The fission yeast *sts5*[+] gene is required for maintenance of growth polarity and functionally interacts with protein kinase C and an osmosensing MAP-kinase pathway

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SUMMARY

Cell morphogenesis is a fundamental phenomenon that involves understanding a number of biological processes including the developmental program, polarity and cell division. Fission yeast *sts5* mutant cells are round rather than cylindrical with cortical actin randomly dispersed. Genetic analyses demonstrate that the *sts5*[+] gene is required for maintenance of cell shape during interphase when the cell normally exhibits polarised growth. The *sts5* mutant is not defective in cell wall integrity. Deletion of *ppe1*[+], which encodes a type 2A-like protein phosphatase, shows similar phenotypes to the *sts5* mutant and these two mutations are synthetically lethal. Multicopy plasmids containing either the protein kinase C-like gene *pck1*[+] or the protein tyrosine phosphatase *pyp1*[+], an inhibitor of an osmosensing Sty1/Spc1 MAP-kinase, are capable of suppressing the *sts5* mutation. Consistent with this, we have found that the *wis1* mutation, which is defective in a MAP-kinase kinase of the pathway, suppresses the *sts5* mutation. The predicted *sts5*[+] gene product exhibits sequence similarity to two yeast proteins, Dis3 and Ssd1 and a nematode

protein, F46E8.6, where the former two yeast proteins have been shown to be involved in cell cycle control and cell morphogenesis. The *sts5*[+] gene is not essential for cell viability, but is absolutely required for polarised growth as the gene disruption showed the same phenotypes as those of the original mutants. Overexpression of the *sts5*[+] gene resulted in altered cell morphology and, cortical actin in these over-producing cells was also abnormal, fainter and often dispersed. Anti-Sts5 antibody specifically detected a 130 kDa protein by western blotting. A green fluorescent protein-Sts5 fusion protein localised in the cytoplasm with a discrete punctate pattern, suggesting that the Sts5 protein is a component of a novel structure. These results have indicated that the Sts5 protein is a crucial determinant of polarised growth and that it functionally interacts with the serine/threonine phosphatase, protein kinase C, and an osmosensing MAP-kinase to maintain cell morphology.

Key words: Fission yeast, Cell morphology, MAP-kinase, Polarised growth, Protein kinase C

INTRODUCTION

To understand how individual cell shape is regulated, we have initiated a genetic analysis in the simple unicellular organism, the genetically amenable fission yeast, *Schizosaccharomyces pombe*. Fission yeast cells are rod-shaped and cell growth occurs only at the cell tips. This offers great advantages for the use of this organism as a system to study cell morphogenesis and the control of polarity (reviewed by Nurse, 1994). The diameter of the fission yeast cell (3 µm) remains constant throughout the cell cycle and a newly born cell grows from an initial length of around 8 µm until it reaches a critical mass (at 14 µm), whereupon mitosis is triggered, followed by a medial septation and cytokinesis (Mitchison, 1970).

Like most other eukaryotes growth polarity in fission yeast is subject to cell cycle regulation. During the first third of the

cell cycle, growth occurs only at the 'old' end which was already in existence in the previous cycle. Cortical actin localises exclusively to this growing old end. A drastic alteration of growth polarity occurs at ~0.3 of the cell cycle when the cell completes DNA replication and reaches a certain cell size. Actin is then seen at both ends and growth at the new end is initiated. This phenomenon is called NETO (new end take off; Mitchison and Nurse, 1985). From this point on, the cell exhibits bidirectional growth. When the cell becomes committed to executing M-phase, cell growth ceases and this coincides with the transient disappearance of cortical actin from both ends. Actin now rapidly reassembles at the medial region of the cell body where the septum is to be formed. Following nuclear division and cytokinesis, actin moves from the middle of the cell, which is now the new end, to the old end where growth will be initiated (Marks and Hyams, 1985; Marks et al., 1986).

The *sts5-7* mutant was originally isolated as one of eleven loci which are supersensitive to the protein kinase inhibitor staurosporine (Tamaoki et al., 1986; Toda et al., 1991) and which showed a round cell morphology even in the absence of the drug (Toda et al., 1993, and this study). Deletion of the *ppe1+* gene, which encodes a type 2A-like protein phosphatase, shows a similar phenotype, namely, round cell morphology and staurosporine-supersensitivity (Shimanuki et al., 1993). The *sts5+* and *ppe1+* genes seem to coordinately act in the same pathway that determines cell shape as, in addition to the phenotypic similarities, a temperature sensitive mutation *ssp1*, which encodes a novel protein kinase, suppresses both *sts5-7* and *Δppe1* mutant phenotypes (Matsusaka et al., 1995). The *ssp1* mutant shows reduced mating efficiency and the mutant cell elongates like an early *cdc* mutant in which only monopolar growth continues without NETO. From these results, we have proposed that growth polarity and cortical actin localisation are regulated by two counteracting mechanisms; one is via the Ssp1 kinase which stimulates the relocalisation of cortical actin, the other is through the Ppe1 phosphatase and Sts5 which act to fix actin localisation and growth polarity (Matsusaka et al., 1995). However, physiological as well as genetic characterisation of the *sts5* mutant and molecular analysis of the *sts5+* gene have not been performed.

To understand the morphological defects in *sts5* and their relation to cell cycle progression, we have characterised the mutant phenotype in detail. This analysis has led us to propose that the Sts5 protein is required for polarised growth during interphase. We have isolated the *sts5+* gene and found that it encodes an evolutionarily conserved protein. In particular the predicted Sts5 protein has a significant similarity to two yeast proteins which are involved in cell morphogenesis and cell cycle control. Genetic analysis has indicated that the Sts5 protein functionally interacts with various protein kinases and phosphatases which are involved in cell morphogenesis and polarised growth.

MATERIALS AND METHODS

Strains, media and chemicals

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

Genetic techniques and nomenclatures

Standard procedures for *S. pombe* genetics were followed according to Gutz et al. (1974) and Moreno et al. (1991). The cell concentration and mass were measured with a Sysmex F-800 (TOA Medical Electronics, Japan). *S. pombe* cells were transformed using the lithium method (Ito et al., 1983). Gene disruptions are abbreviated as the gene preceded by *Δ*, such as *Δsts5*. Protein is denoted by the first letter with a capital, followed by two lower case letters, such as Sts5.

Allelism tests

Several round mutants have been isolated through visual screening or by selecting for mutants which are resistant to an inhibitor of cell wall biosynthesis (Fukui and Yamamoto, 1988; Ribas et al., 1991; Snell and Nurse, 1994). Among a number of mutants tested, we found that *sts5* is allelic to *orb4* which was previously isolated by visual

screening (Snell and Nurse, 1994). Consistent with this finding, *orb4* mutant cells (obtained from V. Snell) exhibited staurosporine-supersensitivity.

Genetic mapping of the *sts5* locus

Tetrad analysis between the *sts5* mutant and known genetic markers showed that *sts5* is tightly linked to *sds21* (PD:TT:NPD=18:0:0, <2.6 cM) and to *wee1* (PD:TT:NPD=61:0:14, 9.3 cM), both of which map to the left arm of chromosome III (Mizukami et al., 1993). Fine mapping using three factor crosses by random spore analysis (Gutz et al., 1974) was performed to assign the precise order of the *sts5* locus in relation to *sds21* and *cut1* which is also tightly linked to *sds21* (Mizukami et al., 1993). The results showed that the order on chromosome III is *sts5-cut1-sds21*.

Glucanase sensitivity

The procedures described by Shiozaki and Russell (1995a) were followed. β-glucanase (100 μg/ml, Zymolyase-20T, ICN) was used for digesting the cell wall.

Nuclei acids 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. Co.).

Cloning of the *sts5+* gene

S. pombe genomic libraries constructed in a vector pDB248 (Beach et al., 1982) or in a cosmid sCOS-LEU2 (a gift from Dr Osami Niwa) were used for isolation of the *sts5+* gene (TP40-5B, Table 1). Six different plasmids (designated pN2, pN15, pTC7-4, pTC10-2, pTC16-1 and pTC16-5) were recovered from transformants using a plasmid library which suppressed both staurosporine-supersensitivity and a cell shape defect. Subsequent physical mapping by hybridisation against an ordered cosmid library (Mizukami et al., 1993) showed that none of them contained the *sts5+* gene as no clones hybridised to cosmids derived from chromosome III where the *sts5+* gene resides (see above). Thus, these six plasmids are multicopy suppressors.

One transformant (TA101), which reverted to both the drug-sensitivity and the cell shape defect, was obtained using a cosmid library. The Leu+ phenotype in TA101 was mitotically stable, suggesting that the cosmid DNA was integrated into the genome by homologous recombination. Genetic crosses between TA101 and a marker (JY6 and cut1, Table 1) showed that the Leu+ phenotype segregated 2:2 and that the Leu+ marker was linked to the *cut1* locus, suggesting that the insert DNA is integrated at the *sts5+* locus and that the insert DNA of the cosmid contains the *sts5+* gene. In order to recover an integrated cosmid DNA containing the *sts5+* gene from TA101, the following method was undertaken. Genomic DNAs were isolated from TA101, completely digested with *Bam*HI (which cleaved at only a single site in the vector, and was used to construct the library by ligating a genomic DNA which had been partially digested with *Sau*3AI into this site and therefore has a low probability of being regenerated in the clones), religated and transformed into *Escherichia coli* to obtain ampicillin-resistant colonies. By these procedures, it was expected that not the whole insert, but two extreme ends of the insert DNAs terminating in a *Bam*HI site, would be isolated as a religated circular plasmid. As expected, a plasmid, designated pTA9, was recovered from *E. coli* and restriction mapping of pTA9 showed that it contained two fragments, of 1.4 kb and 0.3 kb, which were derived from the two ends of the integrated cosmid. Hybridisation using the above two fragments as a probe against an ordered cosmid library (Mizukami et al., 1993) showed that both of them were derived from a contig containing *cut1+* and *sds21+* both of whose genes are tightly linked to *sts5+*. The identity between the cloned fragment and the *sts5+* gene was further confirmed by diploids constructed by crossing *sts5-7* with *Δsts5* (see below), which are still round and staurosporine-supersensitive.

Nucleotide sequence determination

The dideoxy method (Sanger et al., 1977) was performed using double-stranded plasmid DNA as template (Hattori and Sakaki, 1986). A 6.9 kb *Sac*I/*Sal*I fragment from cosmid 1817 was subcloned into pBluescript (KS+, Stratagene) to give plasmid pYN201. The nucleotide sequence of this fragment 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 number D58421.

Gene disruption

Two internal *Bam*HI fragments (1.6 kb and 0.3 kb) were deleted from pYN201 and replaced with the 1.8 kb *ura4*+ fragment (Grimm et al., 1988), yielding pYN701. A 4.6 kb *Cla*I/*Sac*I fragment containing this deleted version of *sts5*+ ($\Delta sts5$; more than 50% of the coding region in the amino-terminal region is missing in this construct) was used to transform a diploid 5A/1D (Table 1). Stable Ura+ transformants were obtained and Southern hybridisation confirmed that the *sts5*+ gene had been deleted as expected.

Overexpression of the *sts5*+ gene

The 6.9 kb *Sac*I/*Sal*I fragment that contains the entire coding region of the *sts5*+ gene was subcloned into pSK248, yielding pYN010. To ectopically express the *sts5*+ gene, the entire ORF of the *sts5*+ gene was amplified by PCR using two primer oligonucleotides. These are 5′AACG<u>GTCGAC</u>GATGGATGAGGAGTGTGAA3′ for the 5′ primer and 5′AACG<u>CCCGGG</u>TCAAAACTCGACAATTGA3′ for the 3′ primer (*Sal*I and *Sma*I sites are underlined). The 3.2 kb amplified fragment was subcloned into the *Sal*I/*Sma*I sites of pREP1, which is a fission yeast expression vector under the control of the thiamine-repressible *nmt1* promoter (Maundrell, 1990). The plasmid was designated pREP-*sts5*+. pREP-*sts5*+ could complement the *sts5-7* mutant in the thiamine-containing repressing conditions, indicating that the PCR fragment of the *sts5*+ gene in pREP-*sts5*+ is functional.

Construction of the GFP-*sts5*+ gene

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Prasher et al., 1992) was inserted into an amino-terminal region of the *sts5*+ gene as follows. A DNA fragment encoding the GFP protein was amplified with PCR by using two oligonucleotide primers: GFP-N1, 5′AAA<u>GTCGAC</u>GATGAGTAAAGGAGAAGAA3′ and GFP-C1, 5′AAA<u>GTCGAC</u>GATTTGTATAGTTCATCCAT3′ where the *Sal*I site is underlined. A 720 bp *Sal*I fragment was inserted into the same site of pREP-*sts5*+, designated pREP-GFP-*sts5*+. Insertion of the GFP protein in the amino terminus does not interfere with the function of the Sts5 protein as pREP-GFP-*sts5*+ behaved in the same manner as pREP-*sts5*+. Transformants carrying pREP-GFP-*sts5*+ were grown in minimal medium containing 25 nM of thiamine and green fluorescence (GFP-Sts5) was observed under immunofluorescence microscopy without fixation.

Antisera and immunochemical analyses

Oligopeptides (GKLEKEENRRRKDPIS) corresponding to a region from the 361st to 376th amino acid residues were synthesised, coupled to Hemocyanin (Calbiochem) and used to raise polyclonal antibodies in rabbits. Antisera were affinity-purified using peptide-coupled gel as recommend by a supplier (Sulfolink, Pierce). Fission yeast cell extracts were prepared according to the method of Matsusaka et al. (1995). Protein samples were run on an SDS-polyacrylamide gel (Laemmli, 1970) and electrically transferred onto nitrocellulose filters (Towbin et al., 1979). Horseradish peroxidase-conjugated Protein A (Bio-Rad) and a chemiluminescence system (ECL, Amersham) were used to detect bound antibody.

Indirect immunofluorescence microscopy

For actin staining, TRITC-conjugated phalloidin (Molecular probes) or anti-actin monoclonal antibody (N350, Amersham) and a Cy3-conjugated sheep anti-mouse IgG secondary antibody (Sigma) were used to visualise actin as described by Alfa et al. (1993) and Matsusaka et al. (1995). A monoclonal anti-tubulin antibody (TAT1, a gift from Dr Keith Gull) was used for tubulin staining.

RESULTS

Cell morphology in the *sts5* mutant

A comparison of the morphology of *sts5-7* mutant and wild-type cells stained with Calcofluor is shown in Fig. 1. A typical cylindrical shape was evident in wild type with stripes consisting of dark division scars and bright growing tips (Fig. 1A, right; note that Calcofluor most brightly stains medial septa; Streiblová and Wolf, 1972). On the other hand, in the *sts5-7* mutant grown at 32°C more than 90% of the cells were round or pear-shaped; the diameter being larger and cell length shorter (Fig. 1B). Nuclear chromatin regions stained with DAPI showed that chromosomal DNA structure was normal; the typical interphase hemispherical morphology was observed (Fig. 1A and B, left).

The effect of the temperature on the cell morphology of the *sts5* mutant was examined. At 25°C cell shape was closer to that of wild type although ellipsoidal cells were often observed (Fig. 1C). In contrast, at 36°C the cell shape became almost completely round (Fig. 1D). The *sts5-7* mutant is not temperature sensitive for growth but the generation time at the higher temperature is 50% longer than wild type. Flow cytometry analysis showed that no specific retardation of the cell cycle was observed except that the percentage of septated cells increased to more than 30% (data not shown).

Distribution of cortical actin in the *sts5* mutant during the cell cycle

To examine localisation and structures of actin in the *sts5*

Table 1. Fission yeast strains used in this study

| Strains	Genotypes	Derivations
HM123	*h⁻leu1*	Our stock
JY6	*h⁺leu1his2*	Our stock
HM126	*mei1-B102arg1lys3*	Our stock
TP40-5B	*h⁻leu1sts5-7*	Toda et al. (1991)
cdc7	*h⁻cdc7-24*	Obtained from Dr Paul Nurse
cdc10	*h⁺cdc10-129*	Obtained from Dr Paul Nurse
cdc25	*h⁺cdc25-22*	Obtained from Dr Paul Nurse
TP221-5A	*h⁻leu1sts5-7cdc25-22*	This study
TP258-6C	*h⁻sts5-7cdc10-129*	This study
TP263-1B	*h⁺sts5-7cdc7-24*	This study
TP170-2B	*h⁻leu1sts6(pck2)::LEU2*	Toda et al. (1993)
cut1	*h⁺leu1cut1-645*	Uzawa et al. (1990)
sds21-Leu2	*h⁻leu1sds21 int::LEU2*	Ohkura et al. (1989)
TPR7-1	*h⁻leu1sds21 int::LEU2sts5-7*	This study
TPR8-1	*h⁻leu1sds21 int::LEU2cut1-645*	This study
TA101	*h⁻leu1sts5-7 int::cosmid (sts5*+)	An integrant of *sts5*+-containing cosmid at the *sts5* locus
5A/1D	*h⁻/h⁺leu1/leu1ura4/ura4 his2/+ade6-M210/ade6-M216*	Ohkura et al. (1989)
$\Delta sts5$	*h⁻leu1ura4stst5::ura4*+	This study
$\Delta wis1$	*h⁹⁰leu1ura4ade6-216wis1::ura4*+	Obtained from Dr J. Millar
TP189-2A	*h⁻leu1ura4sts5-7*	This study
TPR23-3	*h⁻leu1ura4sts5-7wis1::ura4*+ int::pREP41-wis1*+	A segregant between $\Delta wis1$ and TP189-2A
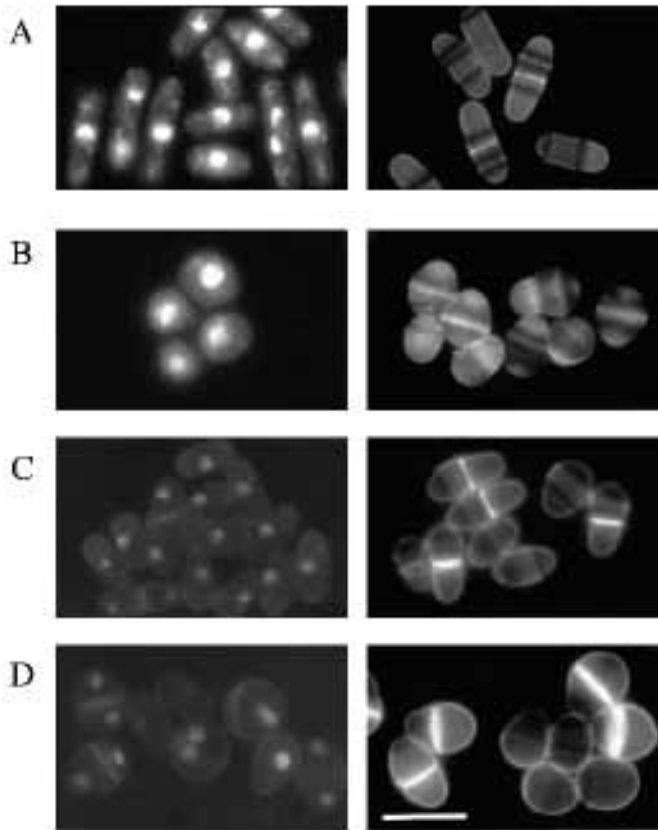
Fig. 1. Cell morphology of the *sts5* mutant. Exponentially growing wild-type (A, HM123, Table 1) or *sts5* mutant cells (B-D, TP40-5B) were collected, fixed and stained with DAPI (left) or Calcofluor (right). Cells were grown at 32°C (A and B), 25°C (C) or 36°C (D). Wild-type cells are rod-shaped at all temperatures. Bar, 10 μm.

mutant, exponentially growing wild-type or *sts5* cells were stained with phalloidin. In wild-type cells cortical actin localised in either growing tips or in the septum as reported previously (Fig. 2A; Marks and Hyams, 1985; Marks et al., 1986). In contrast, in the *sts5* mutant, cortical actin was dispersed within the cell (Fig. 2B). Next actin localisation in the *sts5* mutant during cell cycle progression was examined. As shown in Fig. 3, it was clear that, in interphase cells; namely from the beginning of the cell cycle to just before mitosis (Fig. 3A-D), cortical actin did not localise to the cell tips but appeared dispersed within the round cell. In contrast, during the mitotic and post-mitotic stages (Fig. 3E-H), cortical actin localised in a manner analogous to that in wild type, i.e. it disappeared upon the onset of mitosis (Fig. 3E) and reassembled to a medial region after mitosis (Fig. 3F-H). These results suggested that the *sts5*+ gene is required for localisation of cortical actin to the growing tips before mitosis, but not to a medial region during and after mitosis.

Distribution of microtubules were also examined. It was found that cytoplasmic microtubules also appeared abnormal; they showed criss-crossed structures as reported in other round mutants (data not shown; Snell and Nurse, 1994).

Cell wall integrity in the *sts5* mutant

Why do *sts5* mutant cells lose polarity and become round? One defect could be in cell wall structure. It has been shown that cell shape of mutants defective in the protein kinase C-encoding gene *sts6/pck2* (Toda et al., 1993) is altered and its cell wall composition is impaired (Shiozaki and Russell, 1995a). It was also reported that mutants defective in (1-3)β-D-glucan synthesis exhibit a round morphology (Ribas et al., 1991). To test whether or not cell wall composition is defective in the *sts5-7* mutant, the kinetics of cell lysis upon β-glucanase treatment were examined. As shown in Fig. 4A, no difference between the sensitivity of wild type and the *sts5-7* mutant was evident. Sensitivity to detergents, such as SDS, also did not noticeably differ between wild-type and *sts5* mutant cells.

Defective phenotypes in some morphological mutants have been reported to be suppressed by the addition of osmotic stabilisers (Ribas et al., 1991; Shiozaki and Russell, 1995a). To examine the effect of a high osmolarity on the *sts5* mutant, cell morphology was observed after growing cells in medium containing 1.2 M sorbitol. It was found that the mutant cells were still round (Fig. 4B). These results have strongly suggested that the morphological defect in the *sts5-7* mutant is not due to a defect in cell wall biosynthesis or composition.

Double mutant analysis between *sts5* and known *cdc* mutants

To address the relationship between loss of polarity and cell cycle progression in the *sts5* mutant, double mutants were constructed between the *sts5-7* and temperature-sensitive *cdc* mutants. As discussed by Snell and Nurse (1994), this type of analysis allows us to determine precisely a cell cycle point when the mutant cells lose polarity. The *cdc* mutants employed were *cdc10* which blocks cell cycle progression in G_1, *cdc25* which blocks at G_2 and *cdc7* which bypasses septation without affecting other cell cycle events such as DNA replication and nuclear division (Nurse et al., 1976).

As shown in Fig. 5, all the double mutant cells of either *sts5cdc10*, *sts5cdc25*, or *sts5cdc7* showed abnormal cell morphology, cells being either pear-like, dumbbell-shaped or round (Fig. 5B,D,F). In contrast, single *cdc* mutants were typically elongated (Fig. 5A,C,E). The number of nuclei was the same in single and double mutants, a single nucleus in *sts5cdc10* and *sts5cdc25*, and multiple nuclei in *sts5cdc7*. These results show that the *sts5-7* mutation does not affect the nuclear division cycle. Actin staining showed that in double mutants actin was dispersed as in a single *sts5-7* mutant (data not shown). Thus the *sts5* mutation causes a defect in the maintenance of polarity in both G_1 and G_2 phases of the cell cycle where normal cells elongate in mono- and bipolar manners,
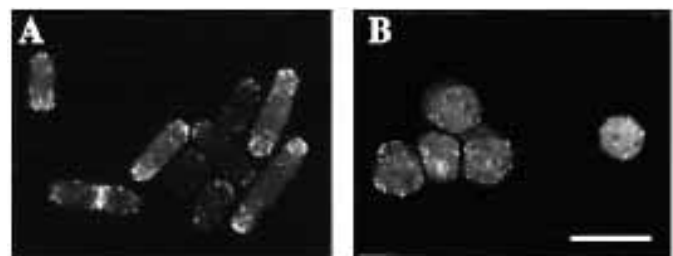

Fig. 2. Actin localisation in the *sts5* mutant. Exponentially growing wild-type (A; HM123, Table 1) or *sts5-7* mutant (B, TP40-5B) cells in YPD at 32°C were fixed and stained with phalloidin. Bar, 10 μm.

respectively. Therefore, the *sts5⁺* gene is needed to maintain polarized growth.

Protein kinase C and tyrosine phosphatase are multicopy suppressors of the *sts5* mutant

As *pck1* and *pck2/sts6* mutants, which encode protein kinase C-like proteins (Toda et al., 1993), are also supersensitive to staurosporine, a multicopy plasmid containing the *pck1⁺* gene was introduced into the *sts5* mutant. This resulted in a reversal of the cell morphology of the *sts5-7* mutant from a round to a rod shape (Fig. 6A). Staurosporine-supersensitivity of *sts5-7* was also suppressed by the introduction of *pck1⁺* or *pck2⁺* (data not shown).

We have isolated six different multicopy suppressor plasmids of the *sts5-7* mutant (see Materials and Methods). Based on restriction maps, none of them appeared to be *pck1⁺* or *pck2⁺*. However, we found that one of them (pN15) contained the *pyp1⁺* gene which encodes a protein tyrosine phosphatase (Ottilie et al., 1991). Fig. 6B shows the morphological suppression of the *sts5-7* mutant phenotype by pN15 (upper panel in Fig. 6B). As reported previously, pN15 resulted in cell elongation when introduced into wild type (lower panel in Fig. 6B, Millar et al., 1992; Ottilie et al., 1992).

Recently it has been shown that *pyp1⁺* acts, at least in part, via the dephosphorylation of a tyrosine residue of a novel MAP-kinase Sty1/Spc1, which is involved in both osmoregulation and mitotic entry (Millar et al., 1995; Shiozaki and Russell, 1995b). To address the question of how high copy *pyp1⁺* gene suppresses the *sts5* mutation, double mutants between *sts5* and *wis1*, which encodes an upstream MAP-kinase kinase of Sty1/Spc1 (Warbrick and Fantes, 1991; Millar et al., 1995; Shiozaki and Russell, 1995b) was constructed. Consistent with the notion that Pyp1 acts through negative regulation of the Sty1/Spc1 pathway, the *wis1* mutation also rescued the *sts5* mutation: the *sts5wis1* double mutant became drug-resistant (Fig. 7A). Different from a single *wis1* mutant (Warbrick and Fantes, 1991), however, cells of the double mutant do not elongate (Fig. 7B), suggesting that the *sts5* mutation also suppresses a G$_2$ delay phenotype of the *wis1* mutation. These results demonstrate that Sts5 and the Sty1/Spc1 pathway regulate cell shape in an opposing manner.

Genetic interaction between *sts5* and other morphological mutants

Although a multicopy plasmid containing *ppe1⁺* did not
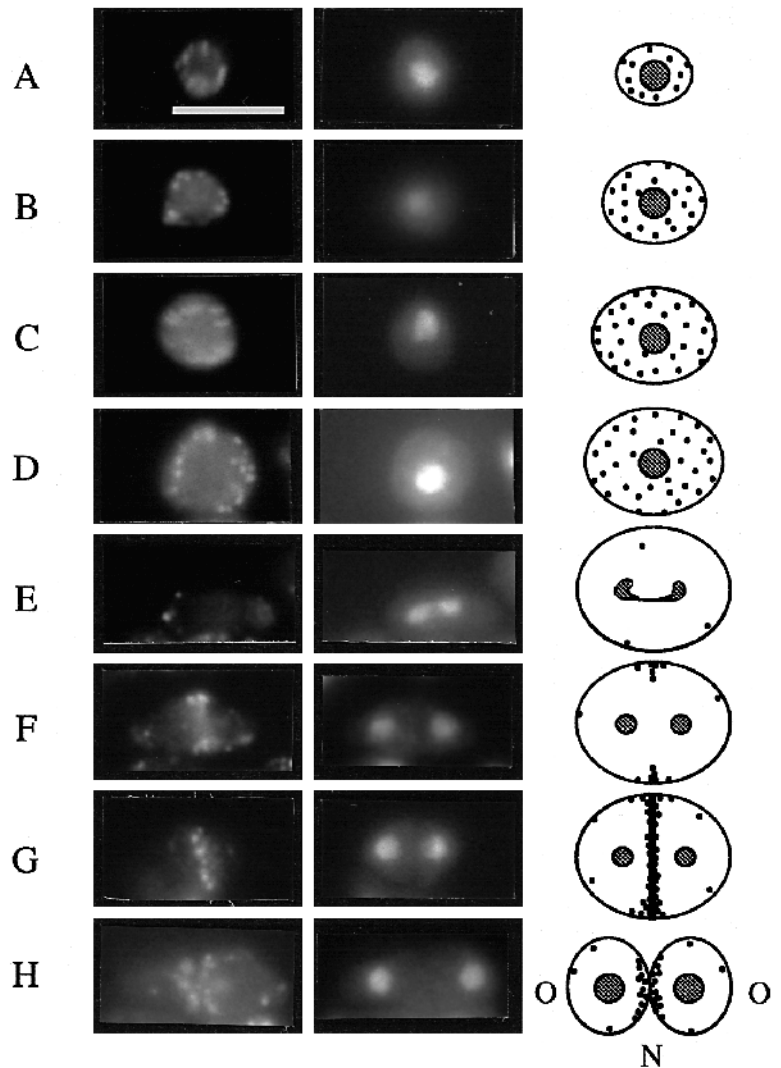

Fig. 3. Localisation of cortical actin in the *sts5* mutant during the cell cycle. Exponentially growing *sts5-7* mutant cells (TP40-5B, Table 1) in YPD at 32°C were fixed and stained with anti-actin antibody (left) and DAPI (middle). Small dots represent localisation of cortical actin and closed circles in the middle of the cell show the nucleus (drawings at right). Actin in D appears to be localised to the cell periphery because of the plane of focus but it is in fact dispersed within the cell. Bar, 10 μm.
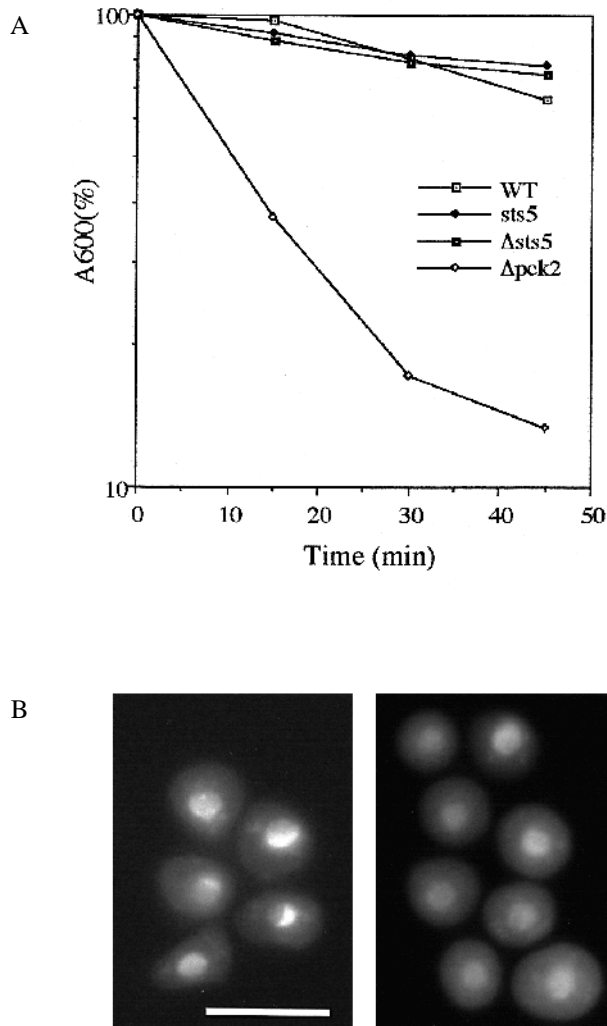
Fig. 4. Glucanase sensitivity and the effect of sorbitol in the *sts5* mutant. (A) Exponentially growing cells cultured in rich YPD medium at 26°C were collected, washed in water, treated with β-glucanase and incubated at 32°C. At the indicated time points, aliquots were taken and the turbidity (at 600 nm) was measured. Turgidity at 0 minutes is expressed as 100% in each sample. Strains used were as follows: wild type (open squares, HM123, Table 1); *sts5-7* (closed diamonds, TP40-5B), Δ*sts5* (closed squares, Δsts5) and Δ*pck2* (open diamonds, TP170-2B). (B) *sts5-7* mutant cells were grown at 32°C either in the rich YPD (left) or in YPD containing 1.2 M sorbitol (right), collected, fixed and stained with DAPI. Bar, 10 μm.

suppress the *sts5* mutant and vice versa, the *sts5* mutant showed synthetic lethality with the Δ*ppe1* mutant. This result is consistent with our previous results which showed that the *ssp1* and *ssp2* mutations, which are defective in the genes encoding novel protein kinases (Matsusaka et al., 1995), are capable of suppressing both *sts5* and *ppe1* mutants. The *sts5* mutant is supersensitive to an immunosuppressive agent, cyclosporin A and showed synthetic lethality with *ppb1* which encodes a catalytic subunit of calcineurin, a type 2B protein phosphatase (Yoshida et a., 1994). A summary of these genetic interactions between *sts5* and other protein kinases and phosphatases is shown in Table 2.
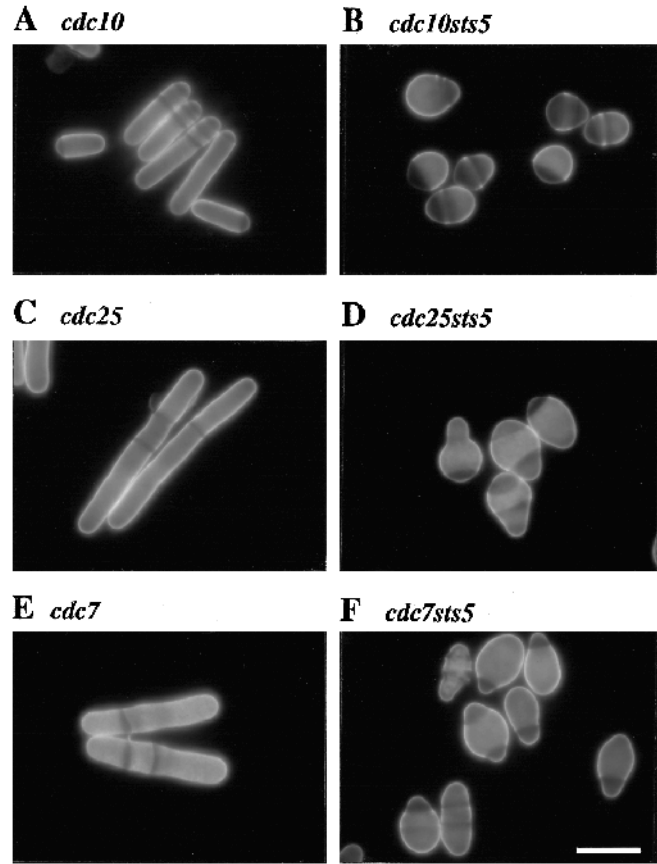

Fig. 5. Cell morphology and cortical actin distribution in the double *cdc* and *sts5* mutants. Cells containing either a single *cdc* mutation or double *sts5cdc* mutations were grown in rich YPD medium at 26°C, shifted to 36°C and incubated for 4 hours (A-F, see Table 1 for genotypes of each strain). Bars, 10 μm.

Isolation of the *sts5⁺* gene and its predicted amino acid sequence

We cloned the *sts5⁺* gene by complementation of the *sts5* mutation from a cosmid library and sequenced the gene (see Materials and Methods). The minimal complementing fragment contained a single uninterrupted open reading frame consisting of 3,198 bp which predicts a protein of 1,066 amino acid residues. Comparison with amino acid sequences in the data bases (EMBL and GenBank) showed that the predicted Sts5 protein shares significant homology with two known proteins, fission yeast Dis3 (Kinoshita et al., 1991) and budding yeast Ssd1/Srk1 (Sutton et al., 1991; Wilson et al., 1991) as well as *Caenorhabditis elegans* F46E8.6 (accession number: U23514). As shown in Fig. 8A and B, two regions exhibit the highest homology. One region 'a', consisting of 170 amino acid residues, shows a 47% identity only between Sts5 and Ssd1/Srk1. No homologous region was found in Dis3. In contrast, another region, 'b', consisting of 680 amino acid residues, shows 21% identity between Sts5 and Dis3, and 33% between Sts5 and Ssd1.

To test whether any of these three genes are functionally exchangeable, multicopy plasmids containing each gene were introduced into each mutant. No suppression was observed; neither *sts5⁺* nor *SSD1* could suppress the *dis3* mutant and

Table 2. Genes interacting with *sts5+*

Suppression of the *sts5* mutant	Genes	Gene products
By multicopy plasmids	*pyp1+*	Tyrosine phosphatase
	pck1+	Protein kinase C-like
By mutations	*ssp1*	Protein kinase
	ssp2	Protein kinase
	wis1	Protein kinase
Synthetic lethality	*ppe1*	Type 2A-like phosphatase
	ppb1	Type 2B phosphatase

References for each gene listed above are as follows: *pyp1+* (Ottilie et al., 1991); *pck1+* (Toda et al. 1993); *ssp1* and *ssp2* (Matsusaka et al., 1995; T. Matsusaka and T. Toda, unpublished results); *wis1* (Warbrick and Fantes, 1991); *ppe1* (Shimanuki et al., 1993); *ppb1* (Yoshida et al., 1994).

neither *dis3+* nor *SSD1* could suppress the *sts5* mutant. Suppression of the *ssd1* mutant by *sts5+* or *dis3+* has not been tested. Thus, these three genes are probably not functional homologues.

Gene disruption of the *sts5+* gene

In order to examine the phenotypes arising upon complete disruption of the *sts5+* gene, one step gene replacement (Rothstein, 1983) of *sts5+* was carried out (Materials and Methods). We found that cells containing a disruption of the *sts5+* gene were viable and showed almost identical pheno-

types to those of the *sts5-7* mutant, including round morphology, temperature-dependence of the morphology, β-glucanase resistance (Fig. 4) and staurosporine-supersensitivity. In addition, multicopy plasmids containing *pck1+*, *pck2+*, or *pyp1+* were capable of suppressing drug-sensitivity of the deleted *sts5* mutant. These analyses show that the *sts5+* gene is not essential for growth in fission yeast but is required for maintenance of cell morphology.

Overexpression of the *sts5+* gene

To examine the phenotypic consequences of ectopic overexpression of the *sts5+* gene, the entire ORF was placed under the control of the thiamine-repressible *nmt* promoter (designated pREP-*sts5+*). Wild-type cells were transformed with pREP-*sts5+* and Leu+ transformants were selected on minimal plates containing thiamine (repressed condition). We found that transformants grew extremely slowly when they were restreaked on the minimal plates without thiamine (derepressed condition) and barely formed colonies (Fig. 9A), showing that ectopic overexpression of the *sts5+* gene is toxic.

The morphology of *sts5+*-overproducing cells was observed in liquid minimal medium in the absence of thiamine. As shown in Fig. 9B, many cells had an ellipsoidal appearance; asymmetrically fat rather than rod-shaped. Actin staining of these cells showed that cortical actin did not localise properly to the cell tips, instead it was often dispersed within the cells. No specific cell cycle arrest was evident in these cells. We
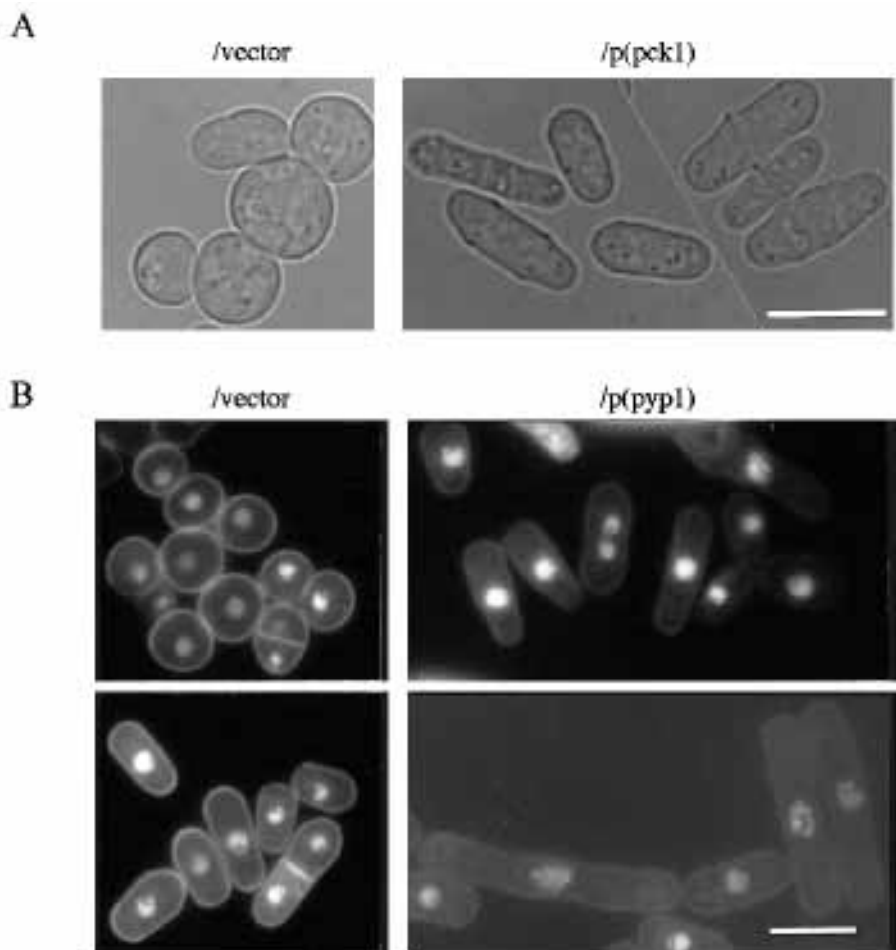

Fig. 6. Multicopy suppression of the *sts5* mutant. (A) The *sts5* mutant (TP40-5B, Table 1) was transformed with vector (left, pSK248) or a multicopy plasmid containing the *pck1+* gene (right, pCK11-2). (B) *sts5-7* mutant (upper panels) or wild-type (lower panels, HM123) cells transformed with vector (left, pSK248) or a multicopy plasmid containing the *pyp1+* gene (right, pN15) are shown. Cells from each exponentially growing culture were fixed and stained with DAPI. Bars, 10 μm.
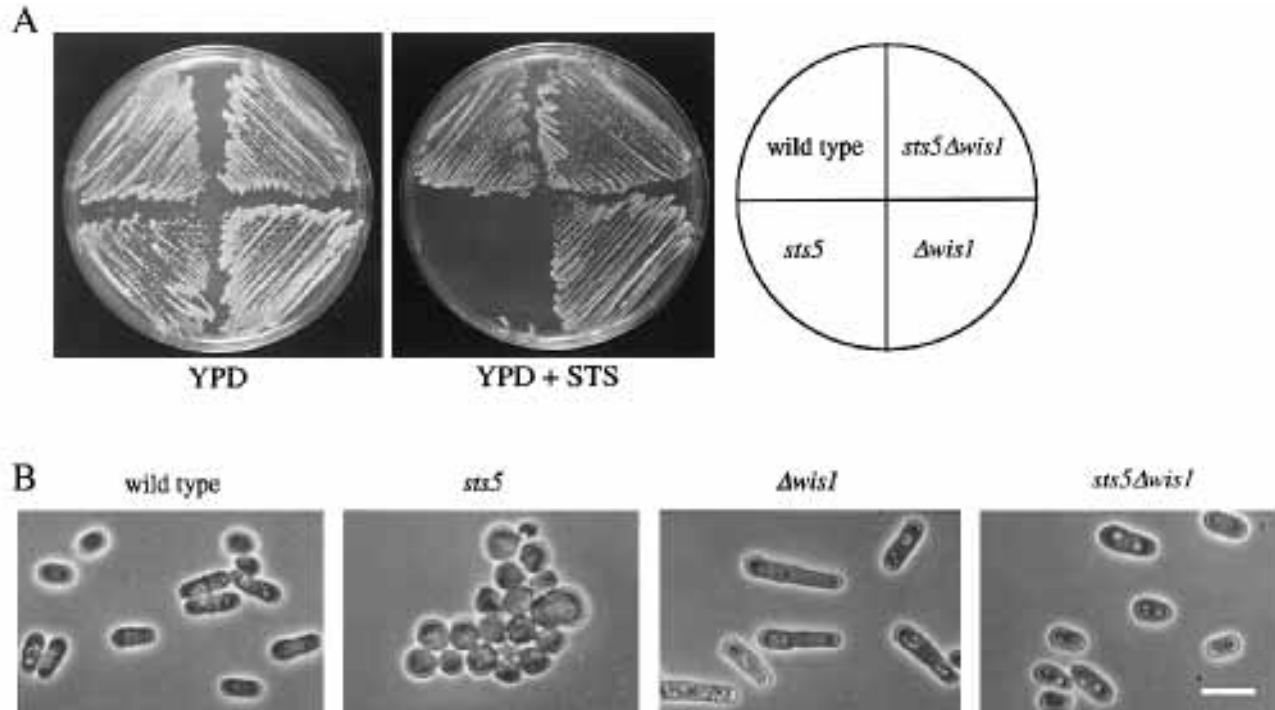
Fig. 7. Suppression of the *sts5* mutant by the *wis1* disruption. (A) Four different strains: wild type (HM123), *sts5-7* (TP189-2A), *Δwis1* (ED1101) and *sts5-7Δwis1* (TPR23-3) were streaked on a rich YPD plate (left) or YPD containing 1 µg/ml staurosporine (middle) and incubated for 3 days at 33°C. (B) Cell morphology of the same strains as in A is shown. Cells grown on a YPD plate at 35°C for 1 day were photographed. Bar, 10 µm.

noticed that the fluorescence intensity of cortical actin in the overproducers was reproducibly weaker than in either wild-type or *sts5-7* mutant cells. The level of the actin protein as judged by immunoblotting remained unchanged upon over-production of the Sts5 protein (data not shown). Thus, ectopic expression of the *sts5+* gene is toxic, leads to a disturbance of cell shape and actin localisation as well as possible alterations of the physical state of cortical actin. The level of the Sts5 protein upon induction was examined using anti-Sts5 poly-clonal antibodies (see Materials and Methods). A specific band of around 130 kDa began to accumulate 12 hours after removal of thiamine and its level peaked at 16 hours and stayed at a high level after 22 hours (Fig. 9C). The kinetics of this increase in protein level is in complete parallel with the morphological alterations seen upon induction. These observations have confirmed that overexpression of the Sts5 protein leads to growth inhibition and alterations of cell morphology.

Cellular localisation of the Sts5 protein

An attempt to determine a cellular localisation of the Sts5 protein with anti-Sts5 antibody has not been successful unless it is highly expressed, which causes morphological alteration of the cell as described before. To address this question, green fluorescent protein from jellyfish (Prasher et al., 1992; Chalfie et al., 1993) was fused in front of the Sts5 coding region which was driven under the thiamine-repressible promoter described before. This plasmid (designated pREP-GFP-*sts5+*) is toxic in the absence of thiamine and capable of suppressing the *sts5* mutant in the presence of thiamine as in the case of pREP-*sts5+* (data not shown). This result indicates that the insertion of GFP does not interfere with the function of the Sts5 protein. Using pREP-GFP-

sts5+, we were able to detect green fluorescence in cells which were grown not only under toxic derepressing condition but also under partially repressed condition (in a low concentration of thiamine; see Materials and Methods) where cell growth was not affected and a normal cell morphology was maintained (Fig. 10A). Consistent with this finding, immunoblotting with anti-Sts5 antibody showed that in the low thiamine condition, the amount of the Sts5 protein was only mildly overexpressed (Fig. 10C). Under this growth condition, the Sts5 protein was found to localise in the cytoplasm as discrete punctate dots (Fig. 10B). These dots appeared not to correlate to those of cortical actin since it did not localise either in the cell tips or septa. It should be stressed that this dotted pattern could be observed without any preparative processes such as fixation or incubation with anti-bodies. It is therefore possible that these dotted bodies represent novel cytoplasmic structures which are involved in determination of growth polarity.

DISCUSSION

In this study we have demonstrated that the *sts5+* gene is required for polarised growth when the cell elongates in defined directions. Cortical actin in the *sts5* mutant did not localise to specific sites in the cell, instead it was randomly dispersed. The round phenotypes of the *sts5* mutant could be due to an indirect effect, e.g. a defect in cell wall synthesis. However, the *sts5* mutant appears not to be defective in cell wall integrity. Ectopic overproduction of the *sts5+* gene also resulted in altered polarity, suggesting a direct role of the Sts5 protein in cell shape control. This notion is further supported by the fact that *ssp1*, an extragenic suppressor of the *sts5*
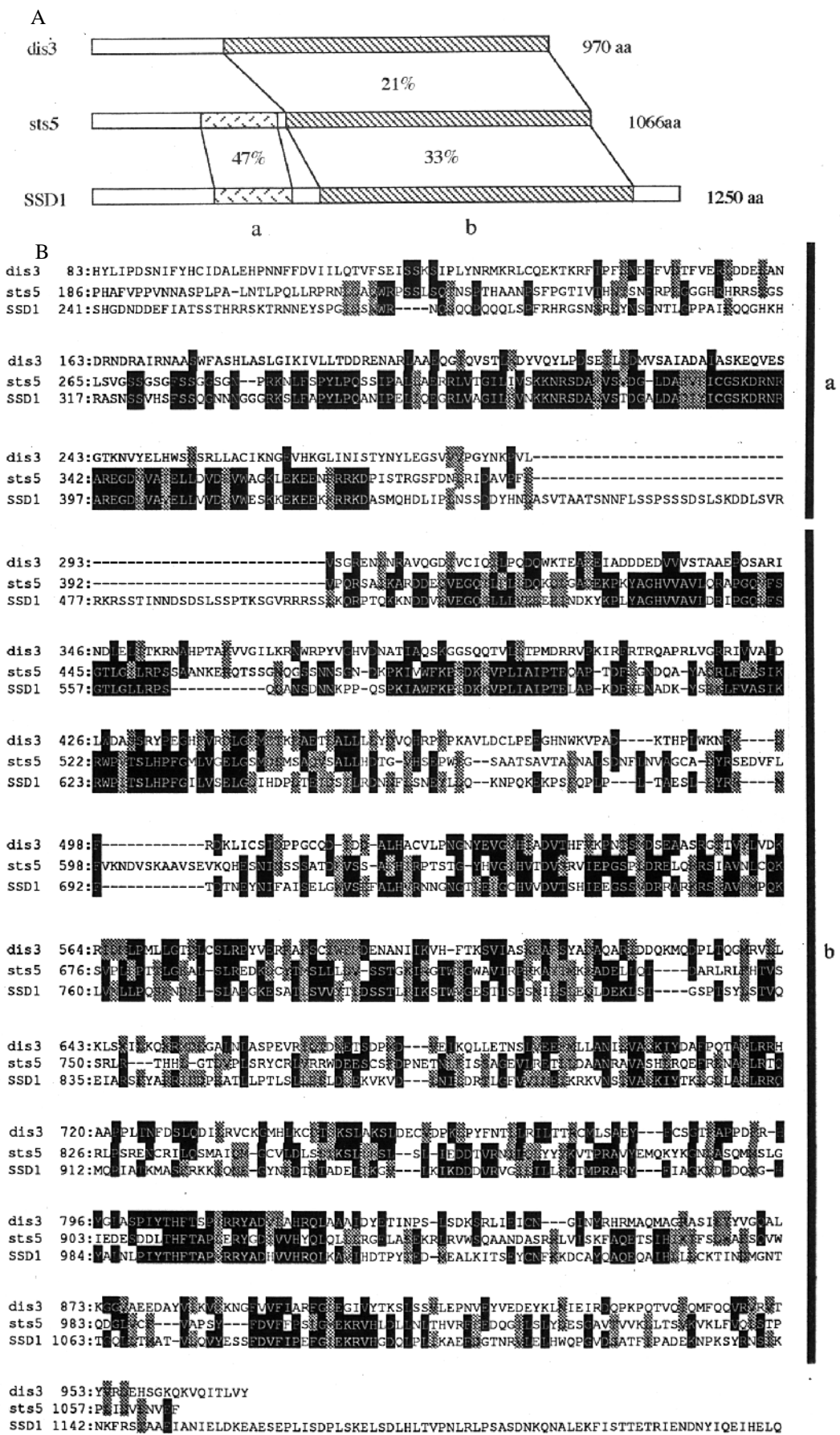
Fig. 8. An amino acid comparison of Sts5, Dis3 and Ssd1. (A) Homology between the Sts5, Dis3 and Ssd1 coding sequences is depicted. The number on the right side shows the number of amino acid residues in each protein. Region 'a' only exists in Sts5 and Ssd1, whilst region 'b' is found in all the three proteins. Percentage shows identity between each pair of proteins in the corresponding region. (B) The amino acid sequences in regions 'a' and 'b' indicated in A are compared among the three proteins. Identical amino acids are marked by filled boxes with white letters and conservative amino acids are shown by shaded boxes with black letters.
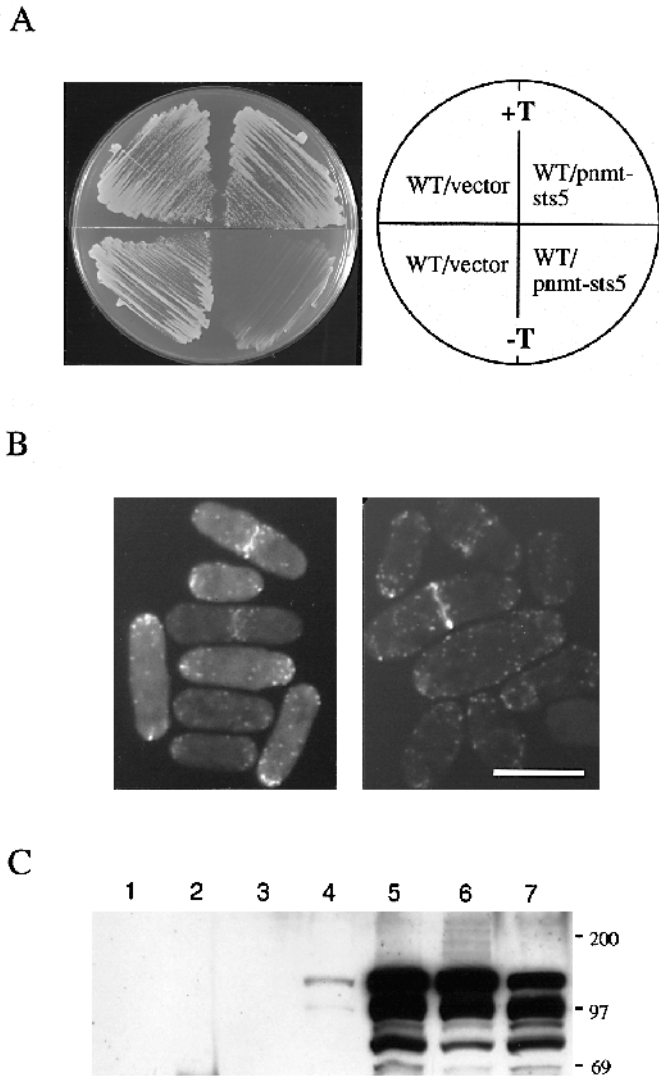
A

B

C

Fig. 9. The consequences of overexpression of the *sts5⁺* gene.
(A) Wild-type cells (HM123, Table 1) containing either the vector
pREP1 (plate on left) or pREP-*sts5⁺* (plate on right) were streaked on
a minimal plate in the presence (+T, repressed, upper two quadrants)
or the absence of thiamine (−T, derepressed, lower two quadrants),
and incubated for 3 days at 32°C. (B) Transformants containing
pREP-*sts5⁺* were grown at 26°C in minimal medium containing
thiamine and, while in an exponentially growing phase, the culture
was washed, divided into two parts and suspended in minimal
medium at a density of 10⁵ cells/ml. Thiamine was added to one of
the two cultures (left panel) and both were subsequently incubated at
35°C with shaking. After 22 hours incubation, cells were fixed, and
stained with phalloidin. Bar, 10 μm. (C) Cell extracts were prepared
from wild-type cells containing a vector (lane 1, HM123), Δsts5 cells
containing a vector (lane 2), or wild-type cells containing pREP-
sts5⁺ grown in the absence of thiamine (derepressed) for 0 (lane 3),
12 (lane 4), 16 (lane 5), 18 (lane 6) or 22 hours (lane 7).
Immunoblotting was performed using an anti-Sts5 antibody. The
positions of protein markers are shown on the right side with the
appropriate molecular mass indicated (kDa).

mutant, also showed polarity defect (Matsusaka et al., 1995).
The *sts5⁺* gene is not required for septum formation and cytoki-
nesis when cortical actin moves from the cell tips to a medial
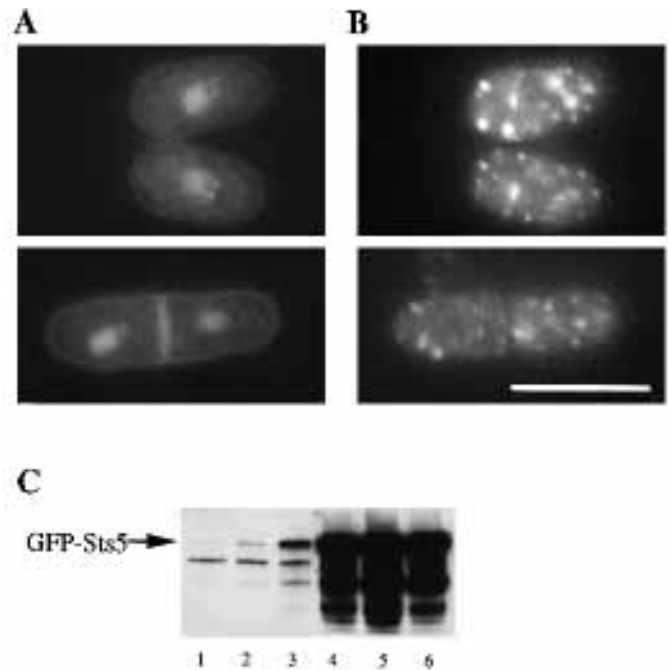
A

B

C

Fig. 10. Cellular localisation of the Sts5 protein by using GFP.
(A,B) Exponentially growing cells containing a multicopy plasmid
carrying pREP-GFP-*sts5⁺* in minimal medium containing 25 nM
thiamine were washed and observed under fluorescence microscopy:
DAPI (A) or GFP (B). The identical punctate pattern of the Sts5
protein was observed in cells grown in the culture before washing.
Note that cell morphology as well as the nuclear chromatin regions is
normal; rod-shaped and hemispherical structures respectively. Bar,
10 μm. (C) Exponentially growing cells containing pREP-GFP-*sts5⁺*
in minimal medium were washed and resuspended in minimal
medium containing various concentrations of thiamine (lane 1, 1,000
nM; lane 2, 100 nM; lane 3, 50 nM; lane 4, 25 nM; lane 5, 8 nM and
lane 6, 0 nM). After 16 hours, cells were collected, extracts prepared
and immunoblotting with anti-sts5 antibody was performed.

region. It is important to note that during mitosis and in the
post-mitotic period when septum formation and cytokinesis
take place, cell length is constant and no polarised growth
occurs (Mitchison, 1970). The *sts5⁺* gene is, therefore, specif-
ically required for polarised growth during interphase.

The sts5 protein functions in polarised growth

Either loss or overproduction of the Sts5 protein leads to
similar, although not identical, morphological defects. Over-
production of the Sts5 protein might absorb interacting
molecules that also regulate polarised growth, which would
lead to a similar morphological phenotype to that caused by a
loss of the *sts5⁺* gene function. Analysis of the localisation of
Sts5 protein by using GFP suggested that the protein exists in
punctate structures in the cytoplasm. We believe that this
punctate localisation is not a mere artifact as we could observe
these structures in living cells. In addition, a similar punctate
localisation of the Sts5 protein was also evident with anti-Sts5
antibody when the protein was overexpressed (unpublished
results). It is interesting to speculate that these structures may
represent novel vesicles or related intracellular bodies which
are responsible for transport of proteins or other materials
required for polarised growth.

In addition to *sts5+*, four other genes are required for maintaining polarised growth in the fission yeast cell cycle. These include *orb1, 6,12,* and *cwg2* (Díaz et al., 1993; Ribas et al., 1991; Verde et al., 1995) where only the *cwg2+* gene has been identified. The *cwg2+* gene encodes the β subunit of a type I geranylgeranyltransferase (Díaz et al., 1993) and *cwg2* mutant cells are supersensitive to staurosporine (D. Hirata and T. Toda, unpublished results). One intriguing possibility is that the Sts5 protein may be involved in a membrane-mediated signal transduction such as a GTPase pathway. At present, however, the exact molecular functions of the Sts5 protein in regulating polarised growth remain to be understood.

Three distinct pathways, Sts5, protein kinase C and the MAP-kinase, regulate coordinately growth polarity and cell morphology

We have shown that *sts5* mutant cells are as resistant as wild-type cells to treatment with a cell wall digesting enzyme. In contrast, *pck2/sts6* mutant cells, defective in one of the two protein kinase C-like proteins, are hypersensitive to this enzyme treatment, leading to cell lysis (Shiozaki and Russell, 1995a). Although *pck2/sts6* and *sts5* mutants are defective in cell shape and supersensitive to staurosporine, a morphological defect could arise from two different mechanisms; one in which cell wall composition is defective and the other when polarised growth itself is defective. However, these two pathways are not functionally independent as we have shown that a multicopy plasmid containing the *pck1+* gene suppresses the *sts5* mutant as well as it does *Δppe1*. It is, therefore, possible that the Sts5 protein regulates polarised growth in concert with Pck1, 2-dependent cell wall architecture. It is known that cell wall synthesis and polarised growth are closely coupled as there are differences in cell wall structure between the growing tips and non-growing parts of the cell body (Kobori et al., 1989, 1994).

Our genetic analyses suggest that the Sts5 protein and the Sty1/Spc1-dependent MAP-kinase pathway coordinately regulate cell shape in an opposing manner. We have shown that overexpression of the *pyp1+* gene was capable of suppressing the *sts5* mutation and that the *wis1* mutation which is defective in an upstream MAP-kinase kinase also suppressed the *sts5* mutation. It should be pointed out that the Sty1/Spc1 pathway is involved in cell shape control as the combination of *Δpyp1Δpyp2* in a double mutant resulted in spherical cell shape and a loss of viability (Millar et al., 1995). These results suggest either that the Sts5 protein negatively regulates the Sty1/Spc1-dependent MAP-kinase pathway or that it regulates cell shape in a parallel but antagonistic manner to the Sty1/Spc1 MAP-kinase pathway. Our results, in fission yeast, indicate that multiple pathways, involving Sts5 and the Ppe1 phosphatase, protein kinase C-like proteins and an osmosensing MAP-kinase, regulate polarised growth and maintenance of cell morphology.

Sts5, Dis3 and Ssd1 constitute an evolutionarily conserved protein family

The predicted amino acid sequence of the Sts5 protein contains regions similar to those in the fission yeast Dis3 and budding yeast Ssd1/Srk1 proteins. Interestingly a multicopy plasmid containing the *dis3+* gene suppresses the morphological phenotype of *Δppe1* which is defective in a type 2A-like

protein phosphatase (Shimanuki et al., 1993). Budding yeast *SSD1/SRK1* is involved in a complex functional network with a number of genes including *SIT4* which encodes a structural and functional homologue of Ppe1 (Sutton et al., 1991; Shimanuki et al., 1993), *IPL2/BEM2* which encodes a GTPase-activating protein (Kim et al., 1994), cAMP-dependent protein kinase (Wilson et al., 1991) and *SPA2* whose product localises at sites involved in polarised growth (Costigan et al., 1992) and G$_1$ cyclins (Cvrcková and Nasmyth, 1993). The Sts5-related gene family seems to be evolutionarily conserved as a homologous protein also exists in higher organisms such as nematodes.

As shown in this study Sts5 closely functions with the Ppe1 phosphatase, as is true for budding yeast Ssd1/Srk1 and the Sit4 phosphatase (Sutton et al., 1991). The Sts5 protein, however, seems not to form physical complexes with Ppe1 in the cell (unpublished results). At present how Sts5 and Ppe1 functionally interact remains to be determined. It should be noted that defective phenotypes of *Δppe1* are more pleiotropic than those of *sts5*. These include cold sensitive growth, sterility and suppression of the *pim1/dcd1* mutant which encodes a RCC1-like protein (Matsumoto and Beach, 1993). In contrast, *sts5+* gene function seems to be limited to the maintenance of cell morphology as the mutant is fertile and does not suppress the *pim1/dcd1* mutant (unpublished results). Further work will be required to identify molecules which physically interact with the Sts5 protein within the cell.

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