The *dmf1/mid1* gene is essential for correct positioning of the division septum in fission yeast

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Little is known about the mechanisms that establish the position of the division plane in eukaryotic cells. Wild-type fission yeast cells divide by forming a septum in the middle of the cell at the end of mitosis. *Dmf1* mutants complete mitosis and initiate septum formation, but the septa that form are positioned at random locations and angles in the cell, rather than in the middle. We have cloned the *dmf1* gene as a suppressor of the *cdc7-24* mutant. The *dmf1* mutant is allelic with *mid1*. The gene encodes a novel protein containing a putative nuclear localization signal, and a carboxy-terminal PH domain. In wild-type cells, Dmf1p is nuclear during interphase, and relocates to form a medial ring at the cell cortex coincident with the onset of mitosis. This relocation occurs before formation of the actin ring and is associated with increased phosphorylation of Dmf1p. The Dmf1p ring can be formed in the absence of an actin ring, but depends on some of the genes required for actin ring formation. When the septum is completed and the cells separate, Dmf1p staining is once again nuclear. These data implicate Dmf1p as an important element in assuring correct placement of the division septum in *Schizosaccharomyces pombe* cells.

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Cells must assure correct spatial and temporal coordination of mitosis with cytokinesis so that each daughter cell will receive a nucleus and an appropriate complement of cytoplasmic material following cell division. A number of studies indicate that the position of the cleavage plane is determined during mitosis, but in most systems very little is known about the nature of the signal or the proteins involved (for review, see Strome 1993). Cytokinesis requires assembly of the division apparatus at an appropriate site on the cell cortex. In higher eukaryotes, a contractile ring forms around the equator of the parent cell at the end of mitosis. The sliding of actin and myosin filaments generates a force that pulls the plasma membrane inward to create the cleavage furrow, which narrows to form the midbody as division is completed (for review, see Satterwhite and Pollard 1992; Fishkind and Wang 1995). A structure called the telophase disc, which forms prior to the appearance of the contractile ring, has been proposed to organize contractile ring formation (Margolis and Andreassen 1993).

The mitotic apparatus plays a critical role in determining where cell cleavage will occur. The site of cell division is defined by the plane of the metaphase plate, and is at right angles to the long axis of the mitotic spindle.

It is generally believed that the asters of the mitotic spindle specify where the cleavage furrow will form. Micro-manipulation experiments in marine invertebrate embryo cells have shown that if the spindle is moved during early anaphase, then the incipient cleavage furrow disappears and is reformed at the new location of the spindle. In late anaphase, displacement of the spindle, or even its complete removal, has no effect on the position of cleavage (for review, see Rappaport 1986; Satterwhite and Pollard 1992; Strome 1993). The nature of the signal is unknown, but calcium has been implicated as a second messenger (Miller et al. 1993).

Proteins that are implicated in contractile ring function, such as actin and myosin II, undergo dramatic rearrangements during mitosis and cytokinesis. At prophase in animal cells, stress fibers break down and the constituent actin and myosin II disperse through the cytoplasm until early anaphase, when they become concentrated at the cell cortex where the cleavage furrow will form. During late anaphase, a fraction of the inner centromere proteins (INCENP) localize to the cortex, which is thought to be an early event in cleavage furrow formation (Earnshaw and Cooke 1991). A number of other proteins are known to localize to the cleavage furrow, among them radixin, which is a barbed end actin filament capping protein, whose function is thought to be to provide a link between the cell cortex and the cytoskeletal elements responsible for cleavage (Sato et al. 1991; for review, see Satterwhite and Pollard 1992).

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Studies in the budding yeast *Saccharomyces cerevisiae* have identified a number of genes that are implicated in selection of the bud site, which becomes the division site. Loss of any of a number of *BUD* gene functions results either in random bud emergence or a failure to maintain a characteristic budding pattern (Chant and Herskowitz 1991; for review, see Kron and Gow 1995; Chant 1996; Zahner et al. 1996). In α and α yeast cells, which bud axially, Bud3p seems to mark the budding site. Immunofluorescence studies suggest that it and the neck filament proteins are linked in a cycle whereby each defines the position of the other's assembly. Thus, Bud3p associates with the neck filament ring in one cell cycle to mark the position of the bud site in the next cycle (Chant et al. 1995). In maize, the *tangled-1* mutation affects the orientation of leaf cell division, resulting in the production of new cell wall that is aberrantly placed or oriented (Smith et al. 1996).

The fission yeast *Schizosaccharomyces pombe* provides a simple eukaryotic system in which to study the temporal and spatial coordination of cytokinesis and mitosis (for review, see Simanis 1995). Fission yeast cells grow mainly by elongation at their tips and divide by binary fission after forming a centrally placed septum, as in higher eukaryotes, initiation of cytokinesis is dependent on the onset of mitosis (Minet et al. 1979). In *S. pombe*, F-actin is seen as patches or dots at sites of cell growth or division. During interphase, it is found at the growing ends of the cell, and, after the onset of mitosis, it relocates to form an equatorial ring whose position anticipates the site of septum formation (Marks and Hyams 1985; for review, see Robinow and Hyams 1989). At the end of mitosis, when the daughter nuclei are well separated and the spindle begins to break down, the septum grows inward from the cell cortex and the appearance of the actin staining changes from a ring to clusters of dots. Secondary septa are formed on either side of the primary septum, which is subsequently dissolved to effect cell separation. F-actin then relocates to the old (pre-existing) end of the cell, from which growth resumes.

Recent studies have identified some of the proteins that control the onset of septum formation and cytokinesis in fission yeast. The products of the *cdc3*, *cdc4*, *cdc8*, *cdc12*, *cdc15*, and *rng2* genes are required for actin rearrangement and/or to stabilize the actin ring (Nurse et al. 1976; Balasubramanian et al. 1992, 1994; Fankhauser et al. 1995; McCollum et al. 1995; Chang et al. 1996), whereas the *mid1/dmf1* gene product is implicated in positioning the ring (Chang et al. 1996; this paper). Immunofluorescence studies have shown that the products of the *cdc3*, *cdc4*, *cdc8*, and *cdc15* genes are associated with the medial ring (Balasubramanian et al. 1992, 1994; Fankhauser et al. 1995; McCollum et al. 1995). Once the actin ring is formed, the *cdc7* kinase and the activities of *cdc11* and *cdc14* are required for septation (Nurse et al. 1976; Fankhauser and Simanis 1993, 1994). The *plo1* kinase appears to be required for both actin ring formation and septation (Ohkura et al. 1995). The nature of the signal that defines the position of the equatorial actin ring at the beginning of mitosis is un-

known, although it is thought to originate from the nucleus. The observation that the actin ring is already present and properly localized in a β -tubulin mutant, which is arrested in pro-metaphase and lacks a mitotic spindle, argues that a functional spindle is not required to specify its position (Chang et al. 1996; for review, see Chang and Nurse 1996). In this paper, we show that *S. pombe* cells lacking *dmf1* function complete mitosis, but misplace the septum that forms subsequently. The *dmf1* gene encodes a novel protein that is nuclear during interphase and, upon entry into mitosis, forms a ring at the position where the actin ring, and later the septum, will form. We propose that Dmf1p is an essential component of the mechanism that establishes the central position of the division septum.

Results

Isolation and sequencing of *dmf1*

When we were cloning the *cdc7* gene by complementation of the *cdc7-24* mutant (Fankhauser and Simanis 1994), we obtained an additional cDNA clone that did not contain the *cdc7* gene, but a multicopy suppressor (see Materials and Methods). Because a multicopy suppressor could encode a potential substrate or regulator of the *cdc7*-encoded protein kinase, we studied it further. Our analysis (see below) indicated that loss-of-function mutations in this new gene cause cells to misplace the division septum frequently, although mitosis occurs normally. We therefore named the gene *dmf1* (division mal foutue) to reflect the unsuccessful attempt at cell division.

Isolation of additional *dmf1* cDNA clones indicated that the original *dmf1* cDNA clone, called *pcdc7sup1*, was truncated at the 5' end; the first in-frame methionine was amino acid 135 in the complete sequence. Moreover, at amino acid 390 there was a 40-bp deletion, which changed the reading frame, adding seven novel amino acids to the putative protein before a stop codon was encountered. There are no sequences conforming to the *S. pombe* splice junction consensus in this region, and because no other cDNA contained such a deletion, we believe it to be a cloning artifact.

Increased expression of either full-length *dmf1* or the truncated cDNA clone does not rescue a *cdc7::ura4⁺* null mutation, indicating that *dmf1* does not bypass the requirement for Cdc7p (not shown). Hybridization against an ordered cosmid library mapped the gene to P1 clone 33H2p and cosmid 4B3c, close to the centromere of chromosome III (probe *cdc7sup1*; Hoheisel et al. 1993). A restriction map of the *dmf1* locus is shown in Figure 1A. Sequencing of genomic and cDNA clones indicated that the *dmf1* open reading frame contains no introns. The DNA sequence has been submitted to the EMBL data base under accession no. Y07599. The gene encodes a 920-amino-acid protein, Dmf1p (Fig. 1B), whose main features are summarized in Figure 1C. Overall, the protein has no close relatives in the data bases; however, a number of previously characterized sequence motifs

Figure 1. Restriction map of the *dmf1* locus, sequence of Dmf1p, and structure of the deletion mutations. (A) Restriction map of the *dmf1* locus. The box represents the coding region. The parts of the gene replaced by *ura4*⁺ to construct the partial (ΔP) and the full deletion (ΔF) are indicated. The phenotypes of the full and partial deletions are similar. The solid region represents the protein fragment of *dmf1* that could be encoded by the truncated cDNA clone that complemented *cdc7-24*, assuming that translation starts from the first available ATG. (B) Predicted sequence of Dmf1p. The 920 amino acid protein has a calculated molecular weight of 102367. Sequences matching the phosphorylation consensus of p34^{cdc2} are underlined. (C) Schematic representation of the main features of Dmf1p. The protein contains two high-scoring PEST-sequences, a basic region containing potential nuclear localization signals (NLS), two sequences conforming to the consensus phosphorylation sequence for p34^{cdc2} kinase and a carboxy-terminal PH domain.

were identified. Amino acids 681–710 of Dmf1p comprise a basic region that contains several putative nuclear localization signals (NLS; Dingwall and Laskey 1986). At the carboxyl terminus (amino acids 805–896) there is a pleckstrin homology (PH) domain (Gibson et al. 1994). The presence of a PH domain, which is often found in signaling or cytoskeletal proteins, may provide an interaction site with other proteins or membrane lipids (for review, see Lemmon et al. 1996). There are also two high-scoring PEST regions, amino acids 253–264 and 276–315 (Rechsteiner 1990), and two p34^{cdc2} kinase consensus phosphorylation sequences (amino acids 28–31 and 603–606). Northern blotting showed that *dmf1* mRNA is present throughout the cell cycle and exhibits only a slight (<3-fold) fluctuation in steady-state level (not shown).

Dmf1 mutants misplace the division septum

To investigate the role of *dmf1* in cell division, we examined the effect of inactivating it during exponential growth. Cells of the heat-sensitive *dmf1-6* mutant (a gift from the Gould laboratory, Nashville TN; see Materials and Methods), were synchronized by elutriation at 25°C, and early G₂ cells were shifted to 36°C. The nuclei remained centrally positioned during interphase, and nuclear division occurred normally in most cells, suggesting that Dmf1p is not required for positioning of the nucleus during interphase or for mitosis (Fig. 2A). However, the positions and angles of the division septa were abnormal: 180 min after shift to 36°C, <3% of the septa were placed normally (Fig. 2C; a normal septum is defined as being in the central fifth of the cell and at an angle >80° to the long axis of the cell; a septum which failed to meet either of these criteria was considered ab-

Figure 2. Mutation of *dmf1* results in aberrant positioning of the division septum. (A) Localization of F-actin and division septa in the temperature-sensitive mutant *dmf1-6*. Cells synchronized in G₂ by elutriation were incubated at 25°C or 36°C, fixed and stained with DAPI, Calcofluor and rhodamine-conjugated phalloidin. (B) Transient inactivation of Dmf1p in G₂ does not result in misplacement of the septum. *Dmf1-6* cells were synchronized in G₂ by elutriation at 25°C and incubated at 36°C. Half of the culture was shifted down to 25°C after 85 min at 36°C, just prior to the onset of mitosis. At the indicated times, cells were fixed and stained with DAPI and Calcofluor. (C) The cultures described in B were scored for positioning of the division septa. See text for definition of a normal septum.

errant). Septa were often eccentrically placed, and were usually formed obliquely, or even longitudinally (Fig. 2A). Though some of these cells completed cell division, ~80% arrested with two nuclei and a septum, indicating that most of the septa that were made were not cleaved immediately (Fig. 2B). In contrast to *cdc16-116*, which arrests with two nuclei but undergoes multiple rounds of septation without cell cleavage (Minet et al. 1979; Fankhauser et al. 1993), *dmf1-6* cells only formed a single septum at 36°C.

Staining of either *dmf1-6* (Fig. 2A) or *dmf1::ura4⁺* (not shown; see below) cells with rhodamine-conjugated phalloidin showed that F-actin was associated with the aberrantly placed septa: No normally placed actin rings were seen in cells with misplaced septa. Staining with anti-Cdc15p antiserum showed similar results: Cdc15p was always associated with the misplaced septa (not shown; S. Utzig, L. Cerutti, M. Sohrmann, and V. Simanis, in prep.).

This phenotype is similar to that produced by the *mid1-366* mutant (Chang et al. 1996). This is a heat-sensitive mutant that was isolated in a screen for mutants defective in actin ring formation or placement (Chang et al. 1996). No recombinants were observed in dissection of 60 tetrads in crosses between *dmf1::ura4⁺* strains (see below) and *mid1-366*, strongly suggesting that *mid1* is allelic with *dmf1*. The allelism of *mid1* and *dmf1* has also been confirmed by Chang et al., (1996).

Down-shift of synchronized *dmf1-6* cells from 36°C to 25°C in late G₂, just before entry into mitosis, showed that the majority of septa that formed were placed normally (Fig. 2C) and cleaved subsequently (Fig. 2B). These data suggest that Dmf1p has no essential role in defining the position of the septum before the onset of mitosis. Similar results were obtained following release of a *cdc25-22 dmf1-6* double mutant from G₂ arrest (data not shown).

To determine the *dmf1* null phenotype, deletions of *dmf1* were constructed in a diploid (see Fig. 1A). Tetrad dissection of either deletion produced four viable progeny, with uracil prototrophy segregating 2:2, demonstrating that *dmf1* is not essential for cell proliferation. The absence of the wild-type allele in the *ura⁺* cells was confirmed by Southern blotting (Fig. 3A). Though the cells carrying the *dmf1::ura4⁺* allele are viable at 25°C, the colonies are smaller than their *dmf1⁺* counterparts and stain dark red on media containing phloxin B, indicative of increased numbers of dead cells. Microscopic examination showed that *dmf1::ura4⁺* cells, like *dmf1-6*, misplace the division septum at high frequency (Fig. 3B). We presume that colonies form at 25°C because a fraction of the septa are formed sufficiently close to the correct position that they can be cleaved, giving rise to viable cells after division. At 36°C, cells accumulate multiple aberrantly placed septa, become branched (Fig. 3B), and are unable to form a colony.

Dmf1p is nuclear during interphase and forms a ring prior to septation

Antisera raised against Dmf1p identified a number of

Figure 3. Cells deleted for *dmf1* are viable, but misplace the division septum. (A) Southern blot analysis of the diploid used to delete *dmf1* (lane 6), a diploid heterozygous for the partial deletion (lane 5), and one of its complete tetrads [lanes 2 and 4, DNA from cells carrying the *dmf1⁺* allele (*ura⁻* progeny); lanes 1 and 3, DNA from cells carrying the *dmf1::ura4⁺* allele (*ura⁺* progeny)]. Genomic DNA was digested with *Bgl*III and *Stu*I. The probe was the *Bgl*III–*Kpn*I fragment. This genomic digest produces a single fragment of ~10 kb in wild-type DNA and two bands of ~6.5 kb and ~2.3 kb in the *dmf1::ura4⁺* DNA, caused by the presence of a *Stu*I site in the *ura4⁺* gene. The genomic *Stu*I site lies ~6 kb to the right of the *Kpn*I site, and is not shown on the map in Fig. 1A. Note the absence of the ~10 kb band in lanes 1 and 3. (B) *dmf1::ura4⁺* cells were grown at 25°C in rich medium, and half of the culture was shifted to 36°C for 8 hr. Cells were then fixed and stained with DAPI and Calcofluor.

polypeptides migrating between 120 and 130 kD on a Western blot (bracketed in Fig. 4A). These increased in abundance in cells overexpressing the *dmf1* gene and were absent from *dmf1::ura4⁺* cell extracts, confirming that they are a result of Dmf1p. Phosphatase treatment of immunoprecipitated Dmf1p showed that changes in Dmf1p phosphorylation state account, at least in part, for the presence of several bands (Fig. 4B).

Indirect immunofluorescence showed that in interphase cells, Dmf1p is predominantly nuclear (Fig. 5A, cells 1–3; Fig. 6E–G). Consistent with this, Dmf1p is also associated with the nucleus in a G₂-arrested *cdc25-22* mutant at the nonpermissive temperature (not shown). The staining overlies both the chromatin and nucleolar domains. In mitotic cells, Dmf1p is seen as a ring at the cell equator (Fig. 5A, cells 4–8; Fig. 6A), where it colocalizes with the actin ring (Fig. 6B–D). At the end of mitosis (Fig. 5A, cell 9), the Dmf1p ring breaks down, and in cells synthesizing a septum, no discrete Dmf1p staining is observed (Fig. 5A, cell 10), although the pro-

Figure 4. Characterization of the antibody against *Dmf1p*. (A) Total protein extracts were prepared, Western blotted, and probed with affinity-purified anti-*Dmf1p* antiserum. (Lane 2) Extract from wild-type cells; (lane 1) extract from cells overexpressing *Dmf1p* from pREP41 for 18 hr at 25°C; (lane 3) extract from cells deleted for the *dmf1* gene. (B) Phosphatase treatment converts the slower-migrating mitotic form of *Dmf1p* in the faster-migrating interphase form. *cdc25-22* cells were synchronized by arrest-release. Proteins were prepared from cells 30 min. after release, immunoprecipitated and CIP-treated as described in Materials and Methods. (Lane 1) Mock-treated extract; (lane 2) CIP-treated extract. Mitotic extract (30 min after release from *cdc25-22* block; (lane 4) and interphase extract (100 min after release from *cdc25-22* block; (lane 3) show the migration of hyperphosphorylated and hypophosphorylated *Dmf1p*, respectively. Western blots were prepared as described in A. Note that the lower band in lanes 3 and 4 is the cross-reacting band seen in the Western blots of total protein shown in A. It does not appear in the immunoprecipitates.

tein is still present at this time (see Fig. 10B below). In cells that have completed the septum and are undergoing cell separation (Fig. 5A, cell 11), *Dmf1p* is once again nuclear. The specificity of the staining was confirmed by competition experiments (Fig. 5B) and staining of *dmf1::ura4⁺* cells (Fig. 5C).

Formation of the Dmf1p ring precedes the appearance of the medial actin ring

Because the *Dmf1p* and actin rings co-localize, we investigated whether the *Dmf1p* ring appears before, coincident with, or after, the actin ring. A synchronous culture of wild-type cells was generated by elutriation and grown at 25°C and, as cells entered mitosis, samples were taken every 4 min. Cells were fixed and stained with antibodies recognizing *Dmf1p* or *Cdc15p*, or with rhodamine-conjugated phalloidin to visualize F-actin. As shown in Figure 7A, the peak of *Dmf1p* rings appeared 8 min before the peak of either actin or *Cdc15p* rings. The appearance of *Cdc15p* rings was not separable from the appearance of actin rings. Figure 7B shows cells stained for *Dmf1p* and actin 84 min after reinoculation. Cells which have entered mitosis, but have not begun to separate the nuclei, show a strong *Dmf1p* ring, while actin is located either at the tips or is dispersed. In no case was a medial actin ring seen in the absence of a *Dmf1p* ring. Cells in which anaphase has been initiated show an actin ring in addition to the *Dmf1p* ring. These results were confirmed in cells synchronized by arrest-release of a *cdc25-22* mutant from 36°C to 25°C; *Dmf1p* rings appeared 10 min before actin and *Cdc15p* rings (data not shown).

Figure 5. Immunolocalization of *Dmf1p*. (A) Localization of *Dmf1p* during the cell cycle. Wild-type cells were synchronized by elutriation, grown at 25°C, fixed and stained as described in Materials and Methods. Top panels show DAPI-stained cells and bottom panels cells stained for *Dmf1p*. The cell-cycle stage is indicated above. (B,C) Specificity of the immunostaining for *Dmf1p*. (B) Anti-*Dmf1p* antiserum was preadsorbed against GST protein (upper panel) or the GST-*Dmf1p* fusion protein used to raise the antibody (lower panels). Note that only the GST-*Dmf1p* fusion protein can compete the signal. (C) Cells deleted for the majority of the *dmf1* gene (ΔF ; Fig. 1A) were stained as in A. Note that the region of *Dmf1p* against which the antibody was raised is missing in these cells. No nuclear signal can be seen in interphase cells (upper panels) and no ring in mitotic cells (bottom panels), confirming the specificity of the staining.

These data show that formation of the *Dmf1p* ring precedes the appearance of the medial actin ring, and are consistent with a role for *Dmf1p* in defining the position of the actin ring.

Formation of a Dmf1p ring at mitosis does not require a spindle, or actin ring formation, but depends on the activity of cdc3, cdc4, cdc8 and cdc12

Because we observed a *Dmf1p* ring at the location of the actin ring early in mitosis, we investigated the effects of mutants defective in actin ring formation and septation on the appearance of a *Dmf1p* ring. Centrifugal elutriation was used to generate a population of mutant cells in early G₂ at the permissive temperature, which was then shifted to the restrictive temperature so that they entered mitosis and cytokinesis synchronously. This protocol was adopted to ensure that none of the cells would

Figure 6. Dmflp colocalizes with the actin ring in mitosis and is nuclear during interphase. (A) Cells were fixed and stained with anti-Dmflp antiserum and Mab414, then viewed by confocal microscopy. The location of the Mab414 nuclear pore antigen is shown in green, Dmflp is in red. Note that in this anaphase cell, Dmflp forms a ring. Bar, 5 μ m. (B–D). Cells were fixed and stained with antiserum to Dmflp and rhodamine-conjugated phalloidin to reveal the position of F-actin. (C) Location of F-actin; (D) location of Dmflp; (B) overlay of the two images, with green for Dmflp, and red for F-actin. Note that the combined image is yellow, indicating that the two staining patterns coincide. Bar, 5 μ m. (E–G). Cells were prepared as described in A. Confocal microscopy was used to examine

300-nm sections through interphase cells, after staining with Mab414 and antiserum to Dmflp. One of these sections is shown. (F) Mab414 staining (detected with DTAF-conjugated secondary antibody: green); (G) Dmflp staining (detected with CY3-conjugated secondary antibody: red); and (E) the merged image, in color. Note that the Dmflp staining falls within the boundary defined by the nuclear pore staining, indicating that Dmflp is a nuclear protein in interphase cells. Similar results were seen in all other sections, eliminating the possibility that Dmflp is found as a band associated with the cell cortex in the region of the nucleus. Bar, 5 μ m.

have a Dmflp ring at the time of shift (though similar results were obtained by shifting asynchronous, exponentially growing cells). We found that mutants in *cdc3*, *cdc4*, and *cdc8*, all of which are required for actin ring formation, did not form a Dmflp ring in mitosis (Fig. 8). In a *cdc12* mutant, which is also defective in actin ring formation, Dmflp formed a diffuse medial band, rather than a discrete ring (Fig. 8). No nuclear staining was observed in mitotic nuclei in any of these mutants. In binucleate interphase cells, Dmflp was once again nuclear, despite the failure to form a septum. In contrast, in *cdc15-140*, which forms neither an actin ring nor a Cdc15p ring at the restrictive temperature (Fankhauser et al. 1995; S. Utzig, L. Cerutti, M. Sohrmann, and V. Simanis, in prep.), a Dmflp ring was formed at the cell equator (Fig. 9), consistent with the observation that the Dmflp ring forms before both the actin and Cdc15p rings (Fig. 7). These data indicate that Dmflp ring formation depends on a number of the proteins implicated in actin ring formation or stability, but does not require Cdc15p or an actin ring.

In mutants defective at a later stage of septum formation, such as *cdc7*, *cdc11*, and *cdc14*, which form actin rings but do not septate (Fankhauser et al. 1995), a Dmflp ring was formed at mitosis (Fig. 9). As in *cdc3*, *cdc4*, *cdc8*, and *cdc12*, nuclear staining was observed in binucleate interphase cells. In a *cdc16* mutant, which forms multiple septa without undergoing cleavage, a Dmflp ring formed at mitosis, and then dissipated at the time of septation, as seen in wild-type cells. Dmflp staining was nuclear in both binucleate (type I cell; Minet et al. 1979) and mononucleate (Type II cell; Minet et al. 1979) septated cells, indicating that Dmflp is nuclear when the septum is completed.

In higher eukaryotes, the mitotic spindle is thought to play a crucial role in determining the position of the

division site. The *nda3-KM311* (β -tubulin) mutation blocks spindle formation at the restrictive temperature, causing a pro-metaphase arrest (Hiraoka et al. 1984). We saw that Dmflp also forms a correctly positioned ring in *nda3-KM311* arrested cells (Fig. 8), showing that intact microtubules are not required for its assembly or positioning. This is also consistent with the finding that an actin ring is present in *nda3-KM311* mutants at the restrictive temperature (Chang et al. 1996).

Genetic interactions of *dmf1* with other genes required for septation

Strains carrying either the *dmf1-6* or *dmf1::ura4⁺* alleles were crossed to strains carrying either *cdc3-6*, *cdc4-8*, *cdc7-24*, *cdc8-110*, *cdc11-136*, *cdc12-112*, *cdc14-118*, *cdc15-140*, or *cdc16-116*. The null allele is synthetically lethal with *cdc4-8* and *cdc3-6*, and the restrictive temperature of double mutants with *cdc7-24* and *cdc15-140* was reduced to 25°C. Double mutants with *dmf1-6* showed less severe effects, but *dmf1-6 cdc4-8*, *dmf1-6 cdc7-24*, and *dmf1-6 cdc15-140* all have a reduced non-permissive temperature compared with the single mutants.

Dmflp undergoes changes in phosphorylation at the time of actin ring formation and septation

To examine whether changes in Dmflp occur upon entry into mitosis, we performed Western blots on protein extracts from cells passing synchronously through mitosis and cytokinesis after release from a *cdc25-22* block. When Dmflp staining changes from nuclear to a medial ring, Dmflp becomes hyperphosphorylated (Fig. 10A,B; 15–45 min). This occurs prior to actin ring formation. Treatment of the slower-migrating Dmflp with phos-

Figure 7. The medial Dmf1p ring is formed before the medial actin ring. (A) Wild-type cells were synchronized by elutriation in yeast extract medium at 25°C. Samples were taken at 4-min intervals, fixed, and stained with rabbit antibodies to either Dmf1p or Cdc15p, or rhodamine-conjugated phalloidin to detect actin. The rabbit sera were detected with CY3-conjugated secondary antibodies. The percentage of cells in each time point with Dmf1p, Cdc15p, or actin rings was determined. Note that Dmf1p rings appear before Cdc15p and actin rings. Actin rings decline before Cdc15p rings because actin is seen as dots when the septum starts to form, while Cdc15p remains as a ring, which contracts as the septum is formed (Fankhauser et al. 1995). (B) Cells from this experiment, 84 min after reinoculation, were stained with DAPI, anti-Dmf1p antiserum, and the anti-actin monoclonal antibody. Rhodamine-conjugated and FITC-conjugated secondary antibodies, respectively, were used to detect the rabbit and mouse antibodies. Note that in early mitotic cells (the left one in each set of panels), Dmf1p forms a ring, while actin is dispersed or at the tips of the cells. In anaphase cells (the right one in each set of panels), both Dmf1p and actin form a ring.

phatase reverses most of the change in apparent molecular weight (Fig. 4B), suggesting that it is caused by changes in its phosphorylation state. The hyperphosphorylated forms are seen until the initiation of septation, when Dmf1p returns to a hypophosphorylated form (Fig. 10A,B; 60 min and later). The appearance of hyperphosphorylated Dmf1p precedes that of hypophosphorylated Cdc15p (Fig. 10B), which is associated with actin ring formation (Fankhauser et al. 1995). Similar results were obtained in synchronous cultures generated by centrifu-

gal elutriation (not shown). Shift of *dmf1-6* to the restrictive temperature for 30 min or 4 hr showed that Dmf1p is not degraded, but is present in the hypophosphorylated form: No discrete Dmf1p staining could be observed in these cells by immunofluorescence (not shown).

Although Dmf1p is present through mitosis and into the next interphase (Fig. 10B), the abrupt transition of Dmf1p from hyperphosphorylated to hypophosphorylated forms and the presence of PEST regions in the protein prompted us to investigate whether the appearance of hypophosphorylated Dmf1p results from rapid degradation and synthesis de novo. We therefore performed an arrest-release experiment and added cycloheximide after 30 min to block further protein synthesis. Cells completed mitosis and then arrested with a septum, indicating that protein synthesis is required to dissolve the septum and complete cytokinesis, in agreement with previous observations (Fantes 1982). Dmf1p underwent phosphorylation changes similar to those seen in the absence of cycloheximide and then persisted in the hypophosphorylated state in the arrested cells (Fig. 10C,D). The steady-state protein level did not change significantly, indicating that the alterations in gel mobility result predominantly from changes in Dmf1p phosphorylation, rather than coupled degradation and resynthesis.

Discussion

Correct temporal and spatial coordination of cell-cycle events is necessary for successful cell proliferation. Mitosis must not begin until S phase is completed and cytokinesis must not occur until the duplicated chromosomes have been segregated into two identical sets. Appropriate positioning and orientation of the plane of cell

Figure 8. The effects of the late septation mutants and loss of microtubules upon Dmf1p ring formation. Cells of the indicated mutants were synchronized by elutriation at 25°C in rich medium and shifted to 36°C. At the time of mitosis (~80 min after shift to 36°C), cells were fixed and stained for Dmf1p.

Figure 9. The effects of mutations in *cdc7*, *cdc11*, *cdc14*, *cdc15*, and *cdc16* on Dmflp ring formation. Cells of the indicated mutants were synchronized by elutriation at 25°C in rich medium and shifted to 36°C. At the time of mitosis (~80 min after shift-up), cells were fixed and stained for Dmflp.

cleavage is also of critical importance. Premature or eccentric cleavage of the cell can cut the genetic information, or produce anucleate compartments (e.g., see Hirano et al. 1986). Choice of the cell division site is also important during development, where it determines overall organization of the embryonic cells and tissues (e.g., Etemad-Moghadam et al. 1995; Rhyu and Knoblich 1995).

In fission yeast and somatic animal cells, the division plane is established equidistant between the two poles of the spindle. Very little is known about the molecules that specify where the cell will divide. In this paper, we have shown that in fission yeast, loss of Dmflp function apparently randomizes the position and angle of septum formation by the cell. The septa that form are associated with F-actin and Cdc15p, as they would be in a normal cytokinesis, suggesting that structural elements of the septum-forming machinery are assembled correctly, but misplaced.

Shifting the *dmf1-6* mutant (or *mid1-366*; Chang et al. 1996) to 36°C results in a high percentage of arrested cells with a septum; this implies either that the septum formed is defective and cannot be cleaved, or that a checkpoint exists that reacts to the misplaced septum and prevents, or delays, cell cleavage. There is some evidence for checkpoints linking mitosis and cytokinesis in budding yeast, in which anaphase does not occur if a bud has not been formed (Lew and Reed 1995) and cytokinesis is delayed if nuclear migration into the bud is delayed (Yeh et al. 1995). It will be interesting to investigate the nature of the defect which prevents cleavage in these cells.

In the course of a normal cell cycle, the actin ring is formed at the cell cortex at the position of the early mitotic nucleus (Marks and Hyams 1985). Our studies

show that Dmflp is nuclear throughout interphase and also in cells arrested in late G₂. As cells enter mitosis, Dmflp forms a cortical ring at the position of the nucleus. This precedes formation of both the actin and Cdc15p rings. Alterations in Dmflp phosphorylation state accompany the changes in its subcellular localization, suggesting this as a potential means of regulating its activity. Entry into mitosis and relocalization of Dmflp to form a ring is correlated with its hyperphosphorylation. This precedes both the appearance of hypophosphorylated Cdc15p, which is implicated in actin rearrangement, and formation of the actin ring. At present, we do not know whether the phosphorylation change is required for Dmflp ring formation, or a consequence of it. In this context, it is noteworthy that phosphorylation has been shown to regulate the NLS activity in some proteins (e.g., Moll et al. 1991; Rihs et al. 1991; Jans et al. 1995). It is possible that phosphorylation of Dmflp may negate the NLS in the protein. This will be tested in future experiments.

When septation is initiated, Dmflp is dephosphorylated, the Dmflp ring dissipates and no discrete staining is seen until the septum is completed and the cells enter the next interphase, when Dmflp is again localized in the nucleus. In this respect, its staining pattern differs from that of F-actin (Marks and Hyams 1985) and contractile ring components such as Cdc3p, Cdc4p, Cdc8p and Cdc15p, which associate with the septum (Balasubramanian et al. 1992, 1994; Fankhauser et al. 1995; McCollum et al. 1995). It will be of interest to determine whether dephosphorylation of Dmflp is required for disassembly of the Dmflp ring.

The identity of the kinases and phosphatases which act on Dmflp is unknown at present. Treatment of cells with okadaic acid or cyclosporin A interferes with sep-

Figure 10. Formation of the actin ring and cytokinesis are correlated with changes in the phosphorylation state of Dmf1p. (A) *Cdc25-22* cells were incubated for 4 hr at 36°C, then rapidly cooled to 25°C. At the indicated times after return to 25°C, cells were fixed and stained with DAPI, rhodamine-conjugated phalloidin, and Calcofluor. (Shaded bar) Actin ring; (open bar) anaphase; (solid bar) septa. (B) Proteins extracted from the cells described in A were Western blotted and probed either with anti-Dmf1p antiserum (top), anti-Cdc15p antiserum (middle), or the TAT1 anti-tubulin monoclonal antibody as loading control (bottom). (C) Dmf1p is not degraded at the end of mitosis. *cdc25-22* cells were treated as in A, except that 30 min after release, cycloheximide was added up to 0.1 mg/ml to inhibit new protein synthesis. (○) Anaphase; (●) septa. (D) Protein extracts prepared from the cells described in C were Western blotted and probed with anti-Dmf1p antiserum and TAT1 monoclonal antibody.

tation and cytokinesis, producing binucleate cells, with or without a septum. In addition, a number of phosphatase mutants cause cell separation defects in *S. pombe* (Kinoshita et al. 1993; Yoshida et al. 1994). It will be of interest to determine whether any of these phosphatases acts on Dmf1p. Two candidates for the Dmf1p kinase at mitosis are p34^{cdc2} and plol (the fission yeast homolog of the *Drosophila melanogaster* polo kinase). The activity of fission yeast plol through the cell cycle has not been investigated, but its activity is required to induce actin rearrangement and septa-

tion (Ohkura et al. 1995). The p34^{cdc2} kinase is active at the time when Dmf1p becomes hyperphosphorylated (Moreno et al. 1989). Mapping and mutagenesis of potential phosphorylation sites are in progress. Since *dmf1* was isolated as a suppressor of a temperature-sensitive *cdc7* mutation, the possibility that it is a substrate of Cdc7p has also been investigated. To date, we have not observed phosphorylation of GST-Dmf1p fusion proteins by *cdc7* kinase in vitro (M. Sohrmann and V. Simanis, unpubl.). The observation that a Dmf1p ring can form in a *cdc7*^{ts} mutant argues that Cdc7p activity is not essential for Dmf1p ring assembly. At present, we do not understand why moderately increased expression of a fragment of Dmf1p rescues *cdc7*^{ts} mutants. Because the division septum is formed normally (but in the wrong place) in *dmf1::ura4*⁺ cells, we consider it unlikely that Dmf1p is an essential subunit of the *cdc7* kinase. Expression of a full-length *dmf1* clone from the *nmt1* promoter does not rescue *cdc7* mutants when uninduced, and is toxic when induced (not shown). Our data suggest that neither the NLS nor the PH domain is essential for rescue of *cdc7*^{ts} mutants, because they are not present in the truncated clone. It is possible that this truncated fragment of Dmf1p titrates a negative regulator of the Cdc7p kinase. Future experiments will address whether Cdc7p and Dmf1p interact directly, and the effects of *dmf1* mutations on Cdc7p kinase activity.

Because loss of Dmf1p abolishes the normal controls over septum position, one possibility is that its role is to prevent a nuclear factor marking the cortex with septation sites during interphase. Because the nucleus is always centrally positioned during interphase, and fission yeast cells do not grow equally at their old and new ends through the cell cycle (Mitchison and Nurse 1985), these sites would be eccentrically located at mitosis. This could lead to aberrantly angled or positioned septa, if the machinery were located at more than one site on the cortex. The down-shift experiments on *dmf1-6* synchronous cultures argue against this hypothesis, because a return to permissive temperature just prior to the initiation of mitosis results in most cells having a normally placed division septum. Thus, loss of Dmf1p function does not result in irreversible marking of the cell cortex during interphase. These data also argue that Dmf1p does not play any essential role in defining the position of the cell equator or the central positioning of the nucleus during interphase. The possibility that a temperature shift in interphase has little effect because the protein is locked in the nucleus and therefore more difficult to denature than the cortical ring form is unlikely, as we observed no discrete Dmf1p staining (ring or nuclear) within 30 min of shift-up of a *dmf1-6* mutant.

In a normal mitosis, the Dmf1p ring precedes, and then colocalizes with, the actin and Cdc15p rings until the onset of septation. The observation that an equatorial Dmf1p ring is formed in *cdc15-140* and *nda3-KM311* mutants at the restrictive temperature indicates that its assembly does not require the presence of either the F-actin ring, microtubules, or Cdc15p function.

Failure to form a normal Dmf1p ring in *cdc3*, *cdc4*,

cdc8, and *cdc12* mutants, which are required for actin ring formation, indicates that these proteins are essential both for formation of the Dmflp ring and of the actin ring, whereas Cdc15p is required only for the latter. Cdc3p and Cdc8p encode profilin and tropomyosin, respectively, which are known to be actin binding proteins (Balasubramanian et al. 1992, 1994), whereas Cdc4p encodes a myosin regulatory light chain-like protein (McCollum et al. 1995). Cdc12p encodes a proline-rich protein, of currently unknown biochemical function (F. Chang and P. Nurse, unpubl.). These proteins are all known to localize to the medial ring that defines the position of the septum. These data present an apparent paradox, in that the Dmflp ring can form in the absence of the actin ring, but its appearance requires actin binding proteins.

The observation that actin rings and septa are formed in *dmf1* mutants (both null and heat-sensitive alleles) argues that Dmflp is not required for assembly of the medial ring, but fixes its location. Dmflp may either provide a preferential assembly site for Cdc3p, Cdc4p, Cdc8p, and Cdc12p (or a subset of these), or Dmflp may coassemble with them, and, by doing so, prevent the ring from drifting away from the middle of the cell. The fact that the null mutant phenotype becomes more severe at elevated temperatures is also consistent with Dmflp stabilizing the position of the medial ring. The published immunofluorescence studies of Cdc3p, Cdc4p, and Cdc8p do not allow any firm conclusion to be drawn about whether they form a medial ring before, coincident with, or after, the Dmflp ring, though they all co-localize with the actin ring. Likewise, no data are yet available concerning the localization of Cdc3p, Cdc4p, or Cdc8p in other genetic backgrounds affecting actin relocalization at mitosis.

The finding that no Dmflp rings are present in *cdc3-6*, *cdc4-8*, and *cdc8-110*, taken together with the genetic interactions between *dmf1* and both *cdc3*, and *cdc4*, argues that if these proteins are mutant, the Dmflp ring is either not formed, or very unstable. In *cdc12-112*, diffuse medial Dmflp staining was observed. This may reflect residual activity of the *cdc12-112* allele at its restrictive temperature. Alternatively, it may suggest that Cdc12p plays a role in stabilizing the medial ring, of which Dmflp is a component, rather than being required for its assembly. Mutants in *cdc15* block formation of the F-actin ring, yet the Dmflp ring is present. This argues that the primary role of Cdc15p is to assure recruitment of F-actin to the site of septum formation, rather than participating in the assembly or stabilization of the medial ring.

It has been suggested that the signal defining the position where the septum will be formed is given by the nucleus at the onset of mitosis (Chang and Nurse 1996). Loss of Dmflp does not block formation of the division septum, but prevents it being correctly located in the middle of the cell. Dmflp is a nuclear protein until the onset of mitosis, when it forms a ring at the position of the interphase nucleus. This centrally located Dmflp ring appears prior to the actin ring, and colocalizes with

it, until the onset of septation, when it dissipates. We therefore propose that Dmflp relocalization from the nucleus to form a cortical ring is a critical step in signaling the position where the actin ring, and subsequently the septum, will form. The carboxy-terminal PH-domain of Dmflp may provide an interaction site with proteins or membrane lipids at the cell cortex. It is noteworthy that in budding yeast, PH domain containing proteins are implicated both in localization of cell growth with respect to the septin ring and cytokinesis (Cla4p; Cvrckova et al. 1995) and in bud emergence (Bem3p; Zheng et al. 1994).

The basic mechanisms that regulate initiation of mitosis have been conserved through evolution, as have many of the structural elements that are required for cytokinesis. It will be of considerable interest to determine whether functional homologs of *dmf1* exist in other eukaryotes.

Materials and methods

Yeast techniques

Standard techniques were used for growth, manipulation, and synchronization of fission yeast (Moreno et al. 1991). Cells were grown in yeast extract (YE) or EMM2 minimal medium, supplemented as required. Other techniques have been referred to previously (Fankhauser et al. 1995). All mutants were outcrossed to wild-type at least twice before use. *Dmf1-6* was crossed to *dmf1::ura4⁺*: no wild-type recombinants were observed in 30 tetrads, and ~1000 progeny in free-spore analysis, showing that *dmf1-6* is an allele of *dmf1*.

Cloning of the *dmf1* gene

Standard methods were used for DNA manipulation (Sambrook et al. 1989). The strain *cdc7-24 leu1-32 h-* was transformed to leucine prototrophy by use of a wild-type *S. pombe* cDNA-library based on the pREP3 vector (Kelly et al. 1993) on a thiamine-containing minimal medium. Colonies were replicated to a minimal medium without thiamine and incubated for 16 hr at 25°C to induce the *nmt1* promoter before shift to 36°C for 24 hr. Plasmids were extracted from colonies capable of growth at 36°C. Upon induction of the *nmt1*-promotor, two different plasmids were able to rescue the mutant strain at 36°C. Integration and mapping, and DNA sequencing, showed that one of these clones did not encode *cdc7*, but a multicopy suppressor.

A *dmf1* genomic clone was obtained by subcloning from cosmid 4B3c (Hoheisel et al. 1993). Comparison of the restriction maps of the cosmid, with those obtained from genomic Southern blots, showed no evidence of rearrangements. Additional cDNA clones were obtained by screening the library originally used for transformation at high density on nitrocellulose filters.

dmf1 expression from pREP41

A *Bam*HI site was introduced 50-bp upstream of the ATG in the genomic clone of *dmf1* in pDW232 (Weilguny et al. 1991), by use of the oligonucleotides CCACAGGATCCCACTCGATA-AGGTATT (*Bam*HI site underlined) and TGTGTAAAGTTT-TTTGTTTGATCA (*Bcl*II site underlined) to amplify a fragment of 228 bp by PCR. This fragment was cloned and sequenced. The *dmf1* genomic DNA was digested with *Bam*HI and *Bcl*II. The

BclI site lies within the *dmf1* gene, while the *BamHI* site resides in the polylinker of pDW232. Cutting the genomic clone with *BamHI* and *BclI*, therefore, truncates the region 5' to the *dmf1* ORF. The 228-bp PCR fragment, digested with the same enzymes, was used to replace the 5' end of the *dmf1* gene in the genomic clone. The *dmf1* gene was then excized as a *BamHI*–*SacI* fragment from the polylinker of pDW232, treated with T4 DNA polymerase, and introduced into the *SmaI* site of pREP41 (Basi et al. 1993), which had been modified so that the polylinker no longer provides an ATG (this laboratory, S. Schmidt and V. Simanis, unpubl.).

dmf1 deletions

To disrupt the *dmf1* gene, we replaced either the *BglII*–*PvuII* or the *MscI*–*PvuII* fragment (see Fig. 1A) with the *ura4* gene. A diploid strain was constructed by mating *ade6M210* and *ade6M216* strains, which complement in *trans*. This diploid was transformed with a linear fragment containing one of the deletions. Correct integration was verified by Southern blot analysis.

Antisera, indirect immunofluorescence, and confocal microscopy

To generate an antiserum recognizing Dmf1p, an *EcoRV*–*EcoRV* fragment of *dmf1* (Fig. 1A), corresponding to amino acids 309–505, was cloned into *SmaI*-digested pGEX-3X (Pharmacia). The Dmf1p–GST fusion protein was expressed in *Escherichia coli* DH5 α , solubilized from inclusion bodies, and purified by preparative SDS–PAGE (Fankhauser and Simanis 1993). Approximately 200 μ g of protein, emulsified with incomplete Freund's adjuvant, were injected monthly into New Zealand white rabbits. Standard procedures were used throughout for antibody production and assay, as well as for affinity purification of the sera (Harlow and Lane 1988). Antisera were affinity purified by chromatography, first on a column of GST coupled to cyanogen bromide-activated Sepharose, followed by passage of the flowthrough from the first column through a column of GST–Dmf1p fusion protein coupled to cyanogen bromide-activated Sepharose. After washing, the bound anti-Dmf1p antibodies were eluted with 0.1 M glycine at pH 2.1, and neutralized.

For Dmf1p immunolocalization, cells were fixed by addition of 1/7 of 30% (w/vol) formaldehyde in 100 mM PIPES (pH 6.9), 1 mM EGTA, and 1 mM MgSO₄ (PEM). After a 30-min fixation, cells were washed three times with PEM and then digested for 60 min at 37°C in 1 ml of PEM plus 1.2 M Sorbitol (PEMS) containing 2.5 mg/ml zymolyase 20T (Seikagaku Corporation, Japan). Cells were washed once in PEMS plus 1% Triton X100, three times in PEM, resuspended in PEM plus 1% BSA (Sigma), 100 mM lysine hydrochloride, and 0.1% sodium azide (PEM-BAL), and gently shaken for 30 min. Cells (5×10^6) were resuspended in 90 μ l of PEMBAL containing affinity purified anti-Dmf1p antibody diluted 1/30 and left on a rotating wheel overnight. After three 10-min washes in PEMBAL, cells were incubated for 4 hr at 37°C in PEMBAL containing secondary antibodies (1/800) coupled either to rhodamine (Pierce), or CY3 (Jackson ImmunoResearch Laboratories), or FITC (Cappel). Cells were then washed once in PEM, once in PBS at pH 8.0, and once in PBS at pH 8.0 containing 1 μ g/ml DAPI, and resuspended in 50 μ l PBS at pH 8.0, containing 0.1% sodium azide. For costaining with actin, rhodamine-conjugated phalloidin was added to a final concentration of 2 μ g/ml. Cells were then mounted in 50% glycerol containing 1 mg/ml *p*-phenylene diamine and observed with a Zeiss Axiophot microscope. Fixation with 3% paraformaldehyde plus 0.2% glutaraldehyde gave sim-

ilar patterns, but the intensity of the signal was diminished (data not shown). For competition experiments, affinity-purified serum (90 μ l of 1/30 dilution) was preincubated 60 min with 3 μ g GST or 3 μ g *dmf1*–GST fusion protein before adding it to the cells. In experiments with the Mab414 nuclear pore marker, a DTAF-conjugated goat–antimouse secondary antibody was used, diluted 1/100 (purchased from Jackson ImmunoResearch laboratories).

For visualization of nuclear pores, Mab414 (Davis and Blobel 1986) was purchased from Babco and used as recommended. Antisera to Cdc15p have been described (Fankhauser et al. 1995). Staining for F-actin with rhodamine-conjugated phalloidin (Sigma) was performed as described (Marks and Hyams 1985). The anti-actin monoclonal antibody was purchased from Amersham and used as recommended; cells were fixed by use of the same protocol as for Cdc15p. Cells were stained with DAPI (4', 6'-diamino-2-phenylindole, Sigma) and Calcofluor (fluorescent brightener no. 28, Sigma) after fixation in 70% ethanol, as described previously (Marks and Hyams 1985).

Confocal microscopy was performed with a Zeiss Axiovert 100 Microscope (Zeiss laser scanning microscope 410), with $\times 63$ Plan-Apochromat objective (1.4 numerical aperture, with oil). Standardized conditions were used for image scanning, averaging, and processing.

Protein extracts and immunoprecipitations

Protein extracts were prepared either according to the method of Yaffe and Schatz (1984), or as described previously (Fankhauser and Simanis 1994). In the latter method, the pooled extracts were, in addition, vortexed with an equal volume of 8 M urea, 0.5 M NaCl, 0.2 M Tris–HCl at pH 8.0, before clarification by successive 1- and 15-min spins. This was necessary because only a minor fraction of the *dmf1* protein is soluble in the absence of chaotropes or SDS (not shown). During the course of these studies, we noted that Dmf1p is labile to proteolysis during extraction, which accounts for bands of variable intensity of relative molecular weight ≤ 110 kD (not shown). Immunoprecipitations were performed as described previously (Fankhauser and Simanis 1994), except that affinity-purified anti-Dmf1p antiserum was used.

For Western blotting, bound antibodies were detected by use of secondary antibodies coupled to alkaline phosphatase. TAT-1 monoclonal antibody was used as a control (Woods et al. 1989).

Calf intestinal alkaline phosphatase (CIP) treatment

cdc25-22 cells were arrested by incubation at 36°C for 4 hr, then shifted to 25°C. Proteins were extracted 30 min after shift-down and Dmf1p was immunoprecipitated as described above. The CIP-treatment was performed as described previously (Fankhauser et al. 1995).

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The *dmf1/mid1* gene is essential for correct positioning of the division septum in fission yeast.

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