Asymmetry of the spindle pole bodies and spg1p GAP segregation during mitosis in fission yeast

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SUMMARY

In the fission yeast Schizosaccharomyces pombe, the onset of septum formation is induced by a signal transduction network involving several protein kinases and a GTPase switch. One of the roles of the spg1p GTPase is to localise the cdc7p protein kinase to the poles of the mitotic spindle, from where the onset of septation is thought to be signalled at the end of mitosis. Immunofluorescence studies have shown that cdc7p is located on both spindle pole bodies early in mitosis, but only on one during the later stages of anaphase. This is mediated by inactivation of spg1p on one pole before the other. The GAP for spg1p is a complex of two proteins, cdc16p and byr4p. Localisation of cdc16p and byr4p by indirect immunofluorescence during the mitotic cell cycle showed that both proteins are present on the spindle pole body in interphase cells. During mitosis, byr4p is seen first on both poles of the spindle, then on only one. This occurs prior to cdc7p becoming asymmetric. In contrast, the signal due to cdc16p decreases to a low level during early mitosis, before being seen strongly on the same pole as byr4p. Double staining indicates that this is the opposite pole to that which retains cdc7p in late anaphase. Examination of the effect of inactivating cdc16p at various stages of the cell cycle suggests that cdc16p, together with cdc2p plays a role in restraining septum formation during interphase. The asymmetric inactivation of spg1p is mediated by recruitment of the cdc16p-byr4p GAP to one of the poles of the spindle before the other, and the asymmetry of the spindle pole bodies may be established early during mitosis. Moreover, the spindle pole bodies appear to be non-equivalent even after division has been completed.

Key words: Mitosis, Cytokinesis, Spindle pole body, Schizosaccharomyces pombe

INTRODUCTION

The fission yeast Schizosaccharomyces pombe is an excellent model system for the study of cytokinesis. S. pombe cells are rod shaped, and grow mainly by elongation at their tips, dividing by binary fission after formation of a centrally placed division septum. The future site of division is defined at the onset of mitosis by the assembly of an F-actin-based medial ring structure at the cell cortex, and the subsequent polarisation of F-actin patches to the ring. At the end of anaphase, the septum grows inwards from this ring. Analysis of S. pombe cell cycle mutants has identified genes whose products are important for different aspects of septum formation and cytokinesis, including specification of the cleavage plane, assembly of the medial ring, polarisation of F-actin patches to the ring, signalling the onset of septum formation, and the subsequent cell separation (for a list of genes implicated in controlling septum formation and a review of the topic, see Gould and Simanis, 1997; Le Goff et al., 1999).

The onset of septum formation is controlled by a signal transduction network involving a small GTPase (spg1p) and a number of protein kinases, including cdc7p, plo1p, sid1p and sid2p (Ohkura et al., 1995; Schmidt et al., 1997; Balasubramanian et al., 1998). The GAP (GTPase-activating protein) for spg1p is a complex of two proteins, cdc16p and byr4p (Furge et al., 1998). The absence of either protein deregulates septum formation, leading to a multiseptate phenotype (Minet et al., 1979; Fankhauser et al., 1993; Song et al., 1996). Biochemical analysis suggests that cdc16p may be the catalytic subunit, and byr4p may act to hold spg1p and cdc16p together to allow cdc16p to activate GTP hydrolysis by spg1p (Furge et al., 1998). Increased expression of byr4p inhibits septum formation, and suppresses heat-sensitive cdc16 mutants, but not a cdc16 null allele (Furge et al., 1998; Jwa and Song, 1998). Immunofluorescence studies have shown that spg1p is present on the spindle pole body at all stages of the cell cycle, while cdc7p is only on the spindle pole body during mitosis. Early in mitosis, cdc7p is observed on both poles of the spindle, while in late anaphase it is seen on only one of the two poles. Localisation of cdc7p requires spg1p function, and previous studies had shown that spg1p was inactivated on one of the spindle pole bodies before the other, thereby mediating the asymmetrical segregation of cdc7p (Sohrmann et al., 1998). This does not require the presence of cdc7p kinase. However, it was not clear whether inactivation of spg1p-mediated signalling was achieved by differential recruitment of the cdc16p-byr4p GAP, or whether the GAP was present on the spindle pole body at all stages of the cell cycle, and activated
at different times. We have used indirect immunofluorescence to analyse the location of both components of the spg1p-GAP during the cell cycle. Our data support a model in which the GAP is recruited to one spindle pole body before the other during mitosis, and that this asymmetry is established early during mitosis, before cdc7p becomes asymmetric. Analysis of the effects of cdc16p inactivation at different stages of the cell cycle suggest that it plays a role in preventing septum formation during interphase, and that the two spindle pole bodies produced during mitosis may not be equivalent, even after division has been completed.

MATERIALS AND METHODS

Standard techniques were used for microscopy (Hagan and Hyams, 1988; Marks and Hyams, 1985), molecular biology (Sambrook et al., 1989) and FACS analysis (Sazer and Sherwood, 1990). Standard techniques were used for growth, manipulation and synchronisation of fission yeast (Moreno et al., 1991). Cells were grown in yeast extract (YE) or EMM2 minimal medium, supplemented as required with 50 μg/ml of adenine, uracil, leucine, histidine or lysine. The vitality stain Phloxin B (Sigma) was added to 5 μg/ml to identify dead cells on plates. Hydroxyurea (HU) was added to a final concentration of 12 mM from a stock of 1.2 M in water. Transformations of S. pombe cells were performed by the lithium acetate method (Okazaki et al., 1990). Selection synchrony was performed as described (Schmidt et al., 1997), using a Beckman JS 5.0 elutriation system. Other techniques have been described previously (Fankhauser et al., 1995). All mutants were outcrossed to wild type at least twice before use. The strains used in this study were as follows: 972 h (Simanis laboratory collection), cdc7-HA (Sohrmann et al., 1998; Simanis laboratory strain collection), mts4-3 (C. Gordon strain collection, Edinburgh), cdc16-116 (Minet et al., 1997; Simanis laboratory strain collection) cdc16-116 cdc2-33 (this study) cdc16-116 cdc25-22 (this strain). The strain SP2198 has the genotype cdc16::ura4+ cdc7-7A20 ade6MT16 leu1-32 ura4D18 h+.

Epitope tagging of cdc16p

The cdc16 cDNA was amplified by PCR from first-strand cDNA with the oligonucleotides VS47 and VS186 (TTAGCGGCCGCACGTTTCTGGTTCTGG), and cloned into pDW232 (Weilguny et al., 1989). The triple HA-tag (Tyers et al., 1992) was cloned into the NotI site introduced by VS186 just before the stop codon. These primers also add the KT3 epitope (MacArthur and Young, 1991). The attenuation of cdc7 alelle rescues the deletion of cdc16 if the strain is grown at 32°C. Full details of its construction will be given elsewhere (L. Cerutti and V. Simanis, manuscript in preparation). Double mutants were constructed by tetrad dissection.

Indirect immunofluorescence and FACS analysis

The following primary antibodies were used: alpha tubulin was detected using TAT-I (Woods et al., 1989), Affinity-purified rabbit anti-byr4p diluted 1/30 (Song et al. 1996), affinity-purified rabbit anti-Spg1p diluted 1/50 (Hagan and Yanagida, 1995), affinity-purified rabbit anti-Cdc7p diluted 1/30 (Fankhauser and Simanis, 1994), affinity-purified rabbit anti-Smp1p diluted 1/30 (SuSu1; Schmidt et al., 1997), and monoclonal anti-HA antibody (12CA5) diluted 1/500 (Babco). The following secondary antibodies were used: goat anti-rabbit CY3-coupled (1/800, Jackson) and FITC-coupled (1/500, Kappel), sheep anti-rabbit CY3-coupled (1/2000, Sigma), goat anti-mouse CY3-coupled (1/500, Jackson) and FITC-coupled (1/500, Sigma), and donkey anti-sheep FITC-coupled (1/500, Sigma). Cells were fixed with 4% (w/v) formaldehyde. After 20 minutes fixation, cells were washed three times with PEM and then digested for 15 minutes at 37°C in PEM plus 1.2 M Sorbitol (PEMS) containing 1 mg/ml zymolyase 20T (Seikagaku Corporation, Japan). Cells were washed once in PEMS plus 1% Triton X-100, three times in PEM, resuspended in PEMS plus 1% BSA (Sigma), 100 mM lysine hydrochloride, 0.1% sodium azide (PEMBAL), and gently shaken for 30 minutes. 5×10⁶ cells were resuspended in PEMBAL containing primary antibody, and left on a rotating wheel overnight at room temperature. After three 10 minute washes in PEMBAL, cells were incubated overnight at room temperature in PEMBAL containing secondary antibodies. Cells were then washed once in PEM, once in PBS, pH 8.0, once in PBS, pH 8.0 containing 1 μg/ml DAPI and resuspended in PBS, pH 8.0. Cells were then mounted in 50% glycerol containing 1 mg/ml p-phenylene diamine and observed with a Zeiss Axiopt microscope or with a Zeiss Axioshot 100 TV coupled with a CCD camera (Quantix).

The sequestration of culutlated cultures was counted using dark-field microscopy. The appearance of type I and type II septated cells, was scored by staining with DAPI (4'-6-diamino-2-phenylindole, Sigma) and Calcofluor (Fluorescent brightener No. 28, Sigma) after fixation in 70% ethanol, as previously described (Marks and Hyams, 1985). Cells were then mounted in PBS pH 8.0 containing 1 mg/ml p-phenylene diamine and observed with a Zeiss Axiopt microscope.

RESULTS

Localisation of byr4p

Exponentially growing wild-type cells were fixed and stained with a polyclonal serum against byr4p (Song et al., 1996). It was found that byr4p is located on the spindle pole body throughout interphase (Fig. 1A). In cells with a short spindle, byr4p was seen on both spindle poles, but by the time that the spindle had elongated to the length of the nucleus, the signal was seen only on one of the two poles (Fig. 1A,B). The byr4p signal in early mitotic cells, when it was seen on both poles of the spindle, was consistently weaker than later in mitosis (Fig. 1A). The asymmetry of byr4p staining on the spindle poles precedes that of cdc7p, as byr4p was seen on only one of the two poles in early anaphase, while cdc7p was clearly present on both of them (Fig. 1C, upper panels). Later in mitosis, when cdc7p was seen on only one of the two poles of the mitotic spindle, byr4p was found on the opposite pole to cdc7p (Fig. 1C, lower panels, D). Staining of cells deleted for cdc16 demonstrated that the byr4p spindle pole staining was absent (Fig. 1E), indicating that stable spindle pole body association of byr4p requires cdc16p. Staining for cdc7p showed that it was present on both spindle pole bodies in these cells (Fig. 1F), consistent with findings described previously for the mutant cdc16::ura4 (Sohrmann et al., 1998).

Localisation of cdc16p

Localisation of cdc16p was performed in cells bearing a plasmid expressing a 12CA5 (HA) tagged copy of the cdc16 gene. The tagged copy is fully functional, as it is able to complement a cdc16::ura4+ null mutant (not shown). The images presented were obtained in a mts4-3 background, at the permissive temperature, when the cells divide normally. The use of a protease mutant and an increased level of cdc16-HA
expression was necessary, as the steady-state levels of the *cdc16* mRNA and protein in wild-type cells are very low. To date, we have not been successful in generating polyclonal sera capable of recognising cdc16p, whether overexpressed or not. Cdc16-HAp was detected on the spindle pole body during interphase, but not in early mitosis (Fig. 2A). Spindle pole body association was confirmed by double staining with antibodies recognising sad1p (Fig. 2B). In late anaphase, it was seen on the opposite pole to cdc7p (Fig. 2D). Staining with the Susu-1 serum, which preferentially recognises GDP-spg1p in vitro (Sohrmann et al., 1998), showed that cdc16-HAp was associated with the pole of the spindle bearing GDP-spg1p (Fig. 2C). Thus, like byr4p, in late anaphase, cdc16-HAp is associated with the opposite spindle pole to cdc7p. In all cases, cdc16-HAp staining was observed on the opposite pole to cdc7p as soon as the staining of the latter became asymmetric on the two spindle poles. The data presented above are consistent with a model which proposes that inactivation of spg1p on one pole before the other during anaphase is mediated by assembly of the byr4p-cdc16p GAP at one pole prior to the other.

In exponentially growing wild-type fission yeast cells, S phase begins around the time that cells complete the division septum and separate. In these interphase cells, cdc16-HAp and byr4p were both observed on the spindle pole body in both daughter cells (Figs 1 and 2), while neither showed spindle pole body cdc7p staining (Sohrmann et al., 1998; not shown).

The staining patterns we have observed for byr4p and cdc16-HAp, together with the previously described segregation of spg1p and cdc7p, are summarised in Fig. 3.

**cdc16p is required to prevent septum formation in early S phase-arrested cells**

The original study of the *cdc16-116* mutant (Minet et al., 1979) found that cells shifted to the nonpermissive temperature at the time of septation divided and gave rise to daughter cells which behaved differently. One (called type I), elongated and then underwent nuclear division before forming multiple septa. The other (called type II) formed another septum immediately after separation, and then grew at only one end, before undergoing mitosis and forming multiple additional septa. It was suggested that this might be due to slow inactivation of the thermolabile cdc16-116 protein. To investigate whether this difference in daughter cells persisted after division, cells were arrested at the start of S phase by the addition of hydroxyurea (HU), and the effect of inactivating cdc16p was examined. The experimental protocol is summarised in Fig. 4A. In order to minimise any effects due to differences in cell size, or time spent at the arrest point, synchronous cell populations were analysed. Newly divided *cdc16-116* cells were selected by centrifugal elutriation. These cells, which are in late S phase/early G2, were allowed to grow for 60 minutes at 25°C to ensure that S phase had been completed, and HU was added to half the culture. Both cultures entered mitosis, formed a septum, and divided. The culture to which HU had been added then arrested at S phase onset.

At various times during the septation peak and after division, samples were removed from both cultures, and incubated at 36°C to inactivate cdc16p. Cells were stained with DAPI and Calceofluor, and the proportion of type I and type II cells in each sample was determined at intervals after shift to the nonpermissive temperature. In sample 1 (225 minutes after inoculation), which corresponds to the septation peak/S phase in the culture without HU, both type I and type II cells were formed in similar proportions (Fig. 4B), as anticipated from previous studies (Minet et al., 1979). Sample 2 (end of S phase/early G2; 275 minutes after inoculation), formed predominantly type I cells in the absence of HU (Fig. 4B), and similar results were obtained for sample 3 (mid-G2; 345 minutes after inoculation), and sample 4 (late G2; 400 minutes after inoculation). Examination of the timing of septum formation indicated that it paralleled entry into mitosis, as judged by the formation of binucleate cells. Thus, in sample 2, type I septated cells were not observed until 180 minutes after shift to 36°C (Fig. 4B, 2-HU; Fig. 4C), while in sample 3, type I septated cells were not observed until 135 minutes after shift to 36°C (Fig. 4B, 3-HU; Fig. 4C).

Samples 2, 3 and 4 from the culture with HU formed only type II cells, and only approximately 50% of cells septated in all three samples. Moreover, the kinetics of septation were similar in all three cases, with septa appearing at the 90 minute time point (sample 2, 3, 4 +HU, Fig. 4B). This contrasts sharply with the result obtained for these samples in the absence of HU, where 80, 90 and 94% type I septated cells, respectively, were formed, and septation coincided with mitotic entry. Note that since these populations are from parallel cultures derived from the same elutriation, this difference in timing is unlikely to be a function of cell size. These data demonstrate that, long after separation has been completed at the permissive temperature, half of the cells arrested early in S phase retain the ability to form an asymmetrically placed septum characteristic of type II cells, thereby eliminating the possibility that this phenotype arises from slow inactivation of the cdc16-116 protein. These points are addressed further in the Discussion.

Previous studies had demonstrated that septum formation induced in interphase by inappropriate expression of spg1p was always associated with recruitment of cdc7p kinase to the spindle pole body (Sohrmann et al., 1998). We therefore examined the localisation of a cdc7p-GFP fusion protein (described in Sohrmann et al., 1998) in HU-arrested *cdc16-116* cells before and after shift to the restrictive temperature. We found that 135 minutes after shift to 36°C, all the cells that had formed a septum had cdc7p on the spindle pole body. In contrast, only 5% of non-septated cells showed spindle pole body localization of cdc7p (Fig. 4D). Analysis of the cells at the permissive temperature showed that only 15% of the HU-arrested cells had spindle pole body-associated cdc7p at 25°C. This result suggests that the spindle pole bodies in the daughter cells differ in their capacity to recruit cdc7p and promote septum formation even after division has been completed, and is addressed further below. Similar HU-arrest followed by temperature-shift experiments on wild-type cells did not produce any septum formation or spindle-pole body recruitment of cdc7p in the early S phase-arrested cells, indicating that the septum formation is triggered by loss of cdc16p function, rather than by the heat shock.

**A role for cdc16p in preventing septation during G2**

Since cdc16p is present on the spindle pole body during interphase, we investigated the effects of inactivation of cdc16p in mutant cells which are unable to undergo the G2-M transition. The double mutants *cdc2-33 cdc16-116* and *cdc25-
22 cdc16-116 were synchronised in early G2 by centrifugal
elutriation at the permissive temperature and samples of the
culture were shifted to the restrictive temperature, 0, 60 and
120 minutes after size selection, and the percentage of type I
and type II septated cells was monitored at intervals thereafter.
The data for cdc25-22 cdc16-116 are shown in Fig. 5. Cells in
all three samples septated with similar kinetics after shift to
36°C (Fig. 5A). Only type II cells were observed at all time
points up to 135 minutes after shift, indicating that cells had
not entered mitosis before septation (Fig. 5B). Consistent with
this, cdc2p kinase was at interphase levels (not shown). As in
the HU-arrested cells, only approximately half the cells formed
a division septum. These data indicate that cdc16p also plays
a role in preventing septum formation during G2 phase.

Similar results were obtained using the mutant cdc2-33
cdc16-116 (not shown). A previous study (Minet et al., 1979)
suggested that the double mutant cdc2-33 cdc16-116 did not
septate. The reason for this discrepancy is unclear, but a
possible explanation may be that in this study we used Calcofluor staining to detect septation, while the earlier study
used dark-field optics, which are less sensitive.

**DISCUSSION**

**cdc7p asymmetry during mitosis is mediated by asymmetrical segregation of spg1p GAP components**

The key regulators of septum formation, spg1p and cdc7p, are
located on the poles of the mitotic spindle during mitosis.
During anaphase, cdc7p is observed on only one of the two
spindle poles, and this asymmetry is mediated by conversion
of spg1p from the GTP- to the GDP-bound form on one pole
before the other (Sohrmann et al., 1998). In this paper we have

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**Fig. 1.** Immunolocalisation of byr4p. Exponentially growing wild-type cells were fixed and stained with the indicated antisera. (A) Localisation of byr4p during interphase (I), mitosis (M) and septum formation/cytokinesis (CK) in wild-type cells. (B) byr4p is asymmetrically localised on one of the two spindle pole bodies late in anaphase. (C) byr4p becomes asymmetrically distributed on the poles of the mitotic spindle in early anaphase, while cdc7p is still present on both poles of the spindle (arrowheads, upper panels). Byr4p is located on the opposite pole of the mitotic spindle to cdc7p late in anaphase (arrowheads, lower panels). (D) Cells bearing a chromosomal copy of cdc7 tagged with the 12CA5 epitope at its C terminus were stained with antisera to byr4p and 12CA5. Note that byr4p stains the opposite pole to cdc7-HAp. (E) byr4p localisation to the spindle pole body requires cdc16p. Cells deleted for cdc16 (SP2198; see Materials and methods) were stained with antisera to sad1p and byr4p. Note that while byr4p shows no discrete staining, sad1p staining is normal. (F) cdc7p stains both poles of the mitotic spindle in cells lacking cdc16p function. SP2198 cells were stained with antiserum to cdc7p.
investigated how this might be achieved. Signaling by spg1p is negatively regulated by a two-component GAP comprising the cdc16p and byr4p proteins. We have used indirect immunofluorescence to localise byr4p and epitope-tagged cdc16p in exponentially growing cells. Both byr4p and cdc16-HAp are observed on the spindle pole body in interphase cells. We have found that during early mitosis, cdc16-HAp cannot be detected on the spindle pole body. In contrast, byr4p is present at low levels on both poles of the spindle initially, then on only one of the two poles. This asymmetrical staining is seen while cdc7p is clearly present on both poles of the spindle. During anaphase, when cdc7p is seen on one spindle pole, cdc16-HAp and byr4p are both located on the pole, which no longer has any cdc7p associated with it. These data are consistent with a model in which the GAP is assembled on one of the two poles before the other to bring about the asymmetric inactivation of spg1p. The finding that the poles of the mitotic spindle become asymmetric with respect to byr4p staining suggests that which of the two poles will be inactive spg1p signaling first is determined by which of them retains byr4p. These data also eliminate an alternative model in which the byr4p-cdc16p GAP is present on both spindle poles throughout mitosis, and is activated on one before

![Fig. 2. Immunolocalisation of cdc16-HAp. mts4-3 cells, transformed with a multicopy plasmid expressing cdc16-HAp, growing exponentially at 25°C were fixed and stained with the indicated antibodies. (A) Localisation of cdc16-HAp in interphase, mitotic and septating cells. Note that double staining for sad1p reveals both spindle pole bodies, while 12CA5 stains only one (late anaphase), or cannot be detected (early mitosis). (B) False-colour images of cells stained with antisera to detect cdc16-HAp and sad1p. (C) cdc16-HAp localises to the same pole as GDP-spg1p in late anaphase. Cells were stained with Susu-1 and 12CA5 and the images were merged. (D) cdc16-HAp is found on the opposite spindle pole body to cdc7p in late anaphase. Cells were stained with a polyclonal serum raised against cdc7p and 12CA5, and the images derived from the three channels were merged.](image)

![Fig. 3. Summary of immunolocalisation of four key regulators of septum formation in fission yeast. The data concerning cdc16-HAp and byr4p are those presented in this paper, the data for cdc7p and spg1p are from Sohrmann et al. (1998).](image)
the other. We have been unable to detect cdc16-HAp early in mitosis, while byr4p is clearly present on the spindle poles, though the level of signal is reduced compared with that seen later in mitosis. Since we are detecting the 12CA5 epitope tag, it is possible that the failure to detect cdc16-HAp early in mitosis is due to masking of this epitope; alternatively, the level of the signal may decrease, like that of byr4p, and thereby fall below the threshold we are able to detect. Staining of

**Fig. 4.** cdc16p is required to prevent septum formation in early S-phase cells. (A) A synchronous population of cdc16-116 cells was generated by centrifugal elutriation and treated as shown in the diagram. Samples were removed at the indicated times (arrows), shifted to 36°C and the percentage of septated cells was determined at intervals thereafter. The percentage of septation was determined by dark-field microscopy. (B) The percentage of septated cells of type I and type II (shown diagrammatically at the bottom of the panel) was determined at the indicated times after shift to 36°C. (C) The percentage of binucleate cells was determined for the –HU samples at the indicated times after shift to 36°C. (D) HU-arrested cdc16-116 cells were shifted to 36°C for 135 minutes. Samples before and after shift were fixed, and stained with antiserum to cdc7p: the percentage of cells with septa and cdc7p staining on the spindle pole body was determined. Note that all the type II cells produced show cdc7p staining on the spindle pole body.
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cdc16::ura4+ cells indicates that byr4p requires cdc16p for stable association with the spindle pole body. We therefore favour the view that the protein is present, but not detectable by indirect immunofluorescence. We are at present attempting to raise a polyclonal antibody to detect cdc16p to distinguish between these alternatives.

The consequence of loss of cdc16p function if cell cycle progression is impeded

We also examined the result of inactivating cdc16p at different stages of the cell cycle. We found that if cells were arrested either in early S phase by addition of HU, or in late G2 by inactivation of either cdc2p or cdc25p, inactivation of cdc16p resulted in the appearance of septated cells, despite the fact that S phase and mitosis, respectively, had not occurred. These data suggest that cdc16p is required to prevent septation during interphase. At first glance, this observation appears to be inconsistent with the fact that shift of cdc16-116 to the restrictive temperature in G2 produces type I cells, and only after entry into mitosis (Fig. 4B). We have considered a number of possible explanations for this.

Inactivation of cdc16p in interphase-arrested cells triggers septation with similar kinetics (septated cells first appear at the 90 minute time point) irrespective of how long cells have been arrested before shift to 36°C to inactivate cdc16p (samples 2, 3, 4, Fig. 4B). The simplest assumption is that this reflects the time taken for cdc16p-byr4p GAP activity to decay, for spg1p to exchange GTP for GDP, thereby signaling the onset of septation, and for the septum then to be synthesised. Thus, in cdc16-116 cells, in which S phase and mitosis can proceed normally, it is conceivable that there is insufficient time between inactivation of cdc16p and the onset of mitosis for formation of a type II cell. Most cells would therefore initiate mitosis before the septum appeared after shift to 36°C, thus producing type I cells. A variation on this theme is that the onset of mitosis may block septum formation, even if it had been signaled during interphase. We consider these possibilities to be unlikely, since the appearance of septa in samples 2 and 3 (−HU) is delayed for significantly longer than in all the +HU cultures (Fig. 4B), suggesting that septation is still tied to the onset of mitosis in these cells.

Alternatively, cells may lose the capacity to form type II cells at some point after the initiation of S phase, so the cell would not be competent to septate in response to loss of cdc16p function during the majority of G2. This could be due to the initiation of bipolar growth (NETO), completion of S phase, the increase in cdc2p kinase activity, which begins early in G2 (Creanor and Mitchison, 1994), or the increase in cdc25p level (Creanor and Mitchison, 1996). We consider NETO or S-phase completion to be an unlikely explanation, since the cdc25-22 cdc16-116 and cdc2-33 cdc16-116 mutants have both initiated bipolar growth and completed DNA synthesis, yet they form type II cells. Therefore, we favour the idea of a role for cdc2p kinase in restraining septation during G2, for the following reason. One important difference between cdc2-33 cdc16-116 and cdc25-22 cdc16-116 mutants, which sepatate in G2, and cdc16-116, which does not, is the activity of cdc2p kinase. The cdc2p kinase is thermolabile in cdc2-33 (Simanis and Nurse, 1986; Moreno et al., 1989), and the activator of the kinase is defective in cdc25-22 (Russell and Nurse, 1986). Thus, at the
restrictive temperature the cdc2p kinase activity will be reduced in these cells. It has been suggested that interphase levels of cdc2p/cdc13p kinase, while insufficient to initiate mitosis, prevents re-replication of the genome (Stern and Nurse, 1996). We propose that interphase levels of cdc2p kinase activity, together with cdc16p-byr4p GAP activity, prevent septum formation in post-S-phase cells. Thus, loss of either cdc2p or cdc16p activity in G2 will not result in septation, but their simultaneous inactivation will do so. In the case of cdc25-22, where cdc2p kinase may accumulate more slowly than in wild-type cells, the level of protein kinase may be insufficient to restrain septum formation in G2 in the absence of cdc16p function. Together, these data support the concept of a ‘window of septation’ (as originally proposed by Minet et al., 1979), which restricts the cell’s potential for septum formation to only some parts of the cell cycle.

**Spindle pole body non-equivalence in S. pombe**

Most interestingly, only about 50% of HU-arrested cells were able to septate after inactivation of cdc16p, and these were all found to have recruited cdc7p to the spindle pole body. Time-lapse analysis, performed in an earlier study, showed that the two daughter cells do not behave identically if cdc16p is inactivated at the time of division (Minet et al., 1979). Together, these data suggest that the spindle pole bodies in the two daughter cells differ in their capacity to recruit cdc7p and promote septum formation, and implies that the spindle pole bodies inherited by daughter cells are non-equivalent. The finding that in cdc25-22 cdc16-116, only approximately half the cells septate suggests that the non-equivalence persists throughout G2. It is unlikely that this difference resides in the activity or presence of the byr4-cdc16 GAP, for two reasons. First, both daughter cells have cdc16p and byr4p associated with the spindle pole body before cell separation is completed, and throughout interphase (Figs 1 and 2). Second, byr4 localisation depends upon functional cdc16p (Fig. 1), so there would be no GAP associated with the poles after shift of cdc16-116 to 36°C.

It is well established that spindle pole body duplication in *S. cerevisiae* is conservative (Thomas and Botstein, 1986; Winey et al., 1991). However, the situation in *S. pombe* is less clear. Electron microscopy has suggested that the material of the first SPB is divided between the two parts of the SPB as it duplicates, and it has been suggested that additional material is acquired by each of the daughter SPBs from a soluble pool of components (Ding et al., 1997). Nonetheless, this does not exclude the possibility of some asymmetry in the duplication process. We have attempted to localise cdc7p and spg1p on the spindle pole body by electron microscopy (in collaboration with R. McIntosh, Boulder), but have not been successful with reagents that are presently available. Analysis of the *S. pombe* cut12 mutant indicates that some spindle pole body components are incorporated conservatively during spindle pole body duplication (Bridge et al., 1998), suggesting that the duplication process may not be symmetrical with respect to all the spindle pole body components. We therefore speculate that the ability to promote septation is acquired by the one of the spindle pole bodies at the time of spindle pole body duplication, and that an asymmetrically distributed component(s) will be responsible for the observed functional difference between the pole bodies. This component is unlikely to be byr4p, since it is observed on both spindle pole bodies at the onset of mitosis. Future studies will address the molecular mechanism by which the asymmetry of byr4p is established.

The data presented in this paper, and our previous studies, imply that the spindle pole bodies are non-equivalent with respect to their interactions with regulators of septum formation, and remain so throughout interphase. We suggest that this is programmed at the time of spindle pole body duplication. It is possible that the ‘new’ spindle pole body is assembled without an inhibitor of septum formation. Alternatively, the ‘old’ spindle pole body may be activated during the duplication process. The molecular basis for this will be the subject of future investigations.

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**REFERENCES**


