and Beach (1992) was followed.

Western Blotting and Immunoprecipitation

S. pombe cells were disrupted by vortexing in the presence of glass beads in buffer 1 (1× PBS, 5 mM EDTA, 1 mM DTT, 10 mM chloroquine, 50 mM NaF). An equal volume of buffer 2 (buffer 1 containing 1% Triton X-100, 0.5% deoxycholate and 0.1% SDS) was added after vortexing. After centrifugation for 30 min at 4°C (25 000 × *g*), the supernatant was precleared by incubation with immobilized protein A (PIERCE) for 30 min. Anti-pim1 antibody was added to the supernatant after centrifugation, and the mixture was rotated for 2 h at 4°C. Immobilized protein A was added, and incubated for 30 min at 4°C. The beads were washed twice with 1:1 mixture of buffer 1 and 2 and washed twice with 3× PBS containing 0.5% Triton X-100. The buffer used for the immunoprecipitation contained 0.1 mM PMSF, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 10 μg/ml soybean trypsin inhibitor. For the experiments shown in Figure 5, immunoprecipitates were incubated in buffer 3 (50 mM NaCl, 20 mM Tris-Cl pH 7.5 and 1 mM DTT) containing Mg^{2+} and/or guanine nucleotide. An additional wash was done in the 1:1 mixture of buffer 1 and 2 at 25°C for 20 min for the experiments in Figure 6.

For Western blotting, cells were disrupted by vortexing in the presence of glass beads in 1× PBS, then SDS was added to a final con-

centration of 1%. After centrifugation, the supernatant was run on SDS-PAGE and blotted on a nitrocellulose membrane (S&S). The membrane was incubated in 1× PBS containing 3% BSA for 4 h at room temperature. Anti-pim1 antibodies were diluted to 1/1500 and anti-spi1 antibodies were diluted to 1/5000. Immunoprecipitation using anti-cdc2 antibody (G8) was carried out by the method described by Lundgren *et al.* (1991).

RESULTS

Isolation of esp1

To isolate extragenic suppressors of *pim1*, the temperature-sensitive *pim1-46* strain was mutagenized as described. From 55 revertants, one was shown to carry an extragenic suppressor of *pim1* by genetic analysis (see MATERIALS AND METHODS) and was designated *esp1-68*. Although the double mutant *pim1-46 esp1-68* grows more slowly than wild-type cells at 36°C (restrictive temperature for *pim1* mutant), it forms colonies after 4 d, whereas *pim1* single mutant does not (Figure 1A). As reported previously (Matsumoto and Beach, 1991 and Figure 2A), *pim1* mutants show tightly condensed chromosomes and central septa after a shift to the restrictive temperature. Microscopic observation revealed that the presence of the *esp1-68* mutation fully suppresses the phenotype of *pim1* (Figure 2B). The single mutant *esp1-68* grows more slowly than wild-type cells, and the cell size is reduced at any temperature tested (36, 32, and 26°C). At 20°C, the *esp1-68* mutant is defective for growth (Figure 1C), although there is no remarkable phenotypic change at this temperature (Figure 2, C and D). In addition to the growth defect, *esp1-68* is defective in its ability to conjugate.

To isolate the *esp1* gene, an *S. pombe* genomic DNA cosmid library (Nakaseko *et al.*, 1986) was introduced into the *esp1-68* mutant, and cosmids were recovered into *E. coli* from yeast transformants. Restriction mapping indicated that all the cosmid clones shared a 10-kb region, suggesting that they represented the same genomic region. After several steps of subcloning, a 1.3 kb *Xba* I-*Mlu* I fragment was found to be active. Genetic analysis confirmed that this fragment maps to the *esp1* locus. A plasmid carrying the *esp1* gene rescues the cold sensitive phenotype and reverts the double mutant *pim1-46 esp1-68* to a temperature sensitive phenotype.

esp1⁺ Encodes a Predicted Phosphatase

Nucleotide sequence analysis of the 1.3-kb fragment revealed an open reading frame of 305 amino acid residues (Figure 3A). A FASTA search (Pearson and Lipman, 1988) of the GenBank data base demonstrated that *esp1* has identity to type 2A-related phosphatases. The sequence was much similar to that of the budding yeast SIT4 gene (Arndt *et al.*, 1989) and PPH3 gene (Ronne *et al.*, 1991) (71 and 57% identity, respectively, Figure 3B) and also showed similarity to mammalian and yeast type 2A phosphatases.

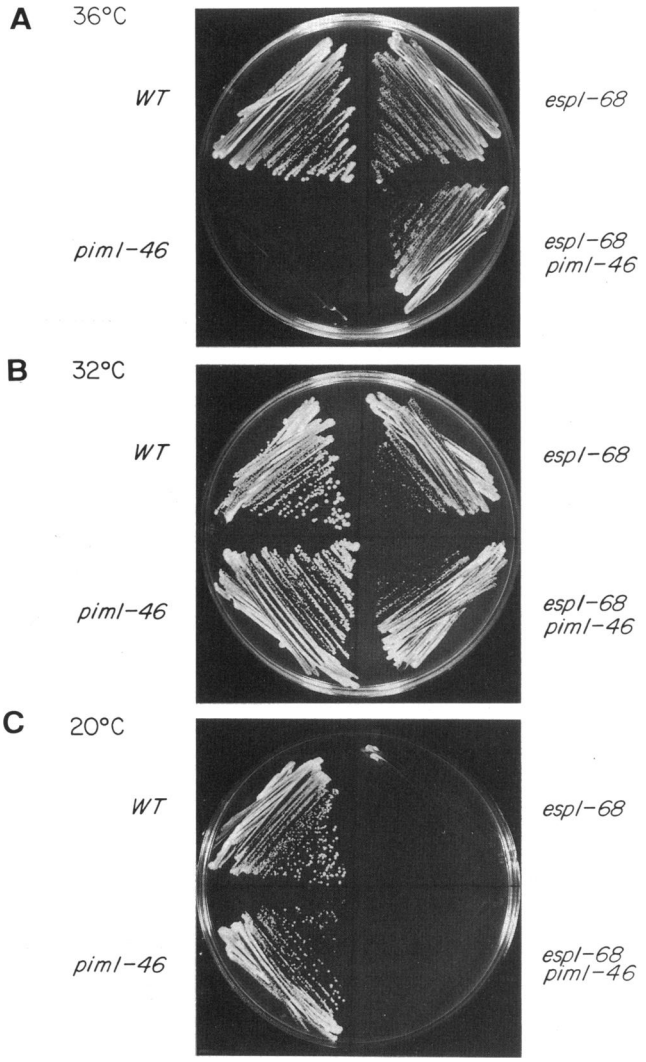

Figure 1. *esp1-68* suppresses *pim1-46*. Each strain was streaked onto a YEA plate, and incubated for 4 d at 36°C (A), 5 d at 30°C (B), or 7 d at 20°C (C).

p58^pim1 and p25^spi1 Proteins

Antibodies against $p58^{pim1}$ and $p25^{spi1}$ were raised in rabbit (see MATERIALS AND METHODS). We estimated the amount of $p58^{pim1}$ and $p25^{spi1}$ in fission yeast. Western blot analysis using anti-pim1 antibodies detected a doublet of 64-kd and 60-kd bands in yeast extracts (Figure 4, left). As reported previously (Matsumoto and Beach, 1991), there are two methionines near the *N*-terminus of the *pim1* open reading frame. We constructed vectors to express two polypeptides in *E. coli* which use either the first or second methionine as a translation start. Molecular weights on SDS-PAGE of these polypeptides match those of the doublet in yeast extracts, suggesting that the doublet in vivo is a result of differential translation initiation. Although the cal-

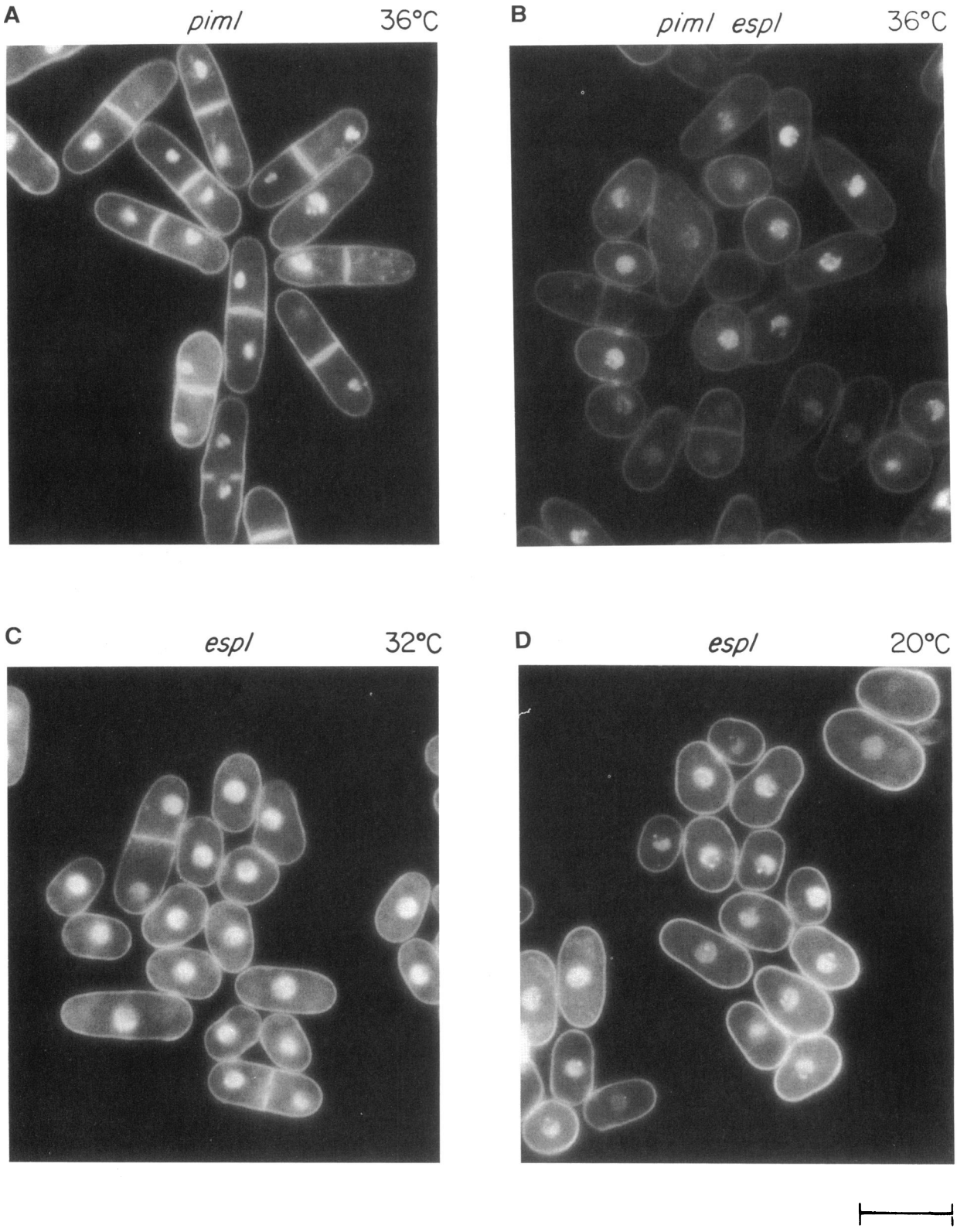

Figure 2. Phenotype of *esp1-68. pim1-46* (SP1027) was shifted to the restrictive temperature (36°C) for 4 h (A). *pim1-46 esp1-68* (SP1122) was cultured at 36°C for 4 h (B). *esp1-68* (SP1123) was cultured at the permissive temperature 30°C (C) then shifted to 20°C for 8 h (D). Bar indicates 10 μm.

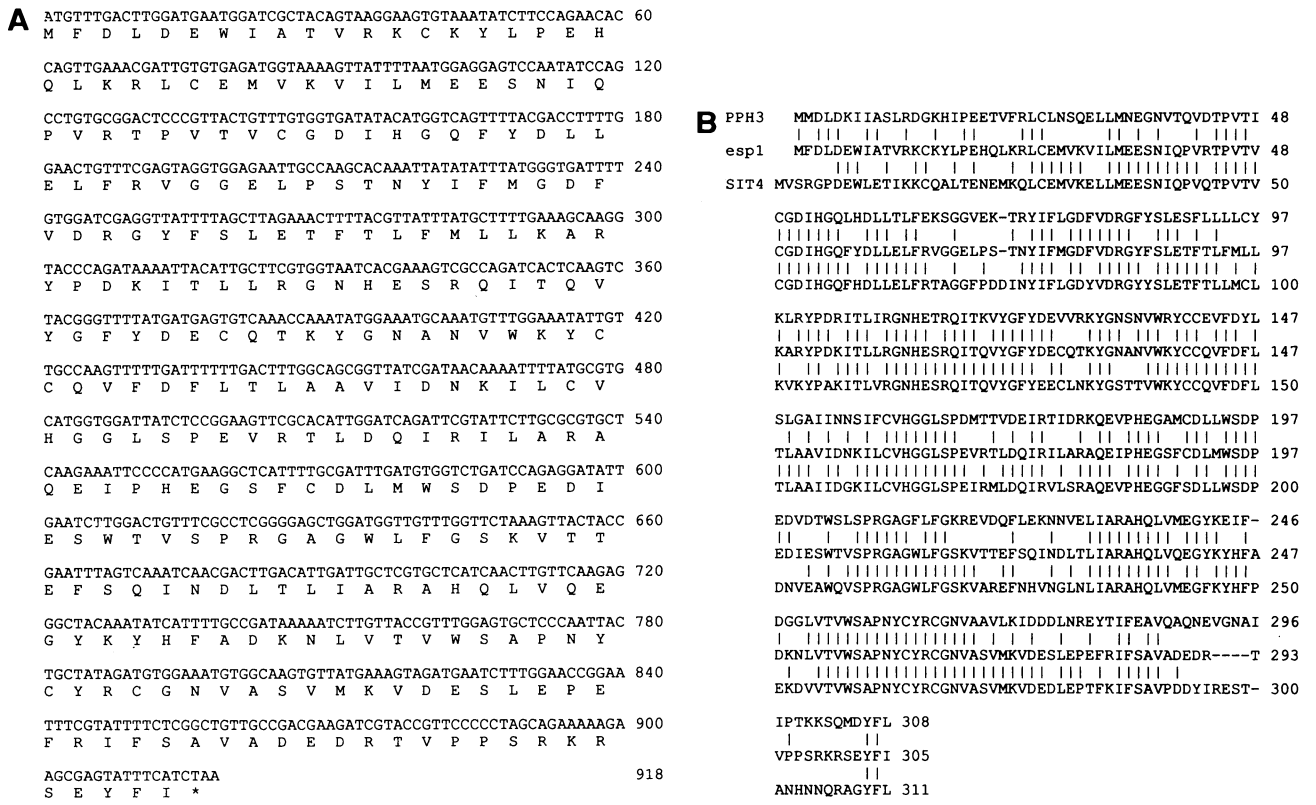

Figure 3. The *esp1* gene. (A) The nucleotide sequence of a 918-bp region within the 1.3 kb *Xba* I-*Mlu* I fragment is shown. The predicted 305 amino acid sequence is indicated in the conventional single-letter code. (B) Homology between *esp1* and SIT4 and PPH3 is shown. Identical amino acids between 2 proteins are marked by vertical lines.

culated molecular weights of $p58^{pim1}$ is 58.4 kd (and 54.4 kd), we concluded that the doublet of 64 and 60 kd probably represents $p58^{pim1}$ because the intensity of the bands increases in extracts prepared from a strain overexpressing $p58^{pim1}$ (Figure 4). By using the bacterially expressed $p58^{pim1}$ as a standard, the total cellular amount of $p58^{pim1}$ was estimated to be ~0.15% of total protein. Western blotting analysis using anti-spi1 antibodies detected a 25-kd band, whose intensity increases in extracts from a strain overexpressing $p25^{spi1}$ (Figure 4, right). We estimated the cellular amount of $p25^{spi1}$ to be ~0.65% of total proteins, using the bacterial expressed protein as a standard. There is no cross-reactivity of either antibody.

$p58^{pim1}$/$p25^{spi1}$ Complex

$p25^{spi1}$, which encodes a 25 kD ras-related GTPase, was isolated previously as a multicopy suppressor of *pim1* (Matsumoto and Beach, 1991). Genetic analysis suggested that the two proteins interacted as $p25^{spi1}$ did not rescue the null allele of *pim1*. To test whether $p58^{pim1}$ and $p25^{spi1}$ interact physically, the products of immunoprecipitations using anti-pim1 antibody were run on an SDS-PAGE gel and transferred onto a nitrocellulose membrane. The top and bottom portions of the blot were western blotted using anti-pim1 and anti-spi1 antibodies, respectively. By this criterion, $p58^{pim1}$ and $p25^{spi1}$ were found to form a complex (Figure 5, top). This complex was resistant to a high salt wash but dissociated in the presence of Mg^{2+} and guanine nucleotides as shown in Figure 5 (top). Guanosine 5′-triphosphate (GTP), guanosine 5′-diphosphate (GDP), and nonhydrolyzable GTP analogs had the same effect, but ATP (1 mM) did not cause dissociation. By using decreasing concentration of GTP, it was demonstrated that in the presence of 0.5 μM of GTP, 80% of the spi1 GTPase was released from the complex (Figure 5, bottom). We have not detected any GTPase activity in the complex.

The nature of the $p58^{pim1}$ mutant protein was tested. Immunoprecipitations were done from wild-type and *pim1-46* extracts. The amount of the $p58^{pim1}$ recognized by the antibody was similar in both extracts at time 0 (top, Figure 6A). However, the amount of $p25^{spi1}$ bound to $p58^{pim1}$ was significantly lower in the *pim1-46* strain than in wild-type cells even at time 0 (middle, Figure 6A). This indicates that the mutant $p58^{pim1}$ has less binding affinity to $p25^{spi1}$ under these conditions (note that the total amount of $p25^{spi1}$ is not altered in *pim1-*

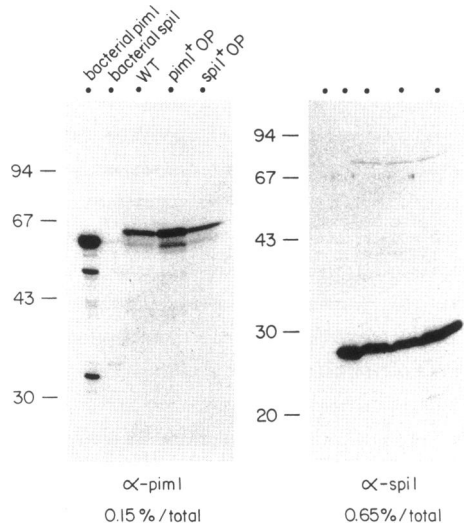

Figure 4. $p58^{pim1}$ and $p25^{spi1}$ proteins. (Right) Extracts from bacteria expressing $p58^{pim1}$, $p25^{spi1}$, yeast cell extracts from wild-type cells, $p58^{pim1}$ overexpressing, and $p25^{spi1}$ overexpressing cells were run on 10% SDS-PAGE and transferred onto a membrane. The membrane was treated for Western blotting using anti-pim1 antibody. (Left) Same samples were run on 12% SDS-PAGE, and Western blotting was done using anti-spi1 antibody.

46 strain; bottom Figure 6A). It is very likely that a high dosage of $p25^{spi1}$ could compensate for the lower affinity of mutant $p58^{pim1}$ in vivo. Similar experiments were done in the *esp1* mutant background (Figure 6B). In the wild type and *esp1-68* mutant cell extracts, $p58^{pim1}$ and $p25^{spi1}$ form a complex. However, the amount of associated $p25^{spi1}$ in *pim1-46* and *pim1-46 esp1-68* cell extract was very low, indicating that the *esp1* mutation does not suppresses the binding defect of the mutant $p58^{pim1}$. In addition to the binding-defect, the mutant $p58^{pim1}$ was found to be degraded after shift to the restrictive temperature (top right Figures 6A and 7). In extracts prepared 6 h after shifting to the restrictive temperature, the $p58^{pim1}$ is hardly detectable. We tested whether the degradation occurs in the double mutant *pim1-46 esp1-68*. As shown in Figure 7, the level of $p58^{pim1}$ in the double mutant decreased after 3 h and does not decrease further for at least another 3 h after the shift to the restrictive temperature. Therefore, the *esp1-68* mutation partially suppresses the lability of the mutant $p58^{pim1}$. We assume that a low level of $p58^{pim1}$ is still necessary in the *esp1-68* background because *esp1-68* does not suppress a null allele of *pim1*.

Tyrosine Phosphorylation on cdc2$^+$

Activation of cdc2 is normally associated with tyrosine dephosphorylation of the protein (Draetta and Beach, 1988; Dunphy and Newport, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989; Lundgren *et al.*, 1991), and it has been shown that tyrosine on cdc2$^+$ is dephosphorylated in the *pim1-46* mutant after a shift to the restrictive temperature (Matsumoto and Beach, 1991). We asked whether the double mutant *pim1-46 esp1-68* shows the normal level of phosphorylation of tyrosine on cdc2$^+$. Immunoprecipitations were done using the anti-cdc2 antibody (G8). In the extract from *pim1-46* mutant prepared 6 h after shift to the restrictive temperature, no phosphorylation on tyrosine is detectable (Figure 8). By contrast, the phosphorylation state of tyrosine on cdc2$^+$ remains constant in the double mutant (Figure 8).

DISCUSSION

Role of $p58^{pim1}$ and $p25^{spi1}$

Human RCC1 and an interacting GTPase (ran/TC4) have been purified biochemically (Bischoff *et al.*, 1990), and RCC1 has been shown to act as a guanine nucleotide exchanger on the GTPase (Bischoff and Ponstingl, 1991). We have shown that a yeast homolog of RCC1 and GTPase ($p25^{spi1}$) forms a complex that dissociates in the presence of Mg^{2+} and guanine nucleotides. Our results supports that $p58^{pim1}$ (yeast homolog of RCC1) plays the same role on the spi1 GTPase. Dissociation of the complex by Mg^{2+} and GTP represents the final step of the exchange reaction.

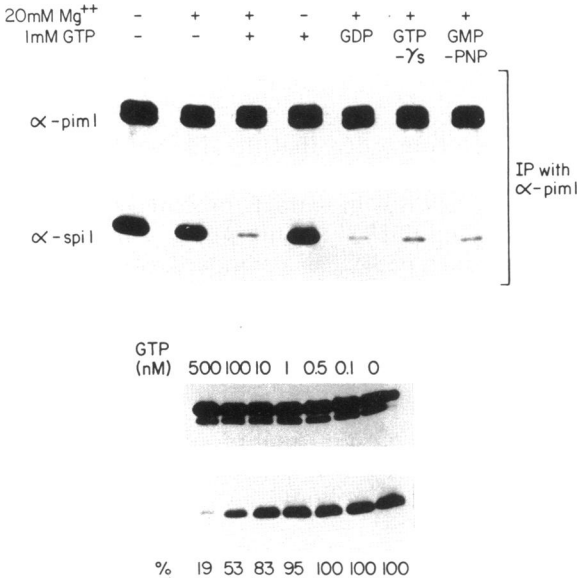

Figure 5. $p58^{pim1}/p25^{spi1}$ complex. (Top) Immunoprecipitates using anti-pim1 antibodies were incubated under the condition shown on the top of each lane. After incubation for 30 min at 25°C, the pellets were run on 12% SDS-PAGE, then transferred to a membrane. Top and bottom portions of the membrane were treated for western blotting using anti-pim1 and anti-spi1 antibodies, respectively. (Bottom) 2 mM Mg^{2+} and GTP to the final concentration shown on the top of each lane were added to immunoprecipitations using anti-pim1 antibodies. After a 30-min incubation, samples were treated as described for the top panel.

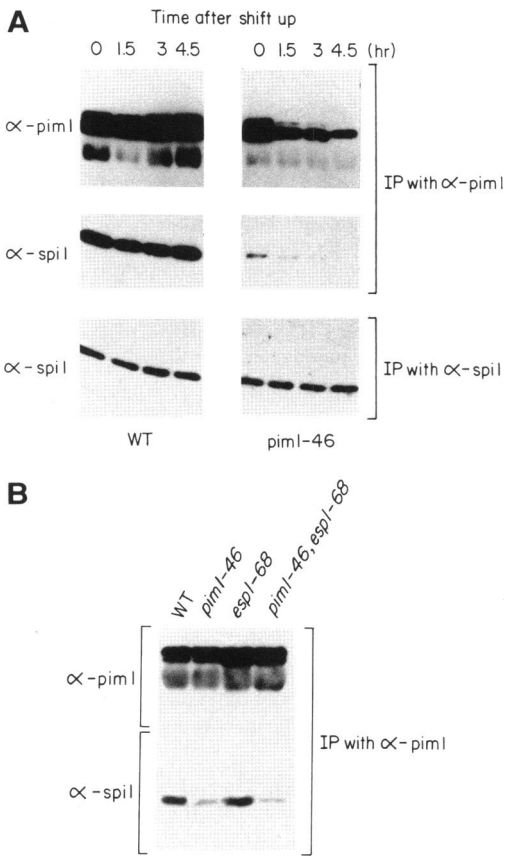

Figure 6. Defect of $p58^{pim1}$ mutant protein. (A) Immunoprecipitates from extracts from wild type (left) and *pim1-46* (right) prepared after 0, 1.5, 3, and 4.5 h after the shift to the restrictive temperature, were run on 12% SDS-PAGE. Top and middle panels were incubated with anti-pim1 antibodies, and the bottom panels were incubated with anti-spi1 antibodies. The amount of $p58^{pim1}$ and $p25^{spi1}$ in each immunoprecipitates were visualized by Western blotting. (B) The extracts from the 4 different strains grown at 26°C (genotypes are shown on the top of lanes) were used for immunoprecipitation using anti-pim1 antibody. The amount of $p58^{pim1}$ (top) and $p25^{spi1}$ (bottom) were visualized by Western blotting.

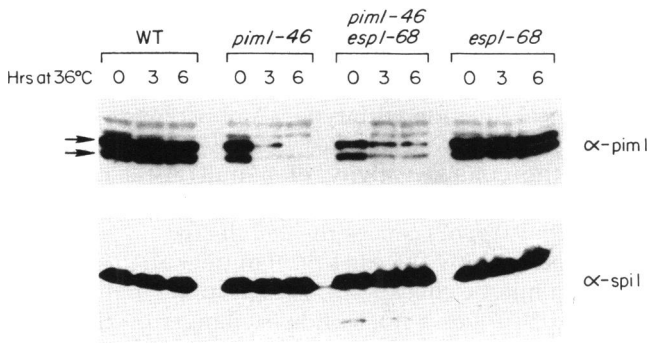

Figure 7. $p58^{pim1}$ in *esp1-68*. Cell extracts from 4 different strains (genotypes are shown on the top of lanes) were prepared after 0, 3, and 6 h of shift to the restrictive temperature and were run on 12% SDS-PAGE. Membrane was treated for western blotting with either anti-pim1 antibody (top) or anti-spi1 antibody (bottom).

In the *pim1-46* mutant, we found that the $p58^{pim1}$ is defective to form a complex with $p25^{spi1}$. Thus it could be imagined that the *pim1* mutant protein is defective in the nucleotide exchange reaction. We propose that active form of the spi1 GTPase (GTP bound form) is a molecular signal that delays the onset of mitosis until the completion of DNA replication. In a *pim1* mutant, mitosis is uncoupled from the completion of DNA replication. $p58^{pim1}$, and an interacting GTPase ($p25^{spi1}$) together act as a mitotic checkpoint.

In the *pim1-46* mutant, tyrosine 15 on $cdc2^{+}$ is dephosphorylated after shift to the restrictive temperature. This suggests that the signal of the pim1/spi1 check point, directly or indirectly, regulates the phosphorylation state of tyrosine 15. In fission yeast, the phosphorylation of the tyrosine is regulated by two parallel pathways, involving either the mik1/wee1 kinases or the cdc25 tyrosine phosphatase. We propose that the signal from the pim1/spi1 checkpoint regulates the activity of the mik1/wee1 kinase because it has been shown that in *pim1-46 mutant*, premature mitosis is not dependent on $cdc25^{+}$ function (Matsumoto and Beach, 1991).

Interaction of a Phosphatase with the Mitotic Checkpoint

Molecular cloning of *esp1*, an extragenic suppressor of *pim1-46*, revealed that the gene encodes a type 2A-related phosphatase. Budding yeast SIT4 or PPH3 show high similarity to *esp1*. In an accompanying paper by Shimanuki *et al.*, a gene encoding a type-2A like phosphatase, *ppe1*, is characterized. Based on the nucleotide

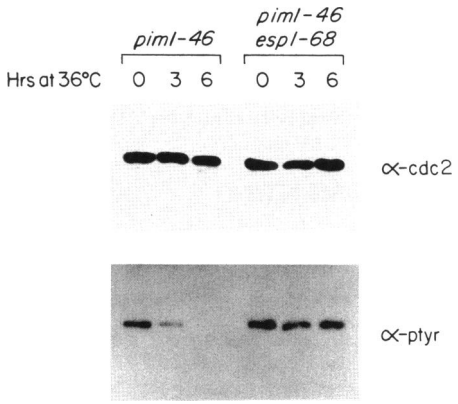

Figure 8. Tyrosine dephosphorylation of $cdc2^{+}$. Cell extracts from *pim1-46* and the double mutant *pim1-46 esp1-68* were prepared after 0, 3, and 6 h of shift to the restrictive temperature. With the use of these extracts, immunoprecipitation using anti-cdc2 antibody (G8) was done. Of each sample 50% was used for Western blotting with anti-cdc2 antibody (G8) (top), and 50% was used for Western blotting with anti-phosphotyrosine antibody (bottom).

sequence and physical mapping on the genome, we conclude that *esp1* and *ppe1* are identical. Shimanuki *et al.* have demonstrated that the *ppe1/esp1* shares a part of biological function with type-2A phosphatases (*ppa1* and *ppa2*). High dosage expression of *ppa1* or *ppa2* suppresses the cold sensitivity of a null allele of *ppe1* (Δ*ppe1*). In addition, a double disruption of *ppe1* and *ppa2* (Δ*ppe1* Δ*ppa2*) causes the cell death.

It is unclear how the esp1 protein phosphatase is involved in mitotic checkpoint. We speculate that the activity of $p58^{pim1}$ as a nucleotide exchanger on the spi1 GTPase is still lower in the double mutant, *esp1-68, pim146,* than in wild-type cell because we found two defects of the mutant $p58^{pim1}$, weaker affinity for the spi1 GTPase, and lability at restrictive temperature, which were not fully suppressed in the double mutant. The *esp1-68* mutation may compensate for a shortage of the active form of the GTPase (GTP bound form) by downregulating the antagonists of $p58^{pim1}$ such as GAP (GTPase activating protein) or by regulating guanine nucleotide metabolism.

In *S. cerevisiae*, the SIT4 protein phosphatase (a homolog of *esp1*) has been shown to be required for the accumulation of G1 cyclin RNAs (Fernandez-Sarabia *et al.*, 1992). Temperature sensitive mutations in SIT4 gene causes cell cycle arrest at G1. By analogy, it was considerable that *S. pombe* $esp1^+$ was required for the accumulation of cdc13 (G2 cyclin) and that mutations in *esp1* caused G2 delay, resulting in the suppression of onset of premature initiation of mitosis in *pim1*. Our preliminary result, however, showed no difference in the level of cdc13 transcript.

Shimanuki *et al.* have shown that loss of function of *ppe1* causes G2 arrest at 20°C. This would suggest that the *ppe1/esp1* mutation delays the onset of mitosis and suppresses the pim1-induced premature entry into mitosis.

Note added in proof: The gene name, *esp1*, has been used for a budding yeast gene. To avoid confusion, the *esp1* will be called *ppe1* as in the manuscript by Shimanuki *et al.*, and will appear as such in EMBL database with the accession number Z18925.

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