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A novel protein kinase gene *ssp1*$^+$ is required for alteration of growth polarity and actin localization in fission yeast

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Temperature-sensitive suppressor mutants were isolated from two fission yeast mutants defective in cell shape control: *ppe1*, encoding a type 2A-like protein phosphatase, and *sts5*, one of 11 staurosporine-supersensitive mutants. Complementation tests showed that suppression was due to two chromosomal loci, *ssp1* and *ssp2*. Cells of the *ssp1* mutant grown at the restrictive temperature arrested uniformly with an elongated cell body and a 2C content of DNA. Interestingly, these mutant cells grew only in a monopolar manner. At a specific point in the G_2 phase of the cell cycle, wild-type cells exhibit a drastic alteration in growth polarity, from mono- to bipolar. This change coincides with the distribution of cortical actin from one end of the cell to both ends. In the *ssp1* mutant cells, cortical actin was localized only at one end, suggesting that the mutant fails to change growth polarity. Nucleotide sequence determination showed that *ssp1*$^+$ encodes a novel protein kinase. Ectopic overexpression of *ssp1*$^+$ resulted in an altered cell morphology and cortical actin was randomly dispersed within the cells. Immunocytological analysis revealed that the protein was primarily localized in the cytoplasm and that half of the protein existed in an insoluble fraction. These results show that the dynamics of actin-based growth polarity during the cell cycle are regulated, at least in part, by a novel set of protein kinases and phosphatases.

Key words: actin localization/cell cycle/fission yeast/growth polarity/protein phosphorylation

Introduction

Actin plays a central role in the maintenance of cell shape and polarized growth (reviewed in Bretscher *et al.*, 1994). Actin is a ubiquitous protein which forms filaments and constitutes a major structural component of the cytoskeleton. It also regulates a variety of dynamic cellular processes, such as muscle contraction, cell motility and cytokinesis (reviewed in Hitt and Luna, 1994). Actin interacts with a number of proteins, collectively called actin binding proteins, which regulate the physical state of actin, such as assembly and disassembly of actin filaments (reviewed in Rozycki *et al.*, 1994; Welch *et al.*, 1994). Upon external signals or during cell cycle progression actin changes its physical state, as well as its subcellular localization. Several molecules which regulate the physical state of actin filaments or cytoskeletal architecture have been identified, including small GTP binding proteins and their regulators (reviewed in Boguski and McCormick, 1993; Lamarche and Hall, 1994) and protein modules such as SH3 domains (reviewed in Pawson, 1995).

Genetically amenable yeasts have been shown to be excellent model systems to study growth polarity control (reviewed in Chant, 1994; Nurse, 1994). Cells of the fission yeast show a cylindrical shape of 8–14 μm length and 3 μm diameter (Mitchison, 1970). The growth of individual fission yeast cells is divided into four distinct stages in terms of growth polarity. The first stage is a monopolar growth phase. At the beginning of the cell cycle all of the cortical actin (or actin patches; Adams and Pringle, 1984; Kilmartin and Adams, 1984; Marks and Hyams, 1985; Marks *et al.*, 1986) is present at the new end of the cell produced at cytokinesis and moves to the opposite old end, which was already in existence in the previous cycle. Following this actin movement, cell growth initiates only from the old end. The second stage is a bidirectional growth phase. At 0.3 of the cell cycle, a rapid and dynamic change in growth polarity takes place, which coincides with actin redistribution; actin moves from the old end to the new end and cortical actin thereafter exists at both ends. This phenomenon is called NETO (new end take off; Mitchison and Nurse, 1985). The third stage is a constant cell length phase which corresponds to the mitotic stage. Actin transiently disappears from both ends upon the onset of mitosis. The fourth stage is a post-mitotic stage, where cortical actin reappears in the central region, followed by septum formation and cytokinesis.

In fission yeast, as well as budding yeast, a single essential actin gene has been isolated (Gallwitz and Sures, 1980; Ng and Abelson, 1980; Mertins and Gallwitz, 1987), although recently several actin-related genes have been isolated and begun to be characterized in other organisms (Lee-Miller *et al.*, 1992; Schwob and Martin, 1992; Herman, 1993; Clark and Meyer, 1994: Muhua *et al.*, 1994). Structural studies of the cortical actin have shown that it is composed of spiral actin filaments which are tightly associated with an invagination of the plasma membrane (Mulholland *et al.*, 1994). This may suggest that actin molecules are anchored in the membrane through a specialized mechanism. However, very little is understood with respect to regulation of growth polarity and actin localization. Molecular analysis of fission yeast *cdc* mutants, which are defective in a late step of septation (Nurse *et al.*, 1976), has revealed that the process of septum formation is regulated by well-conserved actin binding proteins. These include profilin (*cdc3*$^+$; Balasubramanian *et al.*, 1994) and tropomyosin (*cdc8*$^+$;

Balasubramanian *et al.*, 1992). It has also become clear that molecular functions of several gene products, especially a class of small GTP binding proteins, are of importance, and that these regulators are structurally and functionally conserved (Díaz *et al.*, 1993; Chang *et al.*, 1994; Miller and Johnson, 1994). These findings suggest that molecular mechanisms controlling actin-based growth polarity might be common in all eukaryotes.

We have been working on growth polarity control using fission yeast morphological mutants. *ppe1*$^+$, which encodes a novel protein serine/threonine phosphatase, is involved in cell shape control, as disruption of the *ppe1*$^+$ gene (Δ*ppe1*) leads to an aberrant cell shape, pear-shaped or round, rather than cylindrical (Shimanuki *et al.*, 1993). The *sts5* mutant, which was originally isolated as one of 11 staurosporine-supersensitive mutants (*sts1-11*; Toda *et al.*, 1991), also shows a round cell morphology (Toda *et al.*, 1993). *sts5* is allelic to *orb4*, which was independently isolated through visual screening of round mutants (Snell and Nurse, 1994). We showed that cells of the Δ*ppe1* mutant also become supersensitive to staurosporine (Shimanuki *et al.*, 1993), suggesting that these two mutants are functionally related.

In order to understand how growth polarity is regulated in cell cycle progression and to genetically dissect molecules which interact with *ppe1*$^+$ and *sts5*$^+$/*orb4*$^+$, we have started to analyze extragenic suppressors of the *ppe1* and *sts5* mutants. In this study we describe temperature-sensitive (ts) suppressor mutations of the *ppe1* and *sts5* mutants. Consistent with the phenotypic similarities between *ppe1* and *sts5* mutants, we found that the suppressor mutations independently isolated from either *ppe1* or *sts5* mutant are allelic. The ts mutant showed a uniform cell cycle-specific (*cdc*) arrest phenotype which is defective in NETO. Gene cloning and nucleotide sequencing showed that the gene encodes a novel protein kinase. Our analyses suggest that the kinase is one of the critical determinants of growth polarity which functions antagonistically with the ppe1 phosphatase.

Results

Isolation of temperature-sensitive revertants from ppe1 or sts5 mutants

The *ppe1*-deleted mutant (Δ*ppe1*) showed multiple phenotypes, including an abnormal cell shape, sterility, cold-sensitive growth (cs) and staurosporine supersensitivity (ss) (Matsumoto and Beach, 1993; Shimanuki *et al.*, 1993). The *sts5* mutant cells also became round, as well as supersensitive to the drug (Toda *et al.*, 1993). Drug-resistant (ss$^+$) or cold-resistant (cs$^+$) revertants which simultaneously showed ts growth were isolated from these two morphological mutants (see Materials and methods for details). In total, four ts ss$^+$ and two ts cs$^+$ revertants were obtained from Δ*ppe1* and seven ts ss$^+$ revertants were isolated from *sts5-7* (Table II). Four ss$^+$ revertants from Δ*ppe1* also showed a cs$^+$ phenotype and likewise two cs$^+$ revertants showed a ss$^+$ phenotype, confirming that both cs and ss phenotypes are derived from a single Δ*ppe1* mutation. It should be noted that the sterility of the Δ*ppe1* mutant was also suppressed in these ts mutations, although the suppression was weak as the ts suppressors

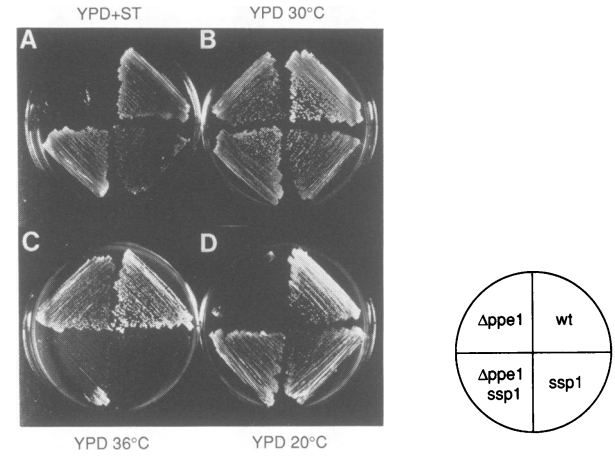


Fig. 1. Suppression of the Δ*ppe1* disruption by the *ssp1* mutation. One set of tetrad segregants tetra type derived from a cross between Δ*ppe1* and *ssp1-068* was streaked on a rich YPD plate with (**A**) or without 0.5 μg/ml staurosporine (**B–D**) and incubated at 30 (A and B) or 36°C (C) for 3 days or at 20°C (D) for 7 days. Strains used are Δ*ppe1* (upper left in each plate, TP228-3D; Table II), wild-type (upper right, TP228-3B), *ssp1-068* (lower right, TP228-3A) and Δ*ppe1 ssp1-068* (lower left, TP228-3C).

by themselves showed a low mating efficiency compared with the wild-type (Materials and methods).

Tetrad analysis between each ts revertant and the wild-type strain showed that all the revertants contained a single chromosomal mutation. In addition, the suppressor phenotype co-segregated with temperature sensitivity. Complementation testing by random spore analysis showed that the suppressor mutations were derived from two genetic loci. One locus, which was composed of all six ts alleles from Δ*ppe1* (four ss$^+$ and two cs$^+$; Table II) and five of the seven ts alleles from *sts5*, was designated *ssp1* (suppressor of *sts5* and *ppe1* mutants) and is described in this study. The other locus, which consists of the two alleles from revertants of *sts5*, was designated *ssp2* and will be reported elsewhere.

Figure 1 shows the temperature and staurosporine sensitivity of segregants from one set of tetrads between Δ*ppe1* and *ssp1*, which consists of four different genotypes (tetra type; see Table II). Cells of Δ*ppe1 ssp1*, *ssp1* and the wild-type (the lower left, the lower right and the upper right in each plate respectively) were capable of forming colonies on plates containing 0.5 μg/ml staurosporine (Figure 1A) and were also cs$^+$ (Figure 1D). In addition, Δ*ppe1 ssp1* and *ssp1* showed ts growth (Figure 1C). In contrast, Δ*ppe1* cells (the upper left in each plate) were ss (Figure 1A), ts$^+$ (Figure 1C) and cs (Figure 1D). A similar suppression of staurosporine sensitivity was observed in *sts5* and *ssp1* mutants. Thus the *ssp1* mutation suppressed the drug sensitivity of Δ*ppe1* and *sts5* and also the cold sensitivity of Δ*ppe1*.

Morphological suppression of Δppe1 or sts5 by the ssp1 mutation

Cell morphology of the Δ*ppe1 ssp1* double or *ssp1* single mutants was examined at either 26 or 36°C. The Δ*ppe1* mutant showed an irregular and often round morphology (Figure 2A; Shimanuki *et al.*, 1993). In contrast, the cell shape of the Δ*ppe1 ssp1* double mutant grown at 26°C

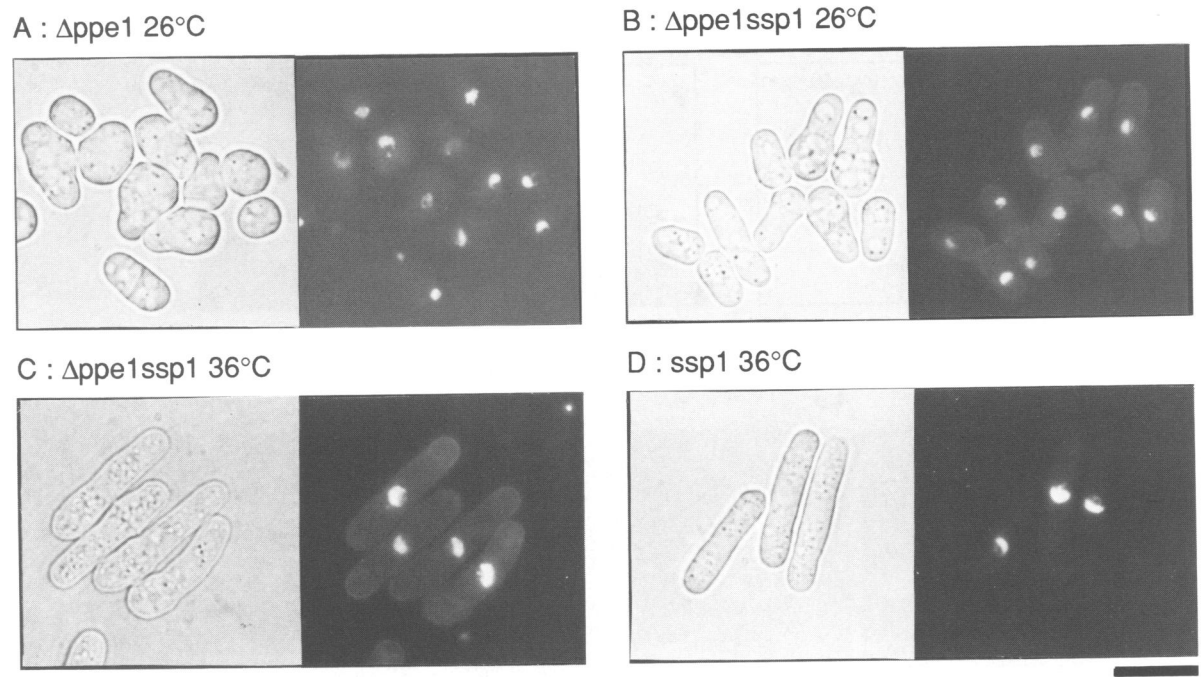


Fig. 2. Cell and nuclear morphology of the Δ*ppe1 ssp1* and *ssp1* mutants. Exponential cells grown in a rich YPD medium at 26°C were shifted to 36°C and incubation continued for 4 h. After fixation with glutaraldehyde, photographs were taken either by phase contrast microscopy (left) or DAPI-stained fluorescence microscopy (right). Cells prepared were (**A**) Δ*ppe1* (26°C, DX1; Table II), (**B** and **C**) Δ*ppe1 ssp1* (26 and 36°C respectively, TP228-3C) or (**D**) *ssp1* (36°C, TP228-3A). The bar indicates 10 μm.

was much closer to that of the wild-type, i.e. cylindrical (Figure 2B). At 36°C cells of the double mutant stopped division and became elongated with a single nucleus, like *cdc* mutants (Figure 2C). The single *ssp1* mutant also showed a cell elongation phenotype (Figure 2D). These observations demonstrated that the *ssp1* mutation suppresses not only cold and staurosporine sensitivity, but also the morphological defect of Δ*ppe1*. The *sts5 ssp1* double mutant also showed a similar cell morphology (data not shown). It should be noted that cells of the Δ*ppe1 ssp1* or *sts5 ssp1* double mutants looked fatter or sometimes more irregularly shaped than those of the *ssp1* mutant or wild-type cells (compare Figure 2C and D), suggesting that *ssp1* may not completely bypass these two mutations.

Monopolar growth of the ssp1 mutant cells at the restrictive temperature

As a single ts *ssp* mutant showed uniform cell elongation with a single nucleus at the restrictive temperature, a detailed physiological analysis on the defective phenotypes of the *ssp1* mutant was performed. When cells of an exponentially growing *ssp1* mutant were shifted from 26 to 36°C, cells divided two or three times and then ceased division (Figure 3). The percentage of septated cells dropped sharply from 17% at 0 h to 3% after 3 h, then slightly increased again and finally became 6% (Figure 3). Wild-type cells shifted in the same manner continued to divide and the percentage of septated cells remained at 15–20%, although a transient decrease in the percentage of septated cells was observed 1 h after the shift, probably because of the heat shock response (Figure 3).

Cell length was measured in the *ssp1* mutant after the shift from 26 to 36°C. As shown in Figure 4A, the average

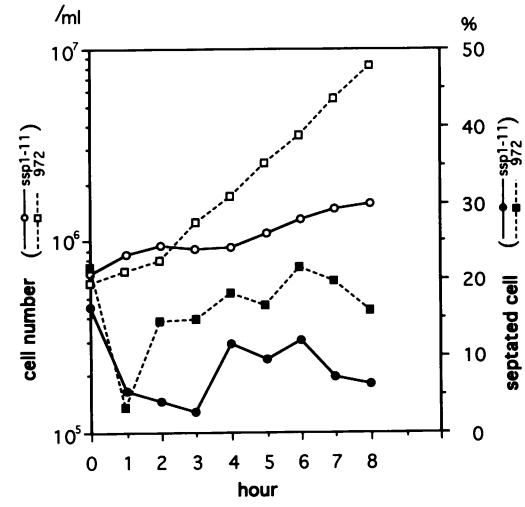


Fig. 3. Cell number increase and percentage of septated cells of the *ssp1* mutant at the restrictive temperature. Exponentially growing wild-type (shown as squares, 972; Table II) or *ssp1* mutant cells (shown as circles, TP235-2C) were shifted from 26 to 36°C and allowed to grow. Cell number (open symbols) and percentage of septated cells (closed symbols) were calculated at each time point.

cell length increased from a value of 10.4 μm before the shift to 14.3 μm after 4 h and to 17.8 μm after 8 h incubation at 36°C. Cell morphology of the *ssp1* mutant was examined by staining with Calcofluor (Streiblová and Beran, 1963). In fission yeast this fluorescent dye stains septa most brightly, but more importantly, it also stains newly synthesized cell wall in the growing end, which can be easily distinguished from the non-growing dark end and division scars (Streiblová and Wolf, 1972; Mitchison and Nurse, 1985). Observation of the *ssp1*

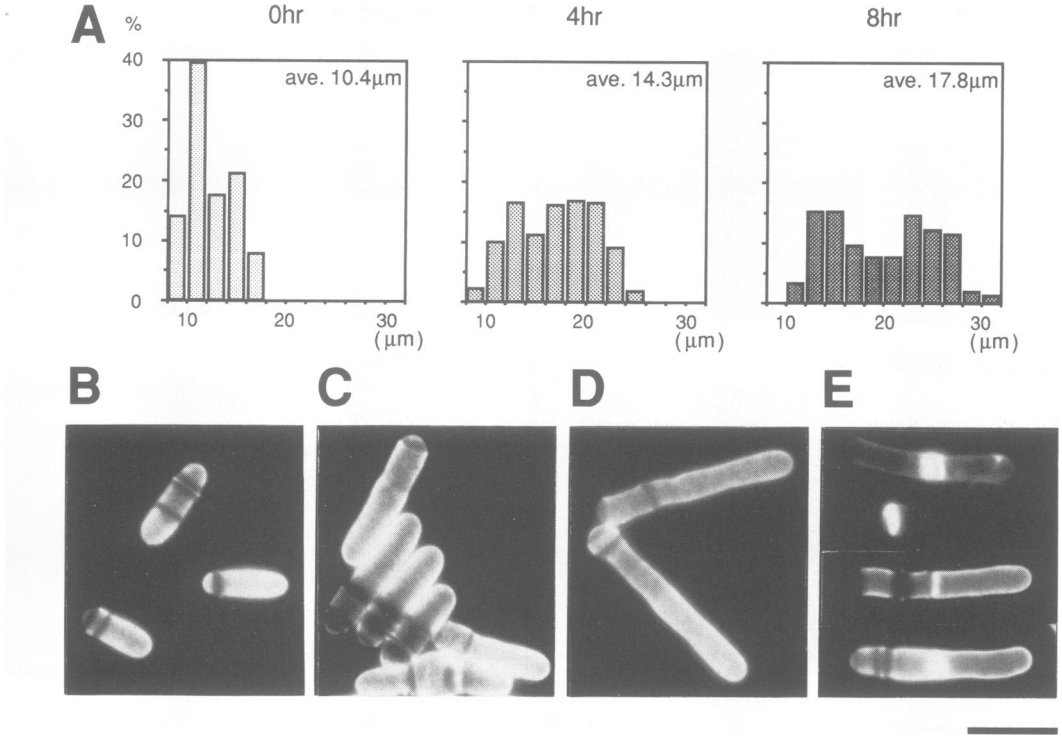


Fig. 4. Cell length and morphology of the *ssp1* mutant. (**A**) The *ssp1* mutant (TP235-2B; Table II) was grown as in the legend to Figure 3. Aliquots were taken at 0, 4 and 8 h after the shift and fixed with glutaraldehyde. At least 100 individual cells were measured for cell length at each time point. (**B–E**) Cells were stained with Calcofluor and fluorescence micrographs were taken. At each time point typical examples of cells are shown; 0 (B), 4 (C) and 8 h (D and E). Abnormal-looking cells which have never been observed in wild-type culture are indicated in (E) (see text for details). Bar indicates 10 μm.

mutant under fluorescence microscopy revealed that most of the *ssp1* cells incubated at 36°C showed monopolar growth, instead of bidirectional elongation (Figure 4C and D). In contrast, the mutant cells incubated at 26°C were shown to have a normal growth pattern, i.e. both mono- and bidirectional growth patterns were observed (Figure 4B). More than 95% of the unseptated cells showed monopolar growth (see Figure 4 and also Table I). These results suggest that in the *ssp1* mutant NETO (Mitchison and Nurse, 1985) does not take place, which leads to monopolar cell elongation at the restrictive temperature.

It should be pointed out that a small population (5%) of the *ssp1* mutant cells showed an abnormal staining pattern with Calcofluor. These included cells containing multiple septa (Figure 4E, top and bottom) or those containing Calcofluor-positive material located in one end of the cell body (the second from the top). Furthermore, septated cells which had multiple division scars with a monopolar growth pattern were observed (Figure 4E, the bottom two cells). These types of cell with an abnormal growth pattern are never observed in the wild-type (Streiblová and Wolf, 1972).

Double mutant analysis between ssp1 and G_2-arrested cdc mutants

Previous results showed that *ssp1* mutant cells continue to elongate in a monopolar manner and finally arrest, like *cdc* mutants. In wild-type cells, NETO, the point when growth polarity changes from mono- to bidirectional, usually occurs at 0.34 in the G_2 phase of the cell cycle (Mitchison and Nurse, 1985). Could *ssp1* be a novel *cdc* mutant which is defective in NETO? To examine the relationship between cell cycle progression and the function of $ssp1^+$, a double mutant was constructed between *ssp1* and *cdc25*, which mutation leads to cell cycle arrest at the G_2/M boundary (Fantes, 1979). The ts *cdc25* mutant arrests at 0.75 of the cell cycle with a bipolar growth pattern after the completion of NETO (Mitchison and Nurse, 1985). Cells of the double *cdc25 ssp1* mutant showed a mixed population of both mono- and bidirectional growth pattern, as shown in Figure 5 [compare cells in Figure 5C (*ssp1 cdc25*) with those in B (*cdc25*) and D (*ssp1*)]. A quantitative measurement of growth pattern indicated that in the double mutant ~50% of cells still showed a monopolar growth pattern after 4 h incubation at 36°C, while the percentages of monopolar cells in the single *ssp1* and *cdc25* mutants were 96 and 24% respectively (Figure 5A). The mixed population of mono-

Table I. Percentage of cells growing in a monopolar pattern after the temperature shift

	26°C	36°C			
		2 h	4 h	6 h	8 h
Wild-type	70	69	76	–	–
ssp1-11	68	–	87	94	97
Δssp1	69	80	94	88	93

Each type of cell (see legend to Figure 6) was fixed and stained with Calcofluor. The percentage of cells which showed monopolar growth was counted in a population of unseptated cells. '–' indicates not determined.

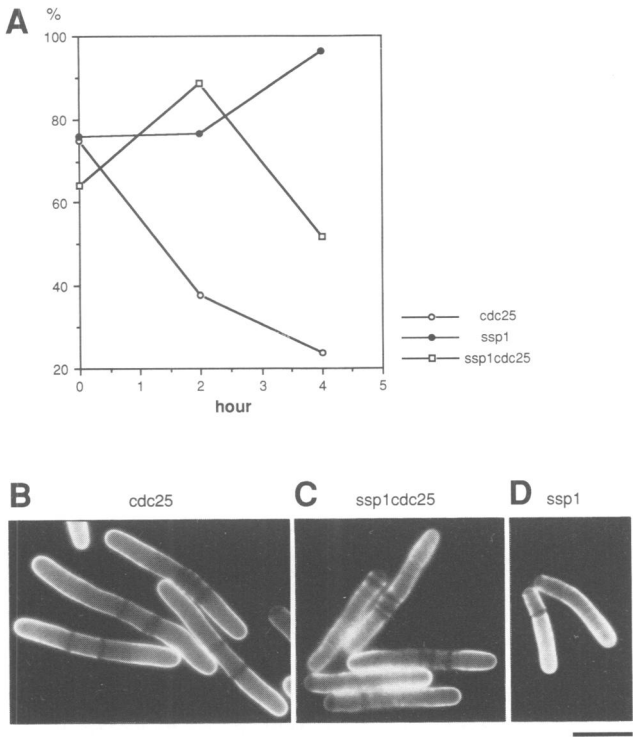


Fig. 5. Growth polarity and cell morphology of the *ssp1 cdc25* double mutant. (**A**) Exponentially growing cells in rich YPD medium at 26°C were shifted to 36°C. At 0, 2 and 4 h aliquots were taken, fixed and stained with Calcofluor. The percentage of monodirectionally growing cells was scored at each time point. (**B–D**) Typical Calcofluor-stained cells are shown (B, *cdc25*; Table II; C, *ssp1 cdc25*; D, *ssp1-31*). Bar indicates 10 μm.

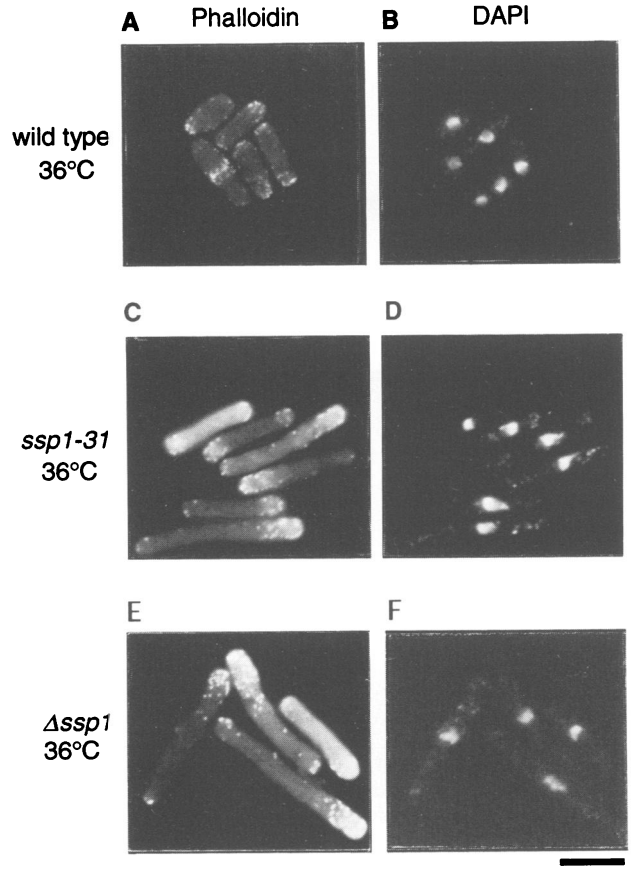


Fig. 6. Cortical actin distribution in the *ssp1* mutant. Distribution of cortical actin in the *ssp1* mutant was visualized with fluorescence microscopy by staining with TRITC-conjugated phalloidin (**A**, **C** and **E**). Cells of wild-type (A and B, HM123; Table II), *ssp1-31* (C and D, TP237-8B) or the *ssp1* disruptant (E and F, MA6) were grown at 26°C and shifted to 36°C for 6 h. DNAs (**B**, **D** and **F**) were stained with DAPI in the same field as in (A), (C) and (E) respectively. The *ssp1* mutant growing in a bipolar manner is shown for comparison in (C) (the upper two cells). Bar indicates 10 μm.

and bipolar growth (50% each) in the *ssp1 cdc25* double mutants is probably due to two points of arrest in the cell cycle; the *ssp1* mutant having an execution point before NETO. This result supported the notion that the *ssp1* mutant is defective or inefficient in traversing NETO in the cell cycle.

Localization of cortical actin in the ssp1 mutant

The results described above, including Calcofluor staining and the double mutant analysis with *cdc25*, strongly suggest that in the *ssp1* mutant actin distribution and movement are defective. To directly examine the behavior of actin in the *ssp1* mutant, fluorescence microscopy was performed by staining actin with phalloidin (Materials and methods). When cells of the wild-type or *ssp1* mutant grown at the permissive temperature were stained with phalloidin, cortical actin was visualized in three regions, namely the septum and the two ends of the cell bodies (Figure 6A and B and Table I). In contrast, in *ssp1* mutant cells shifted to the restrictive temperature, >90% of unseptated cells showed asymmetrical staining; only one end, corresponding to the old end, was stained with a patched pattern (Figure 6C and Table I). These cells were arrested with a single nucleus (Figure 6D). This result clearly supports the notion that the $ssp1^+$ gene is required for the execution of NETO.

The ssp1 mutant and DNA replication

Mitchison and Nurse (1985) previously proposed that two requirements have to be fulfilled for NETO to take place, i.e. completion of S phase and critical cell length. *cdc* mutants defective in 'start' or S phase, such as ts *cdc10* or *cdc22* respectively, cannot execute NETO because of a failure in DNA replication (Mitchison and Nurse, 1985). Thus it is important to examine whether or not DNA replication takes place in the *ssp1* mutant at the restrictive temperature. To this end, two approaches were undertaken. First, flow cytometry analysis (FACS) was performed. As shown in Figure 7B, cells of the *ssp1* mutant incubated at 36°C arrested in G_2 (note that most of the exponentially growing fission yeast cells stayed in the G_2 phase; Figure 7A; Nurse, 1975). The peak of the 2C content of the *ssp1* mutant at the restrictive temperature (Figure 7B) was slightly shifted towards the right, probably because of cell elongation of the mutant. FACS analysis of the 'start' mutant *cdc10* (Nurse *et al.*, 1976) is shown as a control, where two peaks, corresponding to the G_1 and G_2 phases, were evident (Figure 7C). This result suggests that cells of the *ssp1* mutant complete DNA replication and arrest in the G_2 phase of the cell cycle.

To show more clearly the completion of S phase in the *ssp1* mutant, the second approach, namely pulsed-field gel electrophoresis (PFGE) was performed. It was previously shown that PFGE is useful in distinguishing whether or

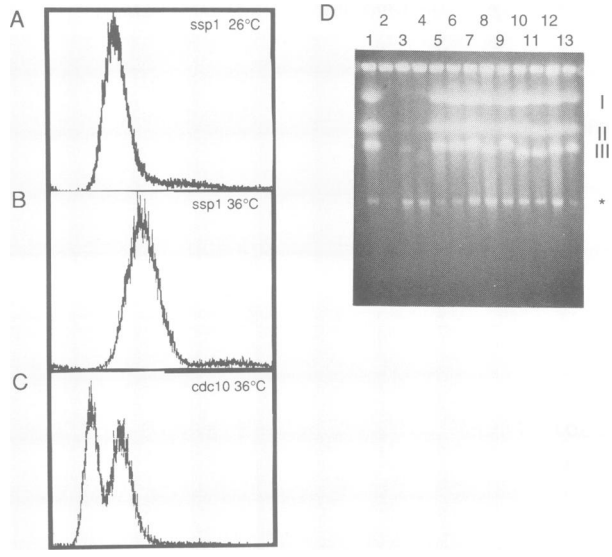


Fig. 7. DNA replication of the *ssp1* mutant under restrictive conditions. (**A–C**) Flow cytometry analysis (FACS) was performed using *ssp1-31* (TP237-8B; Table II) grown at 26 (A) or 36°C for 4 h (B). As a control the pattern of *cdc10* (Table II) shifted from 26 to 36°C and incubated for 1 h is shown (C). (**D**) Pulsed-field gel electrophoresis was performed to show that chromosomal DNA replication was completed in the ts *ssp1* mutant. DNAs were prepared from wild-type cells (HM123; Table I) grown at 30°C (lanes 1, 5–7 and 13) or treated with hydroxyurea for 4 h (lane 2) as a control. Control samples were also prepared from G_1-arrested mutant *nda1* (lane 3; Miyake *et al.*, 1993) or *nda4* (lane 4; Miyake *et al.*, 1993) cells grown at 20°C for 12 h. The *ssp1* mutants used for PFGE were *ssp1-11* (RS5-11, lane 8), *ssp1-12* (RS5-12, lane 9), *ssp1-13* (RS5-13, lane 10), *ssp1-31* (RS5-31, lane 11) and *ssp1-068* (RCX-068, lane 12). Capital roman letters (I, II and III) on the right show the position of individual chromosomes. Asterisk indicates the positions of unidentified DNAs, which were probably derived from ribosomal or mitochondrial DNAs.

not chromosomal DNA replication has been completed (Henessy *et al.*, 1991; Waseem *et al.*, 1992; Kelly *et al.*, 1993). We found that chromosomal DNAs prepared from the *ssp1* mutant grown under the restrictive condition could enter the gel and three individual chromosomal DNAs could be visualized (Figure 7D). These results demonstrate that cells of the *ssp1* mutant arrest in the G_2 phase, after completion of DNA replication. Thus *ssp1* could be assigned as the first *cdc*-type mutant which is defective in NETO while DNA synthesis is completed.

Isolation of the ssp1$^+$ gene

The *ssp1*$^+$ gene was isolated from a fission yeast genomic library (Hirano *et al.*, 1988; see Materials and methods). Six independent complementing plasmids were isolated and restriction mapping indicated that they were classified into two non-overlapping plasmids (designated pSA100 and pSA200 respectively). Integration mapping indicated that the insert of pSA100 (Figure 8A) is derived from the *ssp1*$^+$ locus and pSA200 is a dosage-dependent multicopy suppressor (Materials and methods). Analysis of pSA200 will be described elsewhere. After subcloning, the nucleotide sequence of the insert DNA of pSA100 was determined. One continuous ORF was found which contained an essential *Hin*dIII site and could encode a protein of 412 amino acid residues (Figure 8A and B). This ORF was, however, continuous with a junction between the insert and the vector. Thus a flanking genomic sequence was isolated from a fission yeast cosmid library as a 4.2 kb *Hin*dIII fragment (Mizukami *et al.*, 1993) and the nucleotide sequence was determined. The entire ORF consists of 1956 bp, which encodes a protein of 652 amino acid residues (Figure 8B).

A homology search was performed using known databases (GenBank and EMBL). The predicted ssp1 protein was found to be similar to a number of protein kinases in the databases. The protein kinase which showed the highest score was budding yeast PAK1 kinase, which was isolated as a multicopy suppressor of the DNA polymerase α mutation (P.G.Hovland and R.A.Sclafani, SCU13398). Overall identity reaches 56% among 300 amino acids. Homology was observed in the region beyond a kinase consensus sequence (Hanks *et al.*, 1988). When the same region was compared with that of the cdc2 kinase, the identity was only 20% and was restricted to the kinase consensus region (Figure 9). Therefore, the ssp1 protein is a novel protein kinase, one of a member of a subfamily including PAK1.

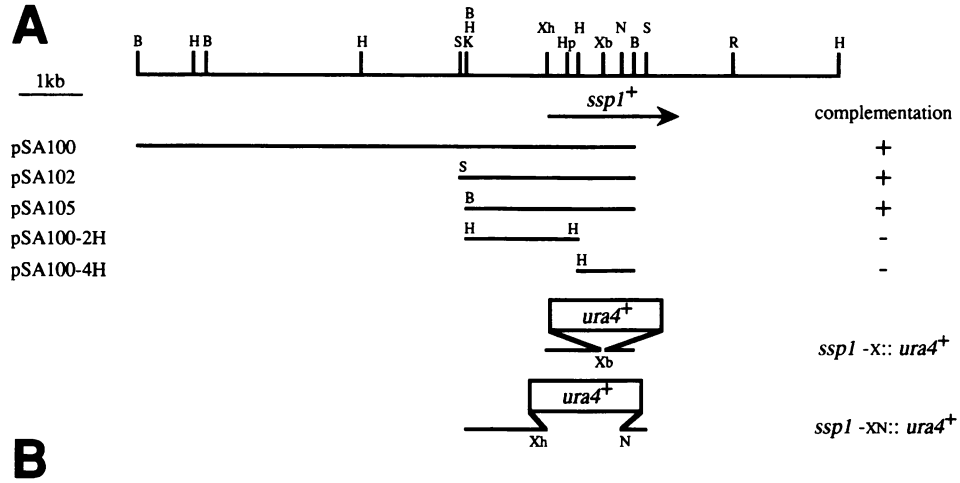
Gene disruption of the ssp1$^+$ gene

In order to examine the phenotypic consequence of disruption of the *ssp1*$^+$ gene, the one-step gene replacement method (Rothstein, 1983) was carried out. Two different constructions were made, one an insertional disruption and the other a complete deletion of the gene (Figure 8A; see Materials and methods for details). In either case, disruption of the *ssp1*$^+$ gene did not lead to cell lethality. Instead, the Δ*ssp1* cells showed a temperature-sensitive growth phenotype, like the original ts isolates. Upon shifting to the non-permissive temperature, cell numbers ceased to increase after a few divisions and arrested in a monodirectional growth pattern. Actin staining showed that the Δ*ssp1* disruptant showed the same asymmetrical monopolar distribution as those of the ts suppressors (Figure 6E and F and Table I); it is defective in NETO. Thus the ssp1 kinase is required for the execution of NETO at 36°C.

Ectopic expression of the ssp1$^+$ gene

To examine the consequences of ectopic overexpression of the *ssp1*$^+$ gene, the entire ORF was placed under the control of the thiamine-repressible *nmt* promoter (designated pREP-*ssp1*$^+$; Maundrell, 1990; see Materials and methods). Wild-type cells were transformed with pREP-*ssp1*$^+$ and Leu$^+$ transformants were selected on minimal plates containing thiamine. Transformants grew slowly when restreaked on minimal plates without thiamine (derepressed condition) and formed smaller colonies compared with those containing a vector plasmid. This growth-inhibiting effect of pREP-*ssp1*$^+$ was more evident when transformants were incubated at 36°C (Figure 10A).

To observe the morphological phenotype due to overexpression of the *ssp1*$^+$ gene, transformants were grown in the presence of thiamine at 36°C. Then thiamine was removed from the medium and cell morphology was observed. After 18–24 h following thiamine removal, the shape of many cells (>50%) became abnormal; fat, ellipsoidal or sometimes round cells appeared. Actin



B

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AAGGAATAGTATCTATATGAGCCTCTTCTCAGCGTACTTCTCGTTAGTGCCACCTATGTTGAAATACCACCCATCCCGTCTACTATCTGGGGAGGAGGATTACGTATAGTAGTGTAATAC  -361
GGAATAGTGAAAGGTACTTCGCCCCCAAATGATCATTCCCAAAAGTGCTCATCGTCATCCCTCACCAGTGTCGTGCTACGGTATAGATGTACCTAACCTTTTCCAACCACCAGTATAACGT  -241
AGGCTTGATTTTAAACAAGATATTAATCAATTTTGGAGATTGAGGACTACTAAAAATCTACAAGTCTTAAAGAAGAAATAAATTTCAGAGTCATTTAGCTTGGTAGCTTTACGACTATAG  -121
                                                                                                                    XhoI
CCCCGCCGAAATACGAGTTGCCTAGTCCCCGATAGAGGAAAAGCTCGATTAGTAAATCCGGTAATTCATGTACTTATTGACTATTGAAGCTATTTAAGAGGACTCGAGGAACACTTCCTG    -1
ATGGGATCAGTGAATAATGAAGAAAAAACTTTAATTGAGCCACAACGTCTTTTACGGAAAAATACTTGGCATCCAGAGGTAGACGATAGCGAAGTTCCTCCTTCTGTCTTTCCTGAATAT  +120
M  G  S  V  N  N  E  E  K  T  L  I  E  P  Q  R  L  L  R  K  N  T  W  H  P  E  V  D  D  S  E  V  P  P  S  V  F  P  E  Y    40

CCCGTCCACAAGGCCATCCAGAAAACGTCCGATTCATTTCGTAAACGGAACTACAGCGCGGGAGATTATGTAATAGCCCCGTTGGGCGGAGAACGTGAAGGAAGTTCGTTAACTCACTCA  +240
P  V  H  K  A  I  Q  K  T  S  D  S  F  R  K  R  N  Y  S  A  G  D  Y  V  I  A  P  L  G  G  E  R  E  G  S  S  L  T  H  S    80

TGGACTTTTCAACCTGGTAAGCATAACCAGCGTCTTTATTCTGATAATTTTCAAGAGGCTCAGCGCCAGTGGAAGCGCCTGCAAGAATGGGGCGAGGTGAAGGAAACAAAAAAGATAAGA  +360
W  T  F  Q  P  G  K  H  N  Q  R  L  Y  S  D  N  F  Q  E  A  Q  R  Q  W  K  R  L  Q  E  W  G  E  V  K  E  T  K  K  I  R    120

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K  R  F  D  R  F  S  G  R  K  Y  I  N  H  Y  E  I  I  K  E  L  G  R  G  M  H  G  K  V  K  L  G  R  D  T  V  T  R  E  L    160

TTGGCGATCAAGATCATCCCGAAGACGGAACGTCGTCCGAAACTGGGCCGAGCCAATGCTTCCAGTCAAAAGGAAAAAGTTCGTCGCGAAATTGCCATCTTAAAGAAATGTGTCCATCCT  +600
L  A  I  K  I  I  P  K  T  E  R  R  P  K  L  G  R  A  N  A  S  S  Q  K  E  K  V  R  R  E  I  A  I  L  K  K  C  V  H  P    200

AATGTCGTCCGCTTGCGCGAGGTCATTGACGATCCATCCTCAACGAAGGTGTATCTTGTTTTAGAGTACATGAGTGGTGGGGAAGTGCCATGGACAGATTGTGACTCCCCCGTACTCTCG  +720
N  V  V  R  L  R  E  V  I  D  D  P  S  S  T  K  V  Y  L  V  L  E  Y  M  S  G  G  E  V  P  W  T  D  C  D  S  P  V  L  S    240
                                  XbaI
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I  S  E  A  R  Q  Y  F  R  D  V  V  L  G  L  E  Y  L  H  Y  Q  G  I  I  H  R  D  I  K  P  A  N  L  L  L  N  S  S  N  C    280

GTCAAAATATCTGATTTTGGTGTTTCGTACATTGCCAACGCTGGCCTGAATGAAGACAACGACGTTGAGCTTGCAAAAACTGTCGGCACTCCTGCTTTTTTCGCCCCTGAATTGTGCTGG  +960
V  K  I  S  D  F  G  V  S  Y  I  A  N  A  G  L  N  E  D  N  D  V  E  L  A  K  T  V  G  T  P  A  F  F  A  P  E  L  C  W    320
                                                                                                          NheI
ACCGATTTGGATCGCCCAAGGCCTAAGATTTCTGAAGCAATAGACGTATGGGCTTTAGGCGTTACTTTGTTTTGCCTTTTGTTTGGTCGTTGTCCATTTAATGCTAGCATGGAGTATGAG  +1080
T  D  L  D  R  P  R  P  K  I  S  E  A  I  D  V  W  A  L  G  V  T  L  F  C  L  L  F  G  R  C  P  F  N  A  S  M  E  Y  E    360

CTTTTTGACAAAATCGTCAATGAGCGTCTGAACATCCCTTCTACACCCGACATTGGTGAAGAGGGTCGAGATTTGCTTAAAAGATTATTATGCAAAGATCCTGAACAACGTATAACACTT  +1200
L  F  D  K  I  V  N  E  R  L  N  I  P  S  T  P  D  I  G  E  E  G  R  D  L  L  K  R  L  L  C  K  D  P  E  Q  R  I  T  L    400

GTAGAAGTAAAACTTCACCCATGGACATTGGATGGCTTGAAGGATCCTGAAAAATGGTTGCAAAACACTGATCCCAGTACCGTCAGTCGTGTTGAGGTTTCTACAGACGAAGTTGCTTCT  +1320
V  E  V  K  L  H  P  W  T  L  D  G  L  K  D  P  E  K  W  L  Q  N  T  D  P  S  T  V  S  R  V  E  V  S  T  D  E  V  A  S    440

GCAATCAGCCTTGTGGGTCGTCTTCGCAGAAAACTTGGTAAGCTGTTTCGTTTCCGCCGTCCAAAAGCGCGAGTGTTTGATTCTTCCTCCTCTGTTCCTTCCGACTCTTCCATTTGTCGG  +1440
A  I  S  L  V  G  R  L  R  R  K  L  G  K  L  F  R  F  R  R  P  K  A  R  V  F  D  S  S  S  S  V  P  S  D  S  S  I  C  R    480

CCTGAGAGCTCTGGTAATTCTTCAATTGGTTTATCCGCGTCCGAGTTGTCCGATTCTTTTAATCGGCTTGCGGTGAATGAGTCTCAGAAGGATCGTGAAAGAAAGCAAGTACATCCTGTC  +1560
P  E  S  S  G  N  S  S  I  G  L  S  A  S  E  L  S  D  S  F  N  R  L  A  V  N  E  S  Q  K  D  R  E  R  K  Q  V  H  P  V    520

GAAATGGGTCGTAATAGTTCTGAGAAAAAGCCTAGATGTGACTTTGGCTGGGACTATGAGGCATTTCCCAATGACAATCAAGATGCTGATGATGCTTGCAGCTATAATACCGGCGACTCT  +1680
E  M  G  R  N  S  S  E  K  K  P  R  C  D  F  G  W  D  Y  E  A  F  P  N  D  N  Q  D  A  D  D  A  C  S  Y  N  T  G  D  S    560

ATTCCACAAGTCTCCAAATCCATTAACGGTCATTTCGAAACGTATAGTCGCACGTCGATGGATACGGATGATGTAGCCTCTTTCGAATCTCCAAATGCGAAGCATGAGGAAAGTGGAATG  +1800
I  P  Q  V  S  K  S  I  N  G  H  F  E  T  Y  S  R  T  S  M  D  T  D  D  V  A  S  F  E  S  P  N  A  K  H  E  E  S  G  M    600

CCTGTCGTTACTTTTCGTAATTATGAGAATTATGATGCTAATCCGTCCAATTTTCATCCAGTGGTTCCTGGTTTTGTATCATCACCTAACCTTCATTTAGCCGGGGGCTCTGACACACCT  +1920
P  V  V  T  F  R  N  Y  E  N  Y  D  A  N  P  S  N  F  H  P  V  V  P  G  F  V  S  S  P  N  L  H  L  A  G  G  S  D  T  P    640

ATCTACTGCATTGAGCATTCCTTCACACCAACTAATTGAGTGCTGTATGAACATTTCCCAATTTTATTCAATTGTTTTTTTTTGGCAATGTAGGTTGCATGTGTCTTTTAATTACTCATT  +2040
I  Y  C  I  E  H  S  F  T  P  T  N  *                                                                                      652

TCTTATTCTTGGCCTTTTTACCTAGTGTTCAATAACACTTTGTCATTCGTTTTCATCAAATCATGTCATCGGACT                                                +2105
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Fig. 8. Subcloning, gene disruption and nucleotide sequence of the *ssp1*$^+$ gene and predicted amino acid sequence of the ssp1 protein. (**A**) A restriction map of the genomic DNA containing the insert of pSA100 is shown along with various subclones and construction of the gene disruption of the *ssp1*$^+$ gene. The position of the *ssp1*$^+$ gene is indicated under the map by a solid line, with the direction of transcription (arrow). Complementation of each subclone derived from pSA100 is indicated as + or –. Two different constructions for disruption of the *ssp1*$^+$ gene (*ssp1-X*::*ura4*$^+$ and *ssp1-XN*::*ura4*$^+$) are also shown. Abbreviations of restriction enzymes used are as follows: B, *Bam*HI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nhe*I; R, *Eco*RI; S, *Sac*I; Xb, *Xba*I; Xh, *Xho*I. (**B**) Nucleotide sequence of the *ssp1*$^+$ gene and predicted amino acid sequence of the ssp1 protein are shown with 5′ and 3′ flanking sequences. Three restriction enzyme sites (*Nhe*I, *Xba*I and *Xho*I) used for the gene disruption (Materials and methods) are also indicated.

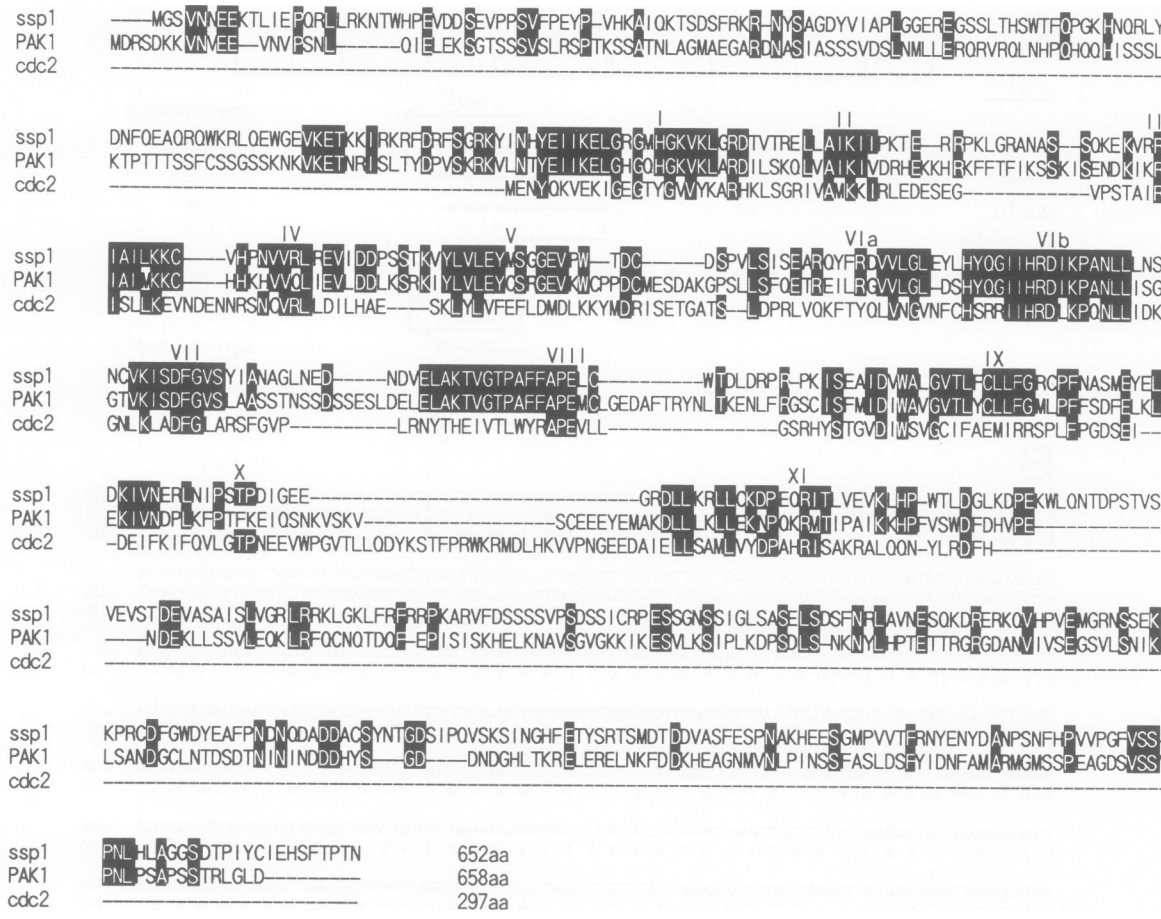

Fig. 9. Amino acid comparison between ssp1 and the cdc2 protein kinase. Homology of amino acid sequences amongst ssp1, PAK1 (P.G.Hovland and R.A.Sclafani, SCU13398) and cdc2 (fission yeast; Hindley and Phear, 1984) is shown. Identical amino acid residues are indicated by filled boxes. Numbers above the amino acids show the kinase consensus sequence, based on Hanks *et al.* (1988).

staining of these abnormal cells showed that the localization of cortical actin became altered; it was not restricted to the cell tips, but was dispersed inside the cells (Figure 10B and C). This result shows a clear contrast to that of the *ssp1* disruption, where cortical actin appeared anchored only in the old end (see Figure 6E), and strongly suggests that the ssp1 kinase determines the cellular localization of cortical actin.

Identification of the ssp1 gene product

As a first step in characterizing the ssp1 protein, the gene product was immunologically identified in a fission yeast cell extract. For this purpose, the HA peptide from influenza coat protein (Niman *et al.*, 1983) was added to the C-terminal region of the ssp1 protein (see Materials and methods for details). The multicopy plasmid containing HA-tagged *ssp1*$^+$ (HA–*ssp1*$^+$) was capable of rescuing the temperature sensitivity of the Δ*ssp1* disruptant. When the HA–*ssp1*$^+$ gene was integrated into the *ssp1*$^+$ locus by homologous recombination in the Δ*ssp1* disruption background, the integrant became ts$^+$, as expected (see Materials and methods and Table II).

Immunoblotting using a monoclonal antibody, 12CA5, against the integrant detected a specific band which exhibited a broad but discrete pattern at ~52–65 kDa (lane 3 in Figure 11A). The predicted value of the tagged ssp1 protein is 58 kDa (520 amino acid residues). This band was absent in the wild-type and parental Δ*ssp1* cells (lanes 1 and 2) and the intensity increased several fold in cells containing multicopy plasmids carrying the HA–*ssp1*$^+$ gene (lane 4). Furthermore, the protein band could be immunodepleted when cell extracts were mixed with an excess amount of the HA peptide epitope during immunoprecipitation (lanes 7 and 8). These results demonstrate that the band detected with 12CA5 corresponds to the tagged ssp1 protein. Solubility of the tagged ssp1 protein was examined in a total cell extract. It was found that ~50% of the ssp1 protein is insoluble (compare lanes 3 and 5 in Figure 11A).

To examine the subcellular localization of the ssp1 protein, immunofluorescence microscopy was performed in cells containing multicopy plasmids carrying the tagged *ssp1*$^+$ gene. As shown in Figure 11B, cytoplasmic staining, but not nuclear staining, was observed. A difference in the intensity among individual cells was probably due to the plasmid copy number in each cell. We have attempted to visualize the ssp1 protein in a single copy integrant, but the signal was too weak to clearly determine the subcellular localization of the ssp1 protein. These data suggest that the ssp1 protein kinase is not a nuclear kinase and possibly functions in the cytoplasm, with half of the fraction existing as insoluble complexes.

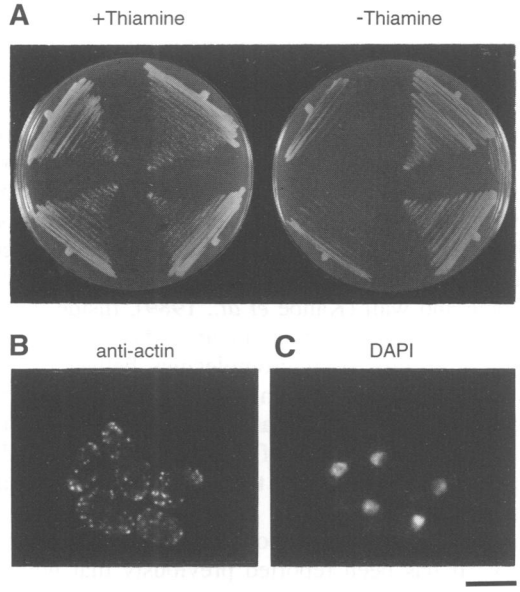

Fig. 10. Ectopic overexpression of the *ssp1*$^+$ gene. (**A**) Wild-type cells (HM123; Table II) were transformed with either vector pREP1 (upper and lower left in each plate) or pREP-*ssp1*$^+$ (upper and lower right). Two independent transformants were streaked on the minimal plate in the presence (repressed, left plate) or absence (derepressed, right plate) of thiamine and incubated at 36°C for 4 days. (**B** and **C**) Transformants containing pREP-*ssp1*$^+$ were grown at 36°C in the minimal medium containing thiamine and then thiamine was removed from the medium (Kumada *et al.*, 1995). After 24 h following thiamine removal, cells were fixed and processed for immunofluorescence microscopy by using TRITC-conjugated phalloidin (B) and chromosomal DNA was also stained with DAPI (C). Bar indicates 10 μm.

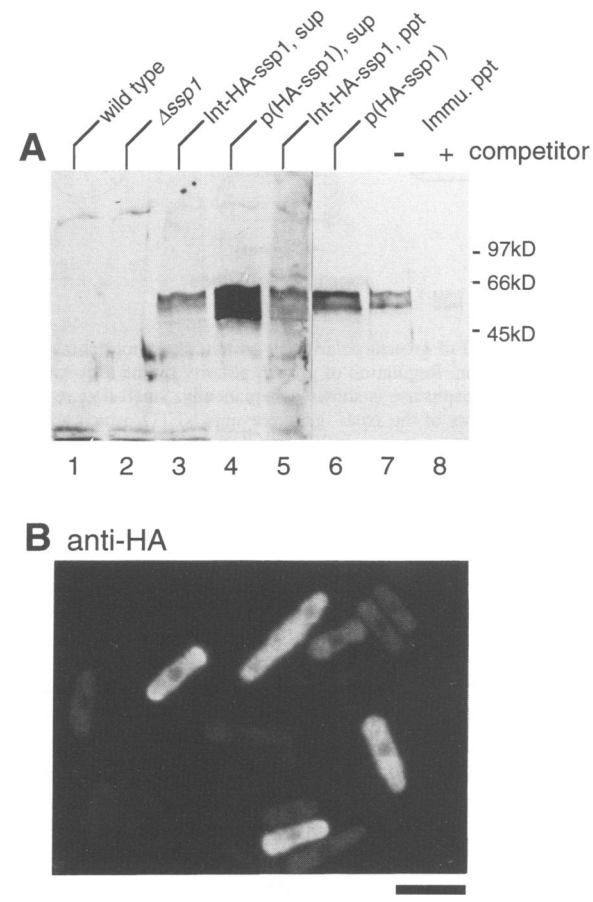

Fig. 11. Identification and subcellular localization of the epitope-tagged ssp1 gene product. (**A**) Total cell extracts were prepared from a culture grown at 32°C of wild-type (lane 1, HM123; Table II), Δ*ssp1* (lane 2, MA6), Δ*ssp1* containing integrated HA-tagged *ssp1*$^+$ (lanes 3 and 5, DMA6) or Δ*ssp1* containing episomal HA-tagged *ssp1*$^+$ (lanes 4, 6, 7 and 8, DMA7). A total of ~50 μg protein were run on the gel (lanes 1 and 2) or extracts were further separated into soluble (lanes 3 and 4) and insoluble fractions (lane 5). Epitope-tagged ssp1 proteins were immunoprecipitated from soluble cell extracts using Δ*ssp1* containing an episomal HA-tagged *ssp1*$^+$ gene in the absence (lane 7) or presence (lane 8) of 9 μg HA peptide as a competitor. Whole cell extract (25 μg) was run as a control (lane 6). Note that lanes 1–5 and 6–8 were run in different gels. Size markers of protein molecular weights are shown on the right. (**B**) Immunofluorescence microscopy was carried out using 12CA5 and Cy3-conjugated anti-mouse IgG. Δ*ssp1* containing episomal HA-tagged *ssp1*$^+$ (DMA7) was used to examine the subcellular localization of the ssp1 protein. Bar indicates 10 μm.

Discussion

In this study we have shown that the temperature-sensitive *ssp1* mutation leads to cell cycle arrest in a monopolar growth pattern with a 2C content of DNA. Actin staining reveals that actin localizes only at the growing old end. These results suggest that one of the primary defects in the *ssp1* mutant is a failure of actin relocalization or alteration in growth polarity in the G_2 phase. A number of *cdc* mutants have been isolated and characterized in fission yeast (Fantes, 1989) but, as far as we know, the *ssp1* mutant is the first G_2-arrest mutant defective in NETO. It is not surprising that the *ssp1* mutant was not detected with previous *cdc* mutants, because the average cell length of the *ssp1* mutant (17.8 μm after 8 h at 36°C) is significantly shorter than that of *cdc* mutants (≥20 μm; Nurse *et al.*, 1976; Nasmyth and Nurse, 1981). The *ssp1* mutant divides two to three times after the shift to the restrictive temperature, which may reduce the cell length of individual arresting mutants.

Growth polarity and regulation of actin localization

Regulation of actin localization is a fundamental process in cell shape and growth polarity control. Two mutually counteractive mechanisms exist to regulate actin dynamics. One mechanism anchors cortical actin in the cell membrane and fixes cell shape and growth polarity. Loss of this mechanism will lead to a failure in anchorage, resulting in dispersion or randomization of actin. The ectopic overexpression of *ssp1*$^+$ or loss of the *ppe1*$^+$ (and *sts5*$^+$/*orb4*$^+$) gene can be understood in these terms (Figure 12). The other opposing mechanism destabilizes cortical actin and induces an alteration in cellular localization and growth polarity. Failure of this process results in the anchorage of cortical actin at a specific site in the cell. The *ssp1* mutant can be considered as this type of mutant (Figure 12). During cell cycle progression or the developmental pathway, these two opposing mechanisms might regulate actin dynamics and cell polarity in a coordinated fashion. How these two counteractive processes are regulated in a cell cycle-dependent manner will be a question to be addressed in the future.

In keeping with the fact that *ppe1*$^+$ encodes a highly conserved protein phosphatase (*SIT4* of budding yeast and

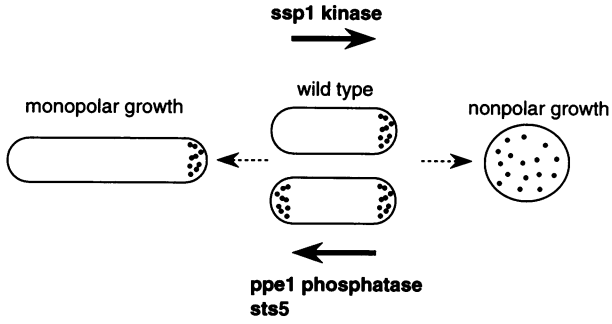

Fig. 12. A model of growth polarity by protein phosphorylation and dephosphorylation. Regulation of growth polarity by the ssp1 kinase and the ppe1 phosphatase is shown schematically. Small dots represent cortical actin. Loss of the *ssp1*$^+$ gene prevents NETO, which leads to an elongated cell with a monopolar growth pattern (left). Overproduction of the *ssp1*$^+$ gene (op-*ssp1*$^+$) or loss of the *ppe1*$^+$ (also *sts5*$^+$) gene results in round cells where growth polarity is lost (right). In wild-type cells, the ssp1 kinase and the ppe1 phosphatase, possibly antagonistically but coordinately, function in growth polarity and actin localization. It is not clear at present how overproduction of the *ssp1*$^+$ gene or loss of *ppe1*$^+$ (also *sts5*$^+$) leads to a round cell morphology.

PPV of fly; Shimanuki *et al.*, 1993), the most intriguing possibility is that the ssp1 kinase and the ppe1 phosphatase work in an antagonistic manner. It is possible that the ssp1 and ppe1 proteins directly phosphorylate and dephosphorylate respectively the same substrates, which in turn regulates fission yeast growth polarity and actin localization. The *sts5*$^+$ gene might be one candidate for such a critical substrate, and molecular analysis of the gene is currently in progress.

Is the *ssp1*$^+$ gene only involved in NETO? Physiological analyses performed in this study suggest that the major defect of the *ssp1* mutant is the failure of NETO, as the mutant showed an almost uniform arrest before NETO at the restrictive temperature. However, cells of the *ssp1* mutant show reduced mating ability; when cells of the *ssp1* mutant were crossed with each other, even at the permissive temperature, no conjugation occurred among ~10^5 cells. This observation suggests that the ssp1 gene product might be required for the conjugation process, where dynamic morphological alterations take place upon exposure to the mating pheromone (Fukui *et al.*, 1986; Leupold, 1987; Davey, 1992; Imai and Yamamoto, 1994).

Immunological analysis of the ssp1 protein showed that half of the protein remains in an insoluble fraction. It is interesting to speculate that the ssp1 kinase exists as large complexes which may interact with cytoplasmic organelles or cytoskeletal components. Although overproduced ssp1 protein from a multicopy plasmid is primarily localized in the cytoplasm, the precise subcellular localization of chromosomally derived ssp1 protein remains to be established. The ppe1 phosphatase was also shown to be localized in the cytoplasm (Shimanuki *et al.*, 1993). Efforts have been made to determine the localization of the ssp1 protein using newly prepared anti-ssp1 antibodies.

A function of NETO

NETO is the point in the fission yeast cell cycle when growth polarity changes from monopolar to bipolar. It has been shown that initiation of bidirectional growth at NETO accelerates the rate of cell elongation (Mitchison and Nurse, 1985). As is evident from Calcofluor staining, the cell wall architecture of the new end is different from that of the old end (Streiblová and Beran, 1963). A period between the beginning of the cell cycle and NETO may be required for some modification of the cell membrane or wall, whereby the new end becomes competent for growth.

It is possible that NETO is a process of equalization of the cytoplasmic material. It is known that cytoplasmic vesicles exist in the growing tips, which are assumed to provide the material required for the synthesis of new cell membrane and wall (Kanbe *et al.*, 1989). Inside the cells which grow in a monopolar manner there must exist a gradient of macro- or micromolecules between the old growing and new non-growing ends. NETO may promote a mixing of cytoplasmic materials on both sides of the cell body. Reorganization of cortical actin upon NETO may promote deposition of these vesicles through actin filament redistribution.

Is actin relocalization upon NETO essential for cell viability? It has been reported previously that not all of the population of growing cells execute NETO (Streiblová and Wolf, 1972), and that in the *wee1* mutant, which enters mitosis with a reduced cell size, NETO has hardly been observed (Mitchison and Nurse, 1985). The fact that Δ*ssp1*-deleted cells are also ts for growth, like the original mutants, suggests that NETO is not absolutely required for cell viability. It is, however, equally possible that other genes may be able to replace a function of the ssp1 kinase at the lower temperature, although genomic hybridization using the *ssp1*$^+$ gene did not show any cross-hybridizing band (data not shown). A multicopy suppressor of the *ssp1* mutant might be a candidate for that functionally redundant gene. We have started to isolate ts mutants which show a similar defective phenotype to that of *ssp1*. Characterization of these new mutants will help our understanding of the molecular mechanisms regulating NETO.

The ssp1 protein is a novel member of a protein kinase family

The *ssp1*$^+$ gene encodes a protein kinase, although final biochemical proof has not been provided. The most related protein kinase is the recently identified budding yeast product of the *PAK1* gene, which was isolated as a multicopy suppressor of the DNA polymerase α mutation (P.G.Hovland and R.A.Sclafani, SCU13398). Although these two genes may not be functional homologs, they comprise a subfamily of protein kinases. One of the most interesting features of these two kinases is histidine in the nucleotide binding glycine-rich region (GXGXHG, histidine underlined; Hanks *et al.*, 1988). Phenylalanine (F) is most common at this site, but there are several kinases, including cdc2 and MAP kinases, which have tyrosine at this position (Hanks *et al.*, 1988). ssp1 and PAK1 are the only two protein kinases which contain histidine in this nucleotide binding domain (Hanks *et al.*, 1988).

A relationship between ssp1 and fission yeast protein kinase C

Our previous work has identified protein kinase C-related genes, *pck*$^+$ and *pck2*$^+$, as regulatory components of cell shape control (Toda *et al.*, 1993; Kobori *et al.*, 1994). We

Table II. Fission yeast strains used in this study

Strains	Genotypes	Derivations
972	*h⁻*	
HM123	*h⁻leu1*	our stock
JY6	*h⁺leu1his2*	our stock
HM126	*mei1-B102arg1lys3*	our stock
TP108-3D	*h⁻leu1ura4*	Toda *et al.* (1991)
TP40-5B	*h⁻leu1sts5-7*	Toda *et al.* (1991)
DX1	*h⁻leu1ura4ppe1::ura4⁺*	Shimanuki *et al.* (1993)
RSX-011, -021, -034, -044, -046	*h⁻leu1ura4ppe1::ura4⁺ssp1*	ts ss⁺ revertants from DX1
RCX-055, 068	*h⁻leu1ura4ppe1::ura4⁺ssp1*	ts cs⁺ revertants from DX1
RS5-11, -12, -13, -14, -31	*h⁻leu1sts5-7ssp1*	ts ss⁺ revertants from TP40-5B
TP68-2	*h⁺leu1ura4his2ssp1-068*	a segregant from cross between RCX-068 and TP108-3D
TP228-3A	*h⁻leu1ura4ssp1-068*	a segregant from cross between TP68-2 and DX1
TP228-3B	*h⁺leu1ura4his2*	as above
TP228-3C	*h⁻leu1ura4ssp1-068ppe1::ura4⁺*	as above
TP228-3D	*h⁺leu1ura4his2ppe1::ura4⁺*	as above
TP235-2C	*h⁻leu1ssp1-11*	a segregant from cross between RS5-11 and JY6
TP237-8B	*h⁻leu1ssp1-31*	a segregant from cross between RS5-31 and JY6
TP335-3B	*h⁻ssp1-31*	a segregant from cross between TP237-8B and cdc25
TP335-1D	*h⁻ssp1-31cdc25-22*	as above
cdc25	*h⁺cdc25-22*	obtained from Dr P.Nurse
cdc10	*h⁺cdc10-129*	obtained from Dr P.Nurse
5A/1D	*h⁻/h⁺leu1/leu1ura4/ura4his2/+ade6-M210/ade6-M216*	Ohkura *et al.* (1989)
MA1-2	*h⁻/h⁺leu1/leu1ura4/ura4/his2/+ade6-M210/ade6-M216/ssp1-X::ura4⁺/+*	see Materials and methods
MA4	*h⁻/h⁺leu1/leu1ura4/ura4/his2/+ade6-M210/ade6-M216/ssp1-XN::ura4⁺/+*	see Materials and methods
MA4-2D	*h⁻leu1ura4ade6ssp1-XN::ura4⁺*	a segregant of tetrad from MA4
MA6	*h⁻leu1ura4ssp1-XN::ura4⁺*	a segregant of tetrad between MA4-2D and TP108-3D
DMA6	*h⁻leu1ura4ssp1-XN::ura4⁺*-pAT201	Leu⁺ts⁺ transformant of MA6 with pAT201 (tagged HA-*ssp1⁺*)
DMA7	*h⁻leu1ura4ssp1-XN::ura4⁺* containing episomal pAT201	

have shown that *sts5* and *ppe1* mutants are supersensitive to staurosporine, like those of *pck1* or *pck2* (allelic to *sts6*), and are suppressed by overexpression of the *pck1⁺* gene (Shimanuki *et al.*, 1993; Toda *et al.*, 1993). This suggests a functional link between the ppe1 phosphatase (and sts5) and the pck kinases. We have attempted to examine a genetic interaction between ssp1 and pck kinases, but contrary to the results with *ppe1* and *sts5* mutants, the *ssp1* mutation could not suppress the *pck2* mutant phenotype (T.Toda, unpublished results). Although the *pck* and *ppe1* (and *sts5*) mutants show an apparently similar abnormal morphology, the defect seems to have arisen by different mechanisms; the *pck* mutants have weak cell walls, like those of the *pkc1* mutant (Levin and Bartlett-Heubusch, 1992; Kobori *et al.*, 1994; Shiozaki and Russell, 1995), whilst cells of *ppe1* (and *sts5*) have a normal cell wall composition (H.Niwa and T.Toda, unpublished results). At present, a relationship between the ssp1 kinase and protein kinase C pathway remains to be established.

Materials and methods

Strains, media and chemicals

The *Schizosaccharomyces pombe* strains used in this study are listed in Table II. Complete medium, YPD (1% yeast extract, 2% polypeptone, 2% dextrose), modified synthetic EMM2 (Moreno *et al.*, 1991) and nitrogen-free SPA medium for conjugation and sporulation (Gutz *et al.*, 1974) have been described. Plates contained 1.6% agar. Staurosporine (provided by Dr H.Nakano, Kyowa Hakko Co.) was used as described (Toda *et al.*, 1991).

Genetic techniques and nomenclature

Standard procedures for *S.pombe* genetics were followed, according to Gutz *et al.* (1974) and Moreno *et al.* (1991). *Schizosaccharomyces pombe* cells were transformed using the lithium method (Ito *et al.*, 1984). Gene disruptions are denoted by lower case letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption, e.g. *ssp1::ura4⁺*. In the text and figures, gene disruptions are abbreviated as the gene preceded by Δ, e.g. Δ*ssp1*. Temperature- and cold-sensitive phenotypes are abbreviated to the lower case letters ts and cs respectively, e.g. ts *ssp1*. Staurosporine supersensitivity is abbreviated to the lower case letters ss. Revertant phenotypes of ts, cs or ss mutations are shown as ts⁺, cs⁺ or ss⁺ respectively.

Isolation of temperature-sensitive revertants from ppe1 or sts5 mutants

Spontaneous revertants were isolated from either Δ*ppe1* or *sts5-7* mutants as follows. Δ*ppe1* (cs and ss, DX1; Table II; Shimanuki *et al.*, 1993) cells were plated on rich YPD plates (10^7 cells/plate) and incubated at 20°C for 2 weeks. Cs⁺ revertants were selected and temperature sensitivity was examined by incubating these revertants at 36°C for 3 days. Also, Δ*ppe1* or *sts5* (ss, TP40–5B; Table II; Toda *et al.*, 1991) mutants were plated on YPD plates containing 0.5 μg/ml staurosporine and incubated at 33°C for 7–10 days. Drug-resistant colonies (ss⁺) were selected and the temperature sensitivity was examined. In either case, spontaneous revertants appeared with a frequency of $2.5–5\times10^{-7}$. In total, ~200 cs⁺ revertants were obtained from Δ*ppe1* and 100 ss⁺ revertants were obtained from either Δ*ppe1* or *sts5*. Among them, two ts cs⁺ and five ts ss⁺ revertants were isolated from Δ*ppe1* (RCX-055 and -068 and RSX-011, -021, -034, -044 and -046; Table II) and seven ts ss⁺ revertants (RS5-11, -12, -13, -14, -31, -32 and -51; Table II) were isolated from *sts5*.

Complementation test

The ts revertants isolated showed a poor mating ability, which hampered the complementation test to assign genetic loci to these revertants. To overcome this difficulty, at first a plasmid (designated pSA200) which

complemented the temperature sensitivity of one revertant (RS5-31; Table II) was isolated from a fission yeast genomic library constructed in a multicopy vector pDB248 (Beach and Nurse, 1981; Beach *et al.*, 1982; Hirano *et al.*, 1988). pSA200, which turned out to be a dosage-dependent multicopy suppressor plasmid (see below), suppressed the temperature sensitivity of all the revertants except one strain (RS5-13; Table II). Furthermore, the poor mating phenotype of those ts revertants was simultaneously suppressed. After transforming each revertant with pSA200, Leu^+ transformants were crossed with a wild-type marker strain and tetrad analysis was performed. The temperature sensitivity of all the ts revertants showed 2:2 segregation, which indicates that the mutations were derived from a single chromosomal locus. In addition, segregants which showed original phenotypes (cs and ss for Δ*ppe1* and ss for *sts5*) appeared in the ratios 0:4, 1:3 or 2:2, where no ts ss segregants were found. These results indicate that a single extragenic suppressor mutation is responsible for the suppression of Δ*ppe1* or *sts5*. Random spore analysis among ts segregants (carrying pSA200) was performed to classify each ts locus into complementation groups. Free spores were spread on a YPD plate and directly incubated either at the permissive temperature, 26°C, or at the restrictive temperature, 36°C. The presence or absence of pSA200 was tested by examining leucine auxotrophy of segregants. Leu^- ts^+ segregants were assigned as wild-type recombinants. The temperature sensitivity of one revertant, RSX-046, was too subtle to judge complementation with other ts revertants and therefore was not examined further.

A haploidization test (Gutz *et al.*, 1974) was performed between an *ssp1* mutant (TP2352C; Table II) and the *mei1-B102* strain (HM126; Table II) to determine the chromosome on which the *ssp1* locus resides. Ts^+ co-segregated with Arg^- of a marker for chromosome III (*arg1*), while ts^- co-segregated with Arg^+, indicating that the *ssp1* locus is located on chromosome III. Subsequent tetrad analysis showed that *ssp1* is linked to *ppe1* (PD:TT:NPD = 24:15:0, 19.2 cM), which is mapped to chromosome III (Mizukami *et al.*, 1993).

Flow cytometry analysis

Procedures described in Shimanuki *et al.* (1993) were followed.

Pulsed-field gel electrophoresis

Whole chromosomal DNAs were prepared as described in Chikashige *et al.* (1989). The CHEF-DR II electrophoresis system (Bio-Rad) was used to separate each chromosome, as previously described in Mizukami *et al.* (1993).

Nucleic acid preparation and manipulation

Standard molecular biology techniques were followed as described (Sambrook *et al.*, 1989). Enzymes were used as recommended by the suppliers (Takara Shuzo Co., TOYOBO Co. and New England Biolabs). Non-RI chemiluminescence (AMPPD, TROPIX Co.) and alkaline phosphatase-conjugated anti-digoxigenin antibodies (Boehringer Mannheim GmbH) were used for detection in Southern hybridizations.

Cloning of the $ssp1^+$ gene

A *S.pombe* genomic library constructed in the vector pDB248 (Beach and Nurse, 1981; Beach *et al.*, 1982; Hirano *et al.*, 1988) was used for the isolation of plasmids which complemented the ts *ssp1* mutant (TP235-2C and TP237-8B; Table II). Two independent ts^+ and Leu^+ transformants were obtained by directly incubating transformants at 36°C. Among 12 000 Leu^+ transformants obtained at 26°C, four independent transformants could form colonies at 36°C after replica plating. All six transformants showed co-segregation between the ts^+ and Leu^+ phenotypes, showing that the suppression is plasmid-dependent. Plasmids were recovered through *Escherichia coli* from individual transformants. Restriction mapping indicated that they were classified into two different plasmids (pSA100 from five transformants and pSA200 from one transformant). Integration of the 4.2 kb *Bam*HI fragment of pSA100 into the *S.pombe* genome with homologous recombination demonstrated that it was tightly linked to *ssp1* (PD:TT:NPD = 20:0:0). In addition, hybridization of the 4.2 kb *Bam*HI fragment containing the $ssp1^+$ gene with a genomic cosmid library (Mizukami *et al.*, 1993) showed that the $ssp1^+$ gene is derived from a contig which is located next to that containing the $ppe1^+$ gene on chromosome III. These analyses demonstrated that the insert of pSA100 contains the $ssp1^+$ gene.

Nucleotide sequence determination

The dideoxy method (Sanger *et al.*, 1977) was performed using double-stranded plasmid DNA as a template (Hattori and Sakaki, 1986). A 2.7 kb *Bam*HI fragment from pSA100, which contains the $ssp1^+$ gene (Figure 8A), was inserted into Bluescript KS+ (Stratagene), designated pSE29A. To assign the stop codon of $ssp1^+$, a 4.2 kb *Hin*dIII fragment containing the C-terminal region of $ssp1^+$ was isolated from a fission yeast cosmid library (Mizukami *et al.*, 1993) and inserted into Bluescript KS+, designated pSE42A. The nucleotide sequence was determined using various restriction enzyme sites, as well as synthetic oligonucleotides as primers to connect individual sequences. These sequence data are available from EMBL/Genbank/DDBJ under accession no. D45882.

Gene disruption

Two different constructs were made to disrupt the $ssp1^+$ gene. For the insertional disruption, a 1.6 kb *Xho*I–*Sac*I fragment was subcloned into Bluescript KS+ and the 1.8 kb $ura4^+$ fragment (Grimm *et al.*, 1988) was inserted into the internal *Xba*I site, yielding p$ssp1$-X::$ura4^+$ (Figure 8A). A 3.4 kb *Xho*I–*Sac*I fragment containing the interrupted *ssp1* gene (*ssp1-X*::$ura4^+$) was used to transform the *ura4* homozygous diploids (5A/1D; Table II). For complete disruption of $ssp1^+$, a 4.3 kb *Hin*dIII–*Eco*RI fragment was subcloned into Bluescript KS+ and the $ura4^+$ fragment was inserted between the internal *Xho*I and *Nhe*I sites, yielding p$ssp1$-XN::$ura4^+$. By this construction, the N-terminal 355 amino acid residues were deleted. A 4.4 kb *Kpn*I–*Eco*RI fragment containing the deleted *ssp1* gene (*ssp1-XN*::$ura4^+$) was used to disrupt the $ssp1^+$ gene (Figure 8A). The disruption was verified by Southern hybridization of Ura^+ diploids and also a Ura^+ haploid, which was derived from a tetrad of the diploid.

Ectopic expression of the $ssp1^+$ gene

The entire ORF of the $ssp1^+$ gene was amplified by PCR using two primer oligonucleotides. These were 5′-GGCATATGGGATCAGTGAATAAT-3′ for the 5′ primer and 5′-GGAGATCTTCAATTAGTTGGTGTGAA-3′ for the 3′ primer, where *Nde*I and *Bgl*II sites are underlined. A 2.0 kb amplified fragment was subcloned into the *Nde*I–*Bam*HI sites of pREP1, which contains the thiamine-repressible *nmt* promoter (Maundrell, 1990). The plasmid was designated pREP-$ssp1^+$. pREP-$ssp1^+$ could complement the ts *ssp1* mutant at 36°C under thiamine-containing repressing conditions (data not shown), indicating that the PCR fragment of the $ssp1^+$ gene is functional. Derepression of the *nmt* promoter by thiamine is known to be incomplete and a similar complementation phenomenon has been previously reported (Kumada *et al.*, 1995).

Epitope tagging of the ssp1 protein

The epitope-tagged $ssp1^+$ gene was constructed as follows. A 99 bp DNA fragment encoding three copies of an epitope sequence consisting of nine amino acid residues derived from the hemagglutinin of the influenza virus (HA1; Wilson *et al.*, 1984) was amplified by PCR using two primer oligonucleotides (5′-GGGAGCTCTACCCATACGATGT-TCC-3′ and 5′-GGGAGCTCCTAAGCGTAATCTGGAACGT-3′, where the *Sac*I site is underlined). These oligonucleotides were designed in such a manner that the amplified sequence contained a stop codon after the epitope peptides. The amplified fragment was cloned into the *Sac*I site of a plasmid pAD200 which contains a 4.2 kb *Bam*HI–*Eco*RI fragment carrying $ssp1^+$ in the vector Bluescript KS+, yielding pAT200. A 4.0 kb *Pst*I fragment containing the budding yeast *LEU2* gene was inserted into pAT200, yielding pAT201. pAT201 was used to transform the *ssp1* disruptant (MA6; Table II). Stable Leu^+ and ts^+ transformants (DMA6; Table II) were isolated, indicating that the truncated and tagged ssp1 protein is functional. Random spore analysis between DMA6 and a marker strain (TP108-3D; Table II) demonstrated that the tagged $ssp1^+$ gene is correctly integrated into the $ssp1^+$ locus, as no ts *ssp1* colonies appeared from 10^4 segregants. An unstable Leu^+ (and ts^+) transformant (DMA7) was also selected and used as control cells which episomally contain the tagged $ssp1^+$ gene.

Immunochemical analyses

Fission yeast cell extracts were prepared according to Hirano *et al.* (1988), with the following modifications. TEG buffer (50mM Tris–HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 30 mM NaCl, 1 mM dithiothreitol and a cocktail of protease and phosphatase inhibitors; Booher *et al.*, 1989) was used as the buffer for disruption of cells by glass beads. Protein samples were separated by centrifugation at 14 000 r.p.m. for 20 min into soluble and insoluble fractions. Immunoprecipitation was done according to Toda *et al.* (1991). Soluble cell extract (4 mg) and 10 μl 12CA5–Sepharose beads (a gift from Dr J.Correa, ICRF) were used for immunoprecipitation.

Proteins were run on SDS–polyacrylamide gels (Laemmli, 1970) and electrically transferred onto nitrocellulose filters (Towbin *et al.*, 1979).

For detection of the tagged sspl protein, monoclonal antibody 12CA5 was used (Niman *et al.*, 1983; Fields *et al.*, 1988). Horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham) was used as the second antibody. Chemiluminescence (ECL system, Amersham) was used to detect bound antibody.

Fluoresence microscopy

TRITC-conjugated phalloidin (Sigma) was used to visualize actin, as described in Alfa *et al.* (1993) with minor modifications. Cells were fixed with formaldehyde at the same temperature as the culture, for 15–30 min at 26°C or 15 min at 36°C. Samples were washed several times with PM buffer (Alfa *et al.*, 1993) before mounting on glass slides. This procedure reduced the background fluorescence. In some cases fixed cells were treated with zymolyase in order to increase the permeability to phalloidin. For subcellular localization of the sspl protein, cells which contained the epitope-tagged *ssp1*$^+$ gene on a multicopy plasmid (DMA7; Table II) were fixed with methanol (Toda *et al.*, 1991), and 12CA5 and Cy3-conjugated sheep anti-mouse IgG (Sigma) were used for the detection of bound antibody.

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