

The role of Plo1 kinase in mitotic commitment and septation in *Schizosaccharomyces pombe*

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Plo1-associated casein kinase activity peaked during mitosis before septation. Phosphatase treatment abolished this activity. Mitotic Plo1 activation had a requirement for prior activation of M-phase promoting factor (MPF), suggesting that Plo1 does not act as a mitotic trigger kinase to initiate MPF activation during mitotic commitment. A link between Plo1 and the septum initiating network (SIN) has been suggested by the inability of *plo1Δ* cells to septate and the prolific septation following *plo1*⁺ overexpression. Interphase activation of Spg1, the G protein that modulates SIN activity, induced septation but did not stimulate Plo1-associated kinase activity. Conversely, SIN inactivation did not affect the mitotic stimulation of Plo1-associated kinase activity. *plo1.ts4* cells formed a misshapen actin ring, but rarely septated at 36°C. Forced activation of Spg1 enabled *plo1.ts4* mutant cells, but not cells with defects in the SIN component Sid2, to convert the actin ring to a septum. The ability of *plo1*⁺ overexpression to induce septation was severely compromised by SIN inactivation. We propose that Plo1 acts before the SIN to control septation.

Keywords: cell cycle/mitosis/polo kinase/*Schizosaccharomyces pombe*/SIN

Introduction

Commitment to mitosis is controlled by the protein kinase complex called M-phase promoting factor (MPF) (Ohi and Gould, 1999). MPF comprises a catalytic subunit p34^{cdc2}/Cdk1 and its binding partner cyclin B. MPF is inhibited during interphase by Wee1- and Mik1-related kinases, which phosphorylate key residues of p34^{cdc2}. The phosphatases Cdc25/Pyp3 can remove these phosphates to produce active MPF. Commitment to mitosis can be viewed as the balance of activity between Cdc25 and Wee1-related molecules. Studies of cell cycle control in *Xenopus* oocyte extracts have identified a positive feedback loop whereby a trigger level of active MPF catalyses the conversion of the remaining MPF in the extract to the

active state. This activation involves phosphorylation of Cdc25 by p34^{cdc2} and at least one other kinase (Hoffmann *et al.*, 1993; Izumi and Maller, 1993, 1995). The co-purification of the polo-like kinase (Plk) Plx1 with Cdc25 in *Xenopus* extracts suggests an involvement of Plks in this activation loop (Kumagai and Dunphy, 1996). Moreover, phosphorylation of Cdc25 during the amplification loop or *in vitro* by Plx1 enables the protein to be recognized by the monoclonal antibody MPM-2. MPM-2 recognizes a phospho epitope found on Cdc25 following amplification loop activation in extracts (Kuang *et al.*, 1994).

Although MPF can directly regulate some mitotic processes, other conserved kinases such as Plks control many mitotic events. The *Drosophila polo* mutant was identified because of the propensity of cells to form monopolar rather than bipolar spindles. Subsequent analyses of Plks in diverse systems suggested that they regulate multiple and distinct mitotic events in every round of cell division (Glover *et al.*, 1998; Nigg, 1998). In addition to a role in the MPF positive feedback loop, these functions include centrosome maturation, spindle formation, anaphase promoting complex (APC) regulation, several aspects of cytokinesis and a crucial role in mitotic exit.

In *Schizosaccharomyces pombe*, only one Plk, Plo1, has been identified to date (Ohkura *et al.*, 1995). Plo1 function is required for spindle formation and cytokinesis. The first stage of cytokinesis in fission yeast is the generation of a cortical ring in the cell equator during prophase (Le Goff *et al.*, 1999). This contains several components, including F-actin and Mid1/Dmf1. Mid1/Dmf1 resides in the nucleus during interphase, but forms a ring around the cortex that predicts the site of F-actin ring formation. The export of Mid1/Dmf1 from the nucleus coincides with its phosphorylation and remodelling into an equatorial ring (Sohrmann *et al.*, 1996). Cells that lack Mid1/Dmf1 function form a highly disorganized and misplaced structure instead of a tight F-actin ring at the cell equator (Chang *et al.*, 1996; Sohrmann *et al.*, 1996). This phenotype is highly reminiscent of some *plo1*^{ts} mutants (Bähler *et al.*, 1998). The ability of Mid1/Dmf1 and Plo1 to interact in the two-hybrid system, and the inability of Mid1/Dmf1 to leave the nucleus in *plo1*^{ts} mutants, suggested that Plo1 may directly phosphorylate Mid1/Dmf1 to regulate ring formation (Bähler *et al.*, 1998). Strains lacking Plo1 altogether do not form a cortical F-actin ring, although it is unclear whether this is a variation on the *mid1/dmf1* mutant phenotype or a further requirement for Plo1 during an earlier stage of ring formation (Ohkura *et al.*, 1995).

Constriction of the cortical F-actin ring is co-ordinated with the generation of the septum at the end of anaphase. Cells lacking *plo1*⁺ cannot form a septum, while *plo1*⁺ overexpression in interphase drives inappropriate

separation (Ohkura *et al.*, 1995). Overproduction of the small GTP binding protein Spg1 or the protein kinase Cdc7 also induces multiple rounds of septation (Fankhauser and Simanis, 1994; Schmidt *et al.*, 1997). Spg1 activity is regulated by a bipartite GAP protein composed of the products of the *byr4⁺* and *cdc16⁺* genes (Furge *et al.*, 1998; Le Goff *et al.*, 1999). Spg1 is found on the spindle pole bodies (SPBs) throughout the cell cycle. GTP-bound Spg1 recruits Cdc7 to the SPB (Sohrmann *et al.*, 1998), which then activates a downstream network containing at least two more kinases and induces septation (Le Goff *et al.*, 1999). There is a striking similarity between this Spg1 'septum initiating network' (SIN) of *S.pombe* and the 'mitotic exit network' (MEN) of *Saccharomyces cerevisiae* (Balasubramanian *et al.*, 2000; Cerutti and Simanis, 2000). All but one of the SIN components identified to date have counterparts that function in a similar manner in the MEN. It is currently unclear whether Plo1 regulates septation by acting before, after or in parallel with the SIN.

Like other Plks, Plo1 associates with the division apparatus. It is recruited to the medial ring, SPB and spindle upon mitotic commitment, and shows a high affinity for the SPB until the onset of anaphase (Bähler *et al.*, 1998; Mulvihill *et al.*, 1999). Spindle and medial ring association is lost early in anaphase B, and the intensity of Plo1 association with the SPB diminishes considerably during the early stages of anaphase B. SPB association requires the activity of Cdc25 and Cdc2, but no SIN components. As Plo1 protein levels remain constant throughout the cell cycle, post-translational modification of Plo1 or the provision of a Plo1 docking site seems to regulate Plo1-SPB interactions (Mulvihill *et al.*, 1999).

We used an *in vitro* assay to monitor changes in Plo1-associated kinase that accompany cell cycle progression and found a mitotic peak in Plo1-associated kinase activity. Mitotic activation involves phosphorylation and is reliant upon prior activation of Cdc25 and Cdc2. We conclude with experiments suggesting that Plo1 primarily regulates septation by acting before the SIN.

Results

In vitro Plo1-associated kinase activity

Cell extracts were prepared from *cdc25-22* cells in which either a wild-type *plo1⁺* (IH1558) or *plo1.K69R* (IH1559) mutant gene had been integrated at the *leu1* locus. *plo1.K69R* carries a point mutation that is predicted to abolish kinase activity (Hanks and Hunter, 1995). In both cases, the integrated gene was tagged at its N-terminus by fusion to three copies of the haemagglutinin (HA) epitope recognized by the antibody 12CA5 (Field *et al.*, 1988), and expression was regulated by the thiamine-repressible *nmt41* promoter. The wild-type gene tagged in this way can substitute for *plo1⁺* (Mulvihill *et al.*, 1999). Expression of the kinase mutant did not arrest growth (see below). The tagged proteins accumulated to levels that were comparable to the abundance of endogenous Plo1 (see below).

Tagged, but not untagged Plo1 was precipitated when 12CA5 was used to pull down HA-Plo1 or HA-Plo1.K69R from whole-cell extracts. This shows that the tagged molecules did not associate with the untagged,

endogenous protein under these conditions. Casein kinase activity was detected in 12CA5 precipitates from mitotic cell extracts expressing HA-Plo1, but not from identical extracts of cells expressing HA-Plo1.K69R, despite the precipitation of similar quantities of HA-tagged Plo1 protein in each case (Figure 1A and B). This shows that the activity we are monitoring is dependent on Plo1 activity. HA-Plo1-associated kinase activity of extracts prepared from mitotic cells considerably exceeded that of the kinase prepared from an asynchronous culture, which, in turn, was greater than the activity detected in cells that were in the G₂ phase of the cell cycle. When the polyclonal anti-Plo1 antibody HN184 was used instead of 12CA5 for immunoprecipitation, both HA-tagged and endogenous Plo1 were pulled down. Mitotic extracts of strains expressing either HA-Plo1.K69R or HA-Plo1 had high kinase activity. The kinase activity in cells expressing HA-Plo1.K69R was due to the presence of the untagged endogenous Plo1 protein in the reaction. Immunoprecipitation with serum from an unimmunized rabbit did not precipitate any casein kinase activity (Figure 1A). These data demonstrated that a casein kinase activity is associated with Plo1.

Plo1.K69R associates with the mitotic apparatus in *plo1⁺* and *plo1Δ* cells

The generation of the *plo1.K69R* allele enabled us to ask whether the association of Plo1 with the spindle and the SPB during mitosis required Plo1 activity. We expressed N-terminally Pk-tagged *plo1⁺* or *plo1.K69R* genes in the diploid strain IH1847. Both tagged proteins showed normal localization patterns (Figure 1C and D). The lack of association between endogenous untagged and tagged Plo1 in the immunoprecipitation reactions in Figure 1A may suggest that it is unlikely that PK-Plo1.K69R is recruited to the spindle by binding to endogenous Plo1. The endogenous molecule could, however, direct spindle association of PK-Plo1.K69R by phosphorylating it or modifying a docking protein. We therefore localized PK-Plo1.K69R in *plo1.d1* cells in which the genomic *plo1⁺* open reading frame has been replaced by the *ura4⁺* gene (hence referred to as *plo1Δ*). As *plo1⁺* is essential, the *plo1Δ* deletion allele was propagated in a heterozygous *plo1Δ/plo1⁺* diploid and the phenotype analysed in germinating haploid *plo1Δ* spores. The heterogeneous phenotypes of germinating *plo1Δ* cells suggest that some Plo1 is carried over from the diploid parent into the spores and that a phenotype only emerges upon exhaustion of this parental protein (Ohkura *et al.*, 1995). We therefore generated a parental diploid in which the *plo1⁺* gene was fused to green fluorescent protein (GFP) (Bähler *et al.*, 1998). This allowed us to use GFP antibodies to monitor the levels of the 'parental Plo1.GFP^{S65T} protein' at the same time as localizing PK-Plo1.K69R with a monoclonal antibody that recognizes the PK epitope.

Germination of a spore mix containing diploid *plo1Δ/plo1.GFP^{S65T}* and haploid *plo1Δ* spores in media lacking leucine and uracil selected for growth of cells containing both the plasmid-borne *plo1.NPKK69R* and the genomic *plo1Δ* alleles. Anti-GFP and anti-PK immunofluorescence signals were simultaneously seen on SPBs in dividing cells (Figure 1E). In contrast, many apparently interphase cells in the same preparation had strong PK but no anti-GFP

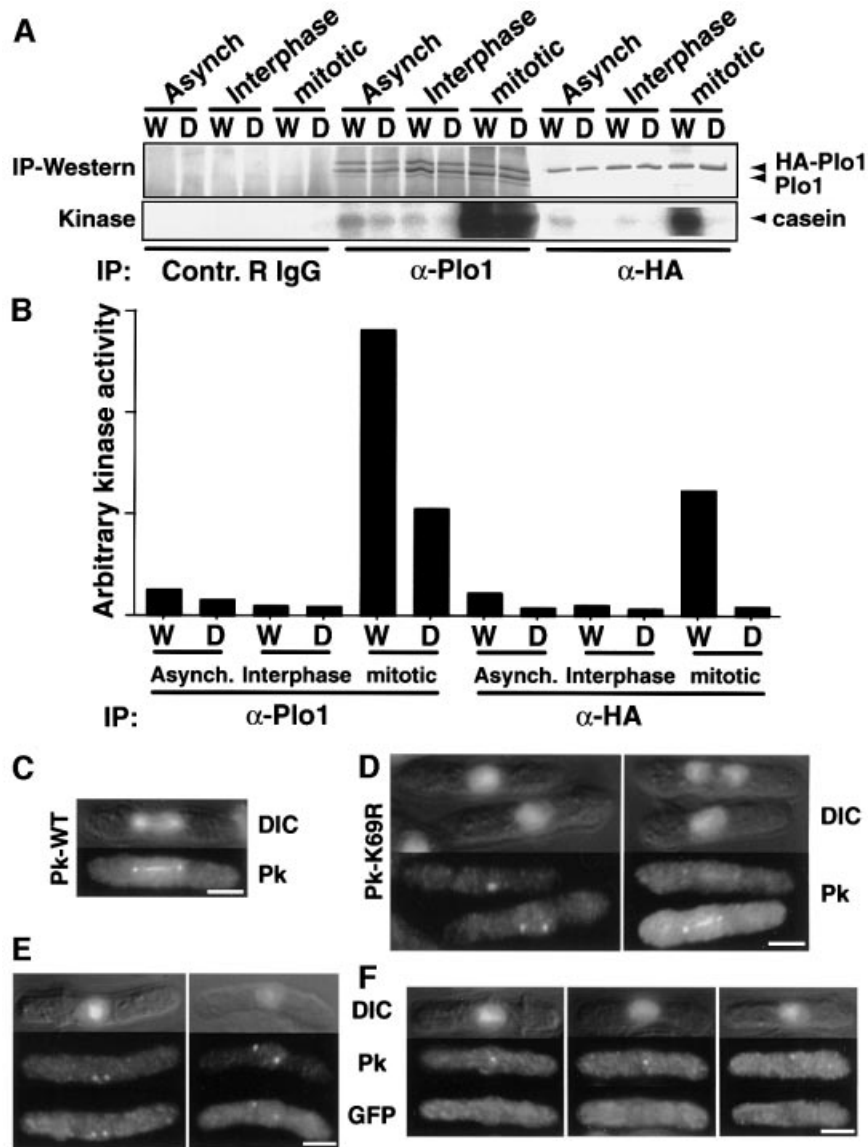


Fig. 1. Plo1.K69R has compromised mitotic kinase activity but still associates with the mitotic apparatus. (A) Cell extracts were prepared from *cdc25.22* mutants harbouring either HA-tagged wild-type *plo1*⁺ (strain IH1558, indicated by W) or mutant *plo1.K69R* (strain IH1559, indicated by D) in pINT541 integrated at the *leu1* locus. Cells were induced to express HA-Plo1 (W or D) for 17 h (Asynch, asynchronous cells) and then shifted to the restrictive temperature of 36°C for 4 h to arrest cell cycle progression at the G₂/M boundary (Interphase). A portion of the arrested culture was returned to 25°C for 40 min to produce a population of mitotic cells (mitotic). Non-specific rabbit IgG, the polyclonal anti-Plo1 antibody HN184 or the 12CA5 monoclonal antibody was used to precipitate the different versions of Plo1. Kinase assay reactions of the precipitates were blotted onto nitrocellulose membranes, monitored for ³²P labelling of casein (kinase) and probed with HN184 (IP-western) to determine Plo1 levels in each reaction. (B) The specific activity of the kinase in each condition was plotted after calculating the kinase activity per unit protein. (C–F) Immunofluorescence images. Each block represents different images of the same cells. The staining in each particular panel is written at its side. DIC indicates combined DAPI/DIC images. Anti-PK immunofluorescence staining of IH1857 (C) and IH1858 (D). (E and F) Combined anti-GFP and anti-PK immunofluorescence staining of spores arising from mating IH1857 with IH1845. Bar, 2.5 μm.

SPB staining (Figure 1F). Thus, the affinity of PK-Plo1K69R for the SPB did not appear to be affected by reducing the abundance of the wild-type protein below our detection limits.

Plo1-associated in vitro kinase activity peaks during mitosis

Before examining the cell cycle regulation of Plo1 activity in great detail, it was necessary to determine the similarity between the profile of kinase activity seen for HA-Plo1 with 12CA5 antibodies and that seen when the endogenous

protein was precipitated by HN184. HA-tagged Plo1-associated activity was assessed in a culture of otherwise wild-type cells (IH1314) that had been synchronized with respect to cell cycle progression by centrifugal elutriation. To minimize any adverse consequences of protein overproduction upon the validity of the kinase assay data in this particular experiment, *plo1.NHA* expression was manipulated so that the levels of the tagged protein approached and did not exceed those of the endogenous wild-type protein at the end of the experiment. The panels in Figure 2B show two read-outs of the same gel. The upper

panel shows phosphorylation of casein, whilst the blots in the lower panel show the amount of Plo1 in each kinase reaction. As with all figures in this study, the plotted kinase activity was calculated by dividing the measured kinase activity by the relative amount of Plo1 precipitated. A sharp peak in Plo1-associated kinase activity was seen during mitosis in both cell cycles (Figure 2A and B).

To determine whether the kinase activity associated with the endogenous protein peaked similarly in mitosis, HN184 was used to precipitate Plo1 from a size-selected wild-type culture (IH365). After correcting for the levels of Plo1 in each assay, the oscillating activity profile was indistinguishable from that with the HA-tagged protein (Figure 2D and E), indicating that the polyclonal antibody gives an accurate measure of Plo1-associated kinase activity. As we have previously shown for the entire population of Plo1 (Mulvihill *et al.*, 1999), soluble Plo1 levels remained constant throughout the cell cycle (Figure 2C and F). Thus, fluctuations in Plo1 activity can not be due to fluctuations in Plo1 abundance. We therefore asked whether the phosphorylation status of Plo1 affected its activity. Active, mitotic Plo1 was incubated with λ phosphatase before its activity was monitored in a kinase assay. Phosphatase treatment virtually abolished Plo1 activity and condensed the broad Plo1 band, which is seen upon running the kinase reactions on large SDS-PAGE gels, to a tighter, more compact band (Figure 2G, compare lanes 3 and 4 of IP-western). Kinase activity was not inhibited when phosphatase inhibitors were included in the phosphatase treatment buffer (Figure 2G). These data show that Plo1-associated kinase activity peaks during mitosis and that this activation may arise from mitotic phosphorylation.

Plo1 is activated after MPF

The kinase activity plots in Figure 2 suggested that Plo1 activity rises early in mitosis. Given the debate about the role played by Plks in MPF activation, we asked whether Plo1 was activated before or after MPF. We used *cdc2.33* mutant cells to address this point because they have temperature-sensitive p34^{cdc2} H1-kinase activity (Moreno *et al.*, 1989). Early G₂ *cdc2-33* cells were collected at 25°C and half of the culture was immediately shifted to 36°C to inactivate p34^{cdc2} function. The other half was kept at 25°C. Plo1-associated activity peaked before septation in the 25°C culture (Figure 3A and B). In contrast, no rise in activity was seen in cells arrested at the *cdc2* execution point by incubation at 36°C (Figure 3A and B). This dependency of Plo1-associated activity upon MPF activation was confirmed by cooling part of the culture that had been incubated at 36°C back down to 25°C, after 150 min at the restrictive temperature, to restore p34^{cdc2.33} function (time 0 in Figure 3C and D). Plo1-associated activity rose dramatically after the return to 25°C (Figure 3C and D; the degree of activation is clearly apparent when one considers that the 0 time point in Figure 3C is between the 140 and 160 min time points in Figure 3B, 36°C).

Given that phosphorylation appears to regulate Plo1-associated kinase activity (Figure 2G) and the *plo1*⁺ nucleotide sequence predicts a single p34^{cdc2} consensus phosphorylation site (Nigg, 1993), we mutated this threonine residue to alanine to ask whether the dependency

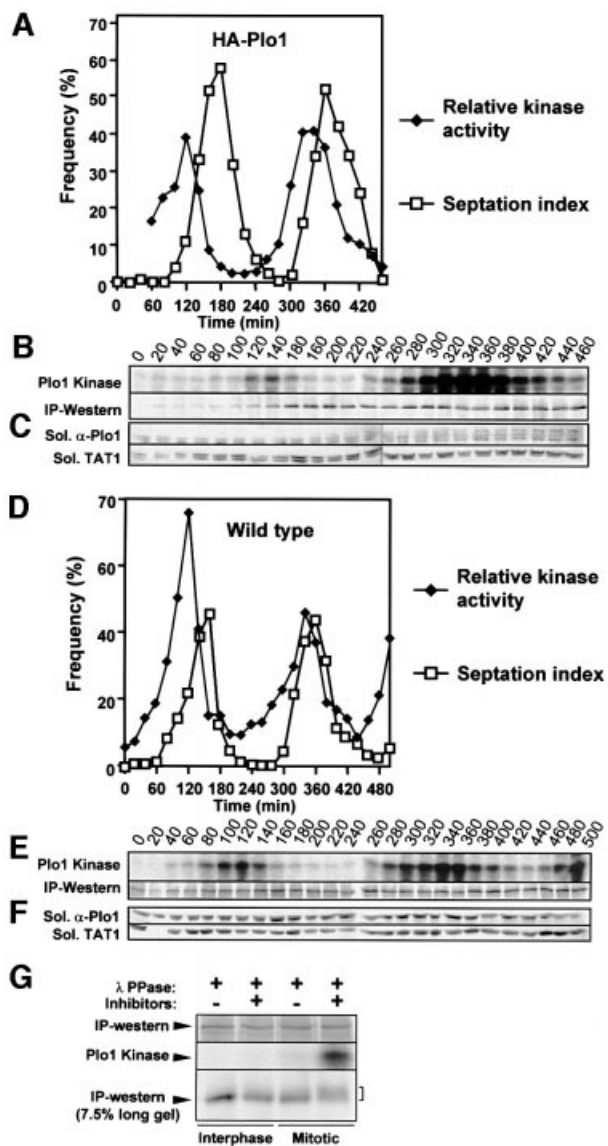


Fig. 2. Plo1-associated kinase activity peaks in mitosis. (A–C) Otherwise wild-type cells (IH1314) were induced to express HA-Plo1 for 13 h and then synchronized with respect to cell cycle progression by elutriant centrifugation size selection at time 0. (A) An aliquot was taken at each time point and processed for a quantitative kinase assay using 12CA5 as in Figure 1. (B) The kinase assay (Plo1 kinase) and HN184 immunoblot of the reaction (IP-western) were used to calculate (A). (C) A parallel aliquot was used to determine the levels of Plo1 and tubulin in the soluble fraction. HA-Plo1 initially appeared at ~60 min and continued to accumulate as the experiment progressed. (D–F) The wild-type strain (IH365) was synchronized and processed as for IH1314 in (A–C) using HN184 to pull down Plo1. Soluble Plo1 protein levels were normalized for variation between samples by determining the ratio of signals from anti-Plo1 and anti- α -tubulin (TAT1) from the same western blot. Three independent sets of blotting data from two independent experiments, each covering two complete cell cycles, showed that levels of soluble Plo1 did not vary during cell cycle progression (data not shown). (G) Interphase or mitotic Plo1 immunocomplexes were prepared from *cdc25.22* cultures as for Figure 1A and treated with λ phosphatase with or without inhibitors before processing in a kinase assay (upper panels) and running on a large 7.5% gel (bottom panel).

of Plo1 activity upon MPF function was due to direct phosphorylation of this site by MPF. The *plo1.T14A* mutant was integrated into the genome at the *leu1*⁺ locus

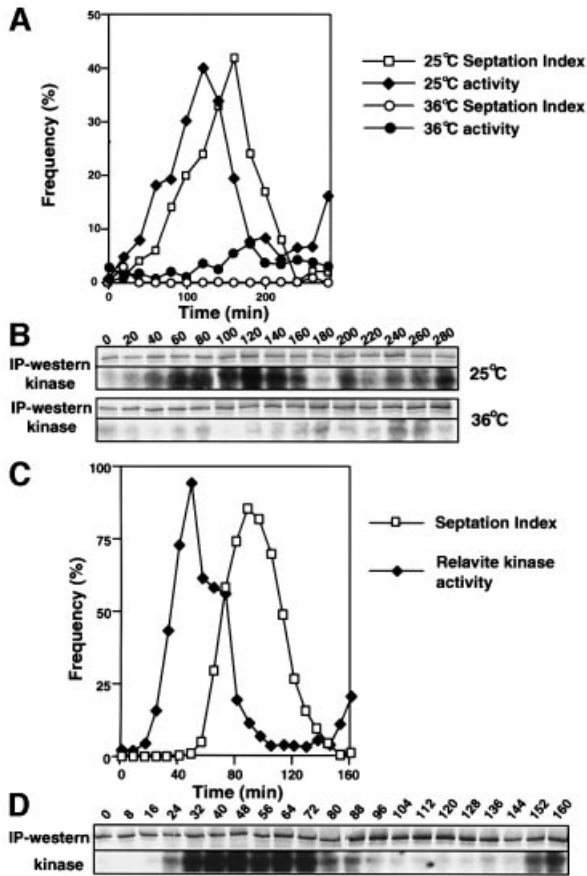


Fig. 3. Plo1-associated kinase activation requires MPF activity. A *cdc2.33* culture that had been grown at 25°C was synchronized with respect to cell cycle progression by elutriant centrifugation before being split into two separate cultures. One was immediately incubated at 36°C whilst the other was kept at 25°C. (A and B) Samples from the 36 and 25°C cultures were processed to assess Plo1-associated kinase activity. Before plotting the data for the figure, the Plo1 kinase assay at each temperature (B) was adjusted according to the amount of Plo1 in the kinase assay. (C and D) At 150 min, half of the 36°C culture was rapidly cooled to 25°C and processed as for (A) and (B).

fused to three HA epitope tags at its N-terminus and regulated by the *nmt41* promoter. The kinase activity of 12CA5 precipitates from the strain expressing HA-Plo1.T14A was indistinguishable from that of the strain expressing HA-Plo1 (Figure 4). As *plo1.T14A* can suppress the *plo1Δ* growth defect (not shown), we conclude that MPF is unlikely to stimulate Plo1-associated kinase by phosphorylating threonine 14.

Plo1 activation and the timing of mitotic events

Established procedures were used to generate cultures that have highly synchronous mitoses in order to relate changes in Plo1-associated kinase activity to distinct mitotic events more precisely. Cells were synchronized by size selection, followed by temporary cell cycle arrest at the *cdc25* execution point. Because Cdc25 triggers mitosis by removing an inhibitory phosphate from p34^{*cdc2*}, cells are unable to divide while Cdc25 activity is missing. Restoring Cdc25 activity results in rapid commitment to mitosis. Using elutriation to select a G₂ population at the start of the experiment and limiting the arrest at the G₂/M

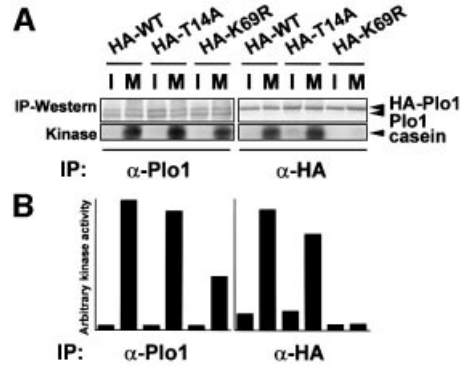


Fig. 4. Mutation of the p34^{*cdc2*} consensus phosphorylation site in Plo1 does not block activation of *in vitro* kinase activity. (A) Cell extracts were prepared as for Figure 1 from *cdc25.22* mutants harbouring HA-tagged wild-type *plo1*⁺ (strain IH1558) (HA-WT), HA-tagged *plo1.K69R* (strain IH1559) (HA-K69R) or HA-tagged *plo1.T14A* (strain IH1724) (HA-T14A). I, interphase extract; M, mitotic extract. In each strain, the tagged gene was integrated at the *leu1* locus under the control of the *nmt41* promoter using pINT541. Kinase assays were performed as for Figure 1. (B) The kinase activity per unit protein for each assay.

boundary to 20 min results in mitotic synchrony of ~70% (Hagan and Yanagida, 1997).

Early G₂ temperature-sensitive *cdc25.22* cells were cultured at 36°C for 160 min, before the return to 25°C. Samples were then taken every 6 min to monitor Plo1 activity (Figure 5C), Plo1 and Cdc13 (cyclin B) protein levels (Figure 5D), and histone H1 kinase activity (Figure 5E). Plo1-associated activity was slightly higher 24 min after release than at the surrounding points, suggesting that there may be an early activation of Plo1, which is distinct from the following, major phase of Plo1-associated kinase activity (Figure 5A, C and E). The start of the second phase of Plo1 activation at 42 min coincided with the initiation of Cdc13 destruction. Cdc13 destruction accelerated at 84 min and continued at this rate until 120 min. The cessation of Cdc13 destruction coincided precisely with the return of Plo1-associated activity to its basal, starting level. The reduction in Plo1 affinity for the SPB and its loss from the spindle also coincided precisely with the return of Plo1-associated kinase activity to its starting, basal level (Figure 6), suggesting that this marks a major, early anaphase control point in mitotic progression.

Plo1 activation and SIN activity

Overproduction of the small G protein Spg1 or mutation of its bipartite Cdc16/Byr4 GAP complex activates the SIN and drives septation in interphase cells (Minet *et al.*, 1979; Fankhauser *et al.*, 1993; Song *et al.*, 1996; Schmidt *et al.*, 1997). As the septation phenotypes associated with manipulation of *plo1*⁺ levels suggest an intimate relationship between Plo1 and SIN function, we asked whether Plo1-associated kinase activity might be regulated by the SIN. Specifically, Plo1-associated activity was followed as the SIN was activated in interphase cells (Cerutti and Simanis, 1999).

The inhibitor of DNA synthesis hydroxyurea was added to a cell-cycle-synchronized *cdc16-116* culture 60 min after these early G₂ cells had been isolated by centrifugal elutriation. Because these cells were maintained at the

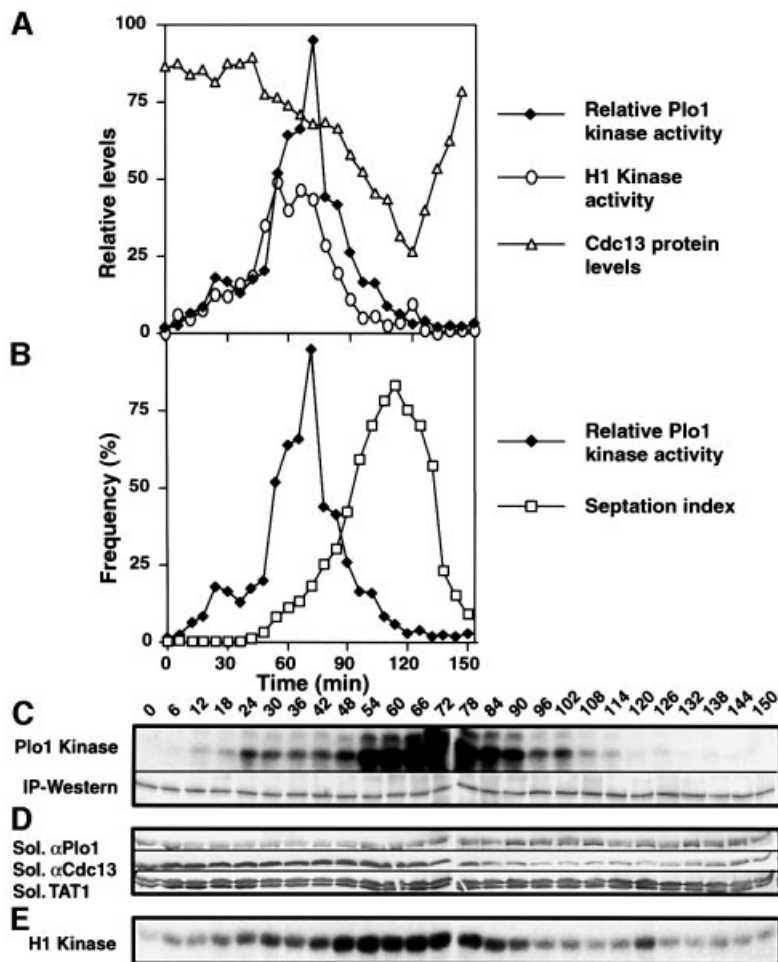


Fig. 5. Plol-associated kinase activity in a highly synchronous mitosis. A *cdc25.22* culture was synchronized by centrifugal elutriation and the early G₂ cells were shifted to 36°C for 160 min before returning to 25°C at time 0. Samples were processed to assay Plol (A–C) and H1 kinase activity (A and E) and blotted to monitor Plol and Cdc13 levels in soluble extracts (A and D) as for Figure 2. The raw data of the Plol-associated kinase assay [(C), Plol-associated kinase] were normalized for the amount of Plol protein in each reaction [(C), IP-western] and are presented in (A) and (B). Plol and Cdc13 levels were calculated by the ratio of the signal from these polyclonal antibodies to that arising from blotting with the anti-tubulin antibody TAT1. Each western blot was performed on a strip cut from the same master blot. These blots were only performed once, but similar data were obtained in parallel experiments (Figure 6).

permissive temperature of 25°C, they went through one mitosis before arresting cell cycle progression in the next S phase. Once the cells were arrested in S phase, the temperature of this culture was shifted to 36°C to inactivate Cdc16 and so activate Spg1 and drive septation (Figure 7A). Although Plol associated with the SPB as a peak of Plol-associated kinase activity preceded the first peak of septation following synchronization, no SPB association (data not shown) or Plol-associated kinase activity accompanied the prolific septation arising from Cdc16 inactivation in G₁/S phase (Figure 7A and B).

We next asked whether the activation of Plol-associated kinase activity was affected when SIN function was abolished. To ensure maximal SIN inactivation, we used a strain containing *ts*⁻ mutations in both the G protein Spg1 and its effector kinase Cdc7. The kinetics of kinase activation in *spg1.B8 cdc7.A20* mutant cells (Figure 7C and D) was comparable to that seen in wild-type cells (Figure 2D). The reduction in kinase activity as cells left mitosis was not as dramatic as that seen in wild-type backgrounds, suggesting that SIN activity may down-

regulate Plol during mitotic exit. The main conclusions we draw from these experiments are that SIN activation in interphase cells did not stimulate Plol-associated kinase activity and that mitotic activation of the kinase was not affected by blocking SIN function.

Activation of Spg1 overcomes the *sin*⁻ phenotype of *plol.ts4*

The ability of inappropriate Spg1 activation to drive septation without stimulating Plol-associated kinase activity suggested that an active SIN may not require Plol to drive septation. If this were the case, activation of the SIN alone should be sufficient to bypass an actin ring activation defect of a *plol* mutant. We therefore activated the SIN with a *cdc16.116* mutation in a *plol.ts4* background. At 36°C, *plol.ts4* cells mimicked a *sin*⁻ defect because they formed a highly misshapen medial F-actin ring but rarely used it to generate a septum (Figure 8A, B and D). If Plol acted before the SIN, the *plol.ts4* mutation should not perturb the ability of the *cdc16.116* mutation to drive

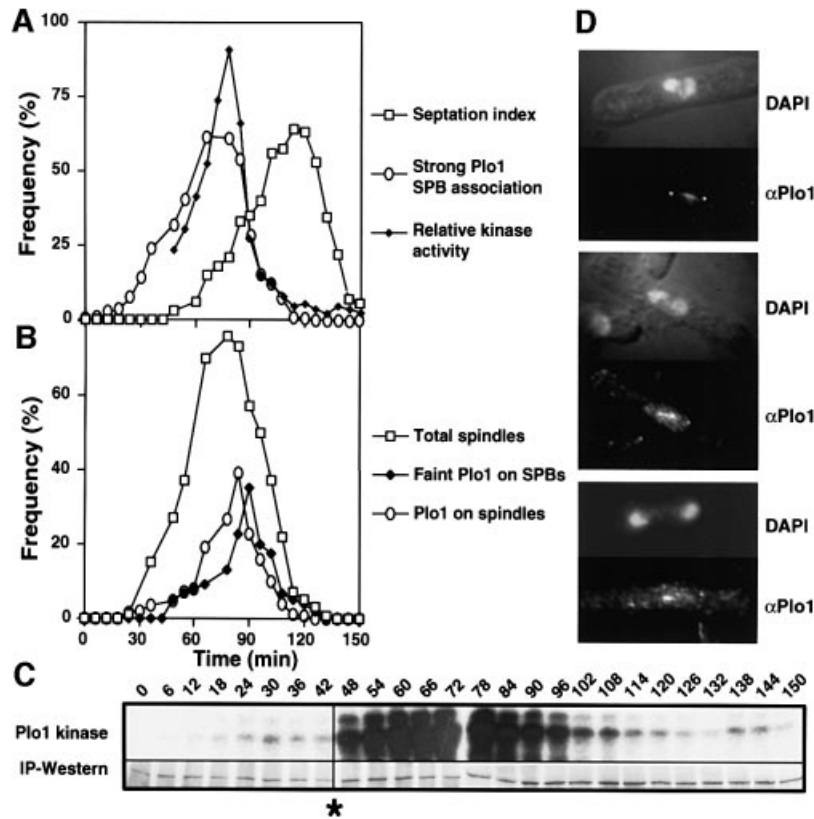


Fig. 6. Maximal Plo1-associated kinase activity coincides with maximal SPB association. The experiment described in the legend for Figure 5 was repeated and one sample was processed for anti-Plo1 (HN1814) immunofluorescence microscopy. (A and B) Graphical representation of the indicated features of the culture. (C) There was a uniform error, which reduced the activity of the first eight kinase assays; however, the trend seen in Figure 5 of an early peak is still apparent. (D) Anti-Plo1 staining 90 min after return to 25°C.

septation. If, however, Plo1 acts after, or is a part of the SIN, the *plo1.ts4* mutation should compromise Cdc16-induced septation.

Asynchronous single *plo1.ts4* and *cdc16.116*, and double *plo1.ts4 cdc16.116* cultures were grown to early log phase at 25°C before shifting the temperature to 36°C. The septation profile of the *plo1.ts4 cdc16.116* mutant mirrored that of the single *cdc16.116* mutant (Figure 8B and D). In contrast, *cdc16.116* could not compensate for the *ts*⁻ septation deficiency of the *sin*⁻ mutant *sid2.250* (Balasubramanian *et al.*, 1998) (Figure 8C and E). Thus, independent SIN activation bypassed the septation defect of *plo1.ts4*.

***plo1*⁺ overexpression can only induce septation when the SIN is functional**

While the data suggested that Plo1 is unlikely to act after Spg1 in the regulation of septation, Plo1 is required to use the actin ring for septation (Figure 8A and D). Thus, Plo1 either acts in concert with the SIN and is rendered redundant by strong activation of the SIN, or acts before the SIN in a progressive decision to septate. To differentiate between these possibilities, we asked whether an active SIN was required for the prolific septation that follows *plo1*⁺ overexpression (Ohkura *et al.*, 1995).

plo1⁺ expression was induced in an *spg1.B8 cdc7.A20* double mutant or an isogenic wild-type culture for 15 h at 25°C. The culture was then split into two, and one half was

maintained at 25°C while the other was incubated at 36°C. The accumulation of septa following *plo1*⁺ overexpression was unaffected by the *spg1.B8 cdc7.A20* mutations at 25°C (Figure 9A and C). In contrast, inactivation of the SIN at 36°C severely compromised the ability of *plo1*⁺ overexpression to induce septation (Figure 9B and D). We conclude that *plo1*⁺ overexpression cannot overcome a defective SIN to induce septation and that Plo1 overproduction predominantly induces septation by activating the SIN.

Discussion

We have used an *in vitro* kinase assay in cultures from wild-type and mutant strains to show that Plo1-associated kinase activity is very low in interphase cells and peaks during mitosis. This mitotic activation is likely to involve phosphorylation as the kinase activity was essentially abolished by phosphatase treatment. The activity of the kinase may not be required for its association with the SPB.

***Polo* kinase and mitotic commitment**

Microinjection of antibodies to Plk into untransformed mammalian cells blocks mitotic commitment (Lane and Nigg, 1996), suggesting that Plks play a key role in regulating the onset of mitosis. The demonstration that *Xenopus* Plk, Plx1, binds, phosphorylates and activates

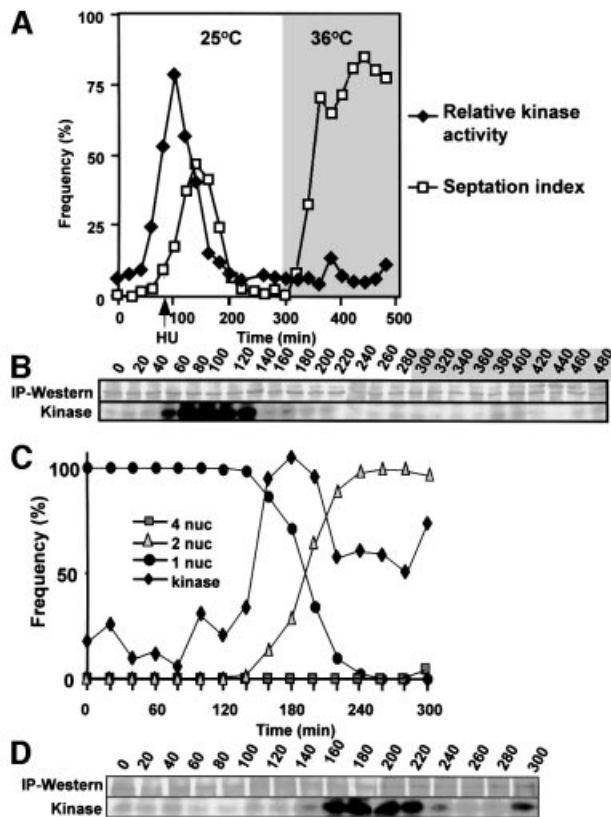


Fig. 7. The dependency of Pl_o1 activation upon SIN function. Pl_o1-associated kinase was assayed and the specific activity was determined as for Figure 1. Septation indices and the number of nuclei per cell were both scored after DAPI/calcofluor staining. (A and B) *G2 cdc16-116* cells were isolated by centrifugal elutriation at time 0 and cultured at 25°C. Sixty minutes later, hydroxyurea (HU) was added to the culture (arrow in A). The temperature was shifted to 36°C at 290 min. The grey shading indicates the period at 36°C. (A) Quantification of the kinase data in (B) and the septation index. (C and D) IH1751 (*spg1.B8 cdc7.A20*) cells were grown at 25°C before small *G2* cells were isolated by centrifugal elutriation at time 0 and half the culture was incubated at 36°C and the other at 25°C. The profile and extent of kinase activation at 36 and 25°C were very similar (not shown).

Cdc25C (Kumagai and Dunphy, 1996) led to the proposal that Plx1 may act as the initial trigger to activate MPF via activation of Cdc25 (Kumagai and Dunphy, 1996; Qian *et al.*, 1999). An alternative interpretation has been that Plx1 functions during the positive feedback loop to amplify this initial trigger MPF kinase to precipitate commitment to mitosis (Abrieu *et al.*, 1998; Karaiskou *et al.*, 1999). Two sets of data support the latter role. First, depletion of Plx1 from *Xenopus* oocytes delays mitotic commitment in a way that can be overcome by addition of excess Cdc25C (Qian *et al.*, 1998a). Secondly, Plx1 activity is not required in *Xenopus* oocyte extracts for mitotic commitment *per se*; rather, its presence ensures abrupt non-linear kinetics of MPF activation (Karaiskou *et al.*, 1999).

The lack of Pl_o1-associated kinase activity in cell-cycle-arrested *cdc2.33* and *cdc25.22* mutants argues against a role for Pl_o1 as the initial, MPF-activating, trigger kinase in *S.pombe*. If Pl_o1 were the primary trigger that initiates MPF activation, we would have expected Pl_o1 to be already active at these mutant

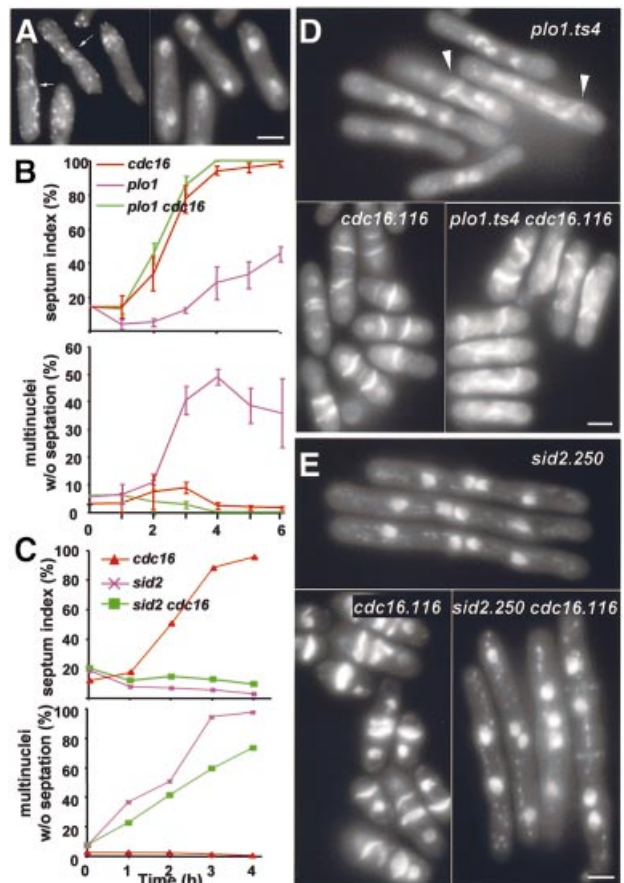


Fig. 8. SIN activation suppresses the *sin⁻* phenotype of *plo1.ts4* but not *sid2.250* mutants. (A) An asynchronous culture of *plo1.ts4* cells was shifted to 36°C for 3 h and stained with TRITC-phalloidin. All anaphase cells ($n = 140$) contained an F-actin ring, although it was generally misshapen or misplaced (left panel, arrows). (D) DAPI/calcofluor staining showed that whilst some cells in the population use the defective F-actin rings to generate septa (arrowheads), most do not and go through multiple cell cycles without cytokinesis. (B and C) Asynchronous early log phase mutant cultures were shifted from 25 to 36°C at $t = 0$ and stained with DAPI/calcofluor to score the septation index (upper panel) and frequency of cells with multiple nuclei and no septa (lower panel). (D and E) Representative micrographs of calcofluor staining of each culture 5 h (D) or 4 h (E) after the shift to 36°C. Bar, 2.5 μ m.

arrest points. The dependency of Cdc25 activation upon prior activation of p34^{cdc2} in *S.pombe* (Kovelman and Russell, 1996) suggests that, like *Xenopus*, mitotic commitment in *S.pombe* is regulated by a positive feedback loop. Thus, if Pl_o1 does play a role in mitotic commitment in *S.pombe*, our data favour a role in this positive feedback loop (Figure 10A).

Mutation of the sole MPF consensus phosphorylation site in Pl_o1 did not block mitotic activation of Pl_o1, suggesting that MPF is unlikely to activate Pl_o1 by direct phosphorylation. Phosphorylation of Pl_o1 is, however, required to stimulate/maintain mitotic activity as treatment of mitotic Pl_o1 precipitates with λ phosphatase abolished kinase activity. The Ste20-related kinase xPlkk has been identified through its ability to phosphorylate and activate Plx1 (Qian *et al.*, 1998b). Whilst several Ste20-related kinases can be found in many systems, including *S.pombe* (data not shown), it is currently unclear how universal

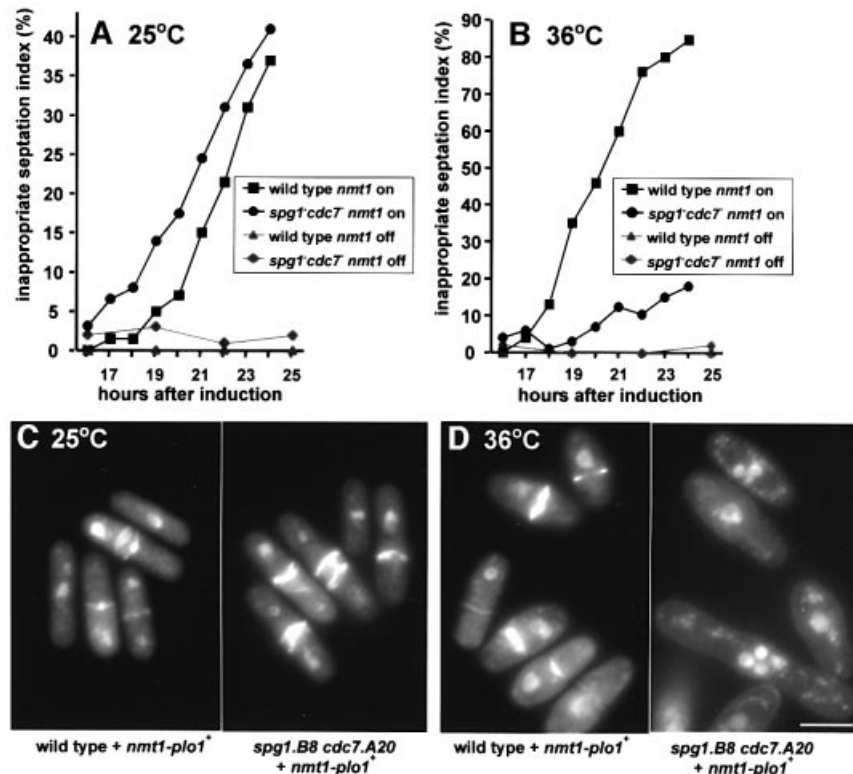


Fig. 9. SIN function is required for the induction of septation by *plo1*⁺ overexpression. *spg1.B8 cdc7.A20 pHN204* and an isogenic *spg1⁺cdc7⁺ pHN204* strain were grown to early log phase in EMM2 + thiamine at 25°C, washed three times in EMM2 – thiamine, split into two, and one half was resuspended in EMM2 + thiamine while the other half was resuspended in EMM2 – thiamine to give early log phase cultures 15 h later. Each of the cultures was then further divided; one half was incubated at 25°C, the other at 36°C. DAPI/calcofluor staining (lower panels) identified inappropriate septation events (cells with multiple septa, or a single septum and a single nucleus). (A and C) 25°C; (B and D) 36°C. Although the double mutant displayed a minor septation defect at 25°C, the kinetics of septation following *plo1*⁺ induction was very similar to that in a wild-type background. All micrographs were captured and printed at the same scale. The widening of the cell girth during *plo1*⁺ overexpression may indicate that excess Plo1 inhibits the re-establishment of tip growth following mitosis (Mitchison and Nurse, 1985). This is consistent with genetic interactions between *plo1ts* and *pom1* mutants (Bähler *et al.*, 1998). Bar, 2.5 μm.

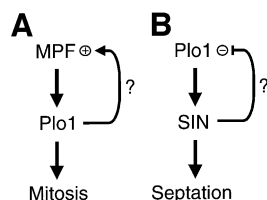


Fig. 10. Plo1 function during mitotic commitment and exit. Plo1 activation during mitotic commitment requires MPF activity. It is currently unclear whether Plo1 functions in the positive feedback loop that controls commitment to mitosis in *S.pombe* (A). Plo1 acts before the SIN. Some data suggest that the SIN may down-regulate Plo1 activity (B).

regulation by xPlkk-related kinases will be and whether it applies to mitotic as well as meiotic systems.

In addition to promoting Plo1 activity by triggering a phosphorylation cascade, MPF may activate Plo1 by regulating partner or docking proteins. It may be significant in this respect that Plo1 is prematurely recruited to the interphase SPB in cells that contain a mutation in the SPB component *Stf1/Cut12*, which confers the ability to bypass the requirement for Cdc25 function (Bridge *et al.*, 1998; Mulvihill *et al.*, 1999).

Plo1 activity and mitotic progression

The profile of Plo1 activity in highly synchronous mitotic cultures suggested that there may be distinct phases in Plo1 activation or that discrete subpopulations execute distinct roles at different stages of mitosis. It may be significant that Cdc13 degradation started just after the first phase of Plo1 activation, and that the rate of Cdc13 destruction accelerated when Plo1-associated kinase activity peaked at metaphase. A similar biphasic breakdown of cyclin in *S.cerevisiae* has been attributed to successive activation of Cdc20 and Cdh1 (Yeong *et al.*, 2000). The relationship between Plo1 activity and APC function awaits further analysis, but it is clear that, unlike mammalian Plk (Golsteyn *et al.*, 1995; Hamanaka *et al.*, 1995; Lee *et al.*, 1995) and budding yeast Cdc5p (Charles *et al.*, 1998; Shirayama *et al.*, 1998), Plo1 is not degraded towards the end of mitosis (Mulvihill *et al.*, 1998).

Plo1 acts before SIN in the regulation of septation

The central role played by the small GTP protein Spg1 in controlling septation has been effectively demonstrated by the prolific septation in interphase cells arising from Spg1 overproduction, or inactivation of its Cdc16/Byr4 GAP complex (Minet *et al.*, 1979; Fankhauser *et al.*, 1993; Fankhauser and Simanis, 1994; Song *et al.*, 1996; Schmidt

Table I. Strains used in this study

Strain number	Genotype	Source
IH164	<i>h⁻ cdc2.33</i>	Nurse <i>et al.</i> (1976)
IH365	<i>h⁻ ura4.d18 leu1.32</i>	laboratory stock
IH403	<i>h⁺/h⁻ ade6-M210/ade6-M216 plo1.d1/plo1 leu1.32/leu1.32 ura4.d18/ura4.d18</i>	Ohkura <i>et al.</i> (1995)
IH634	<i>h⁻ cdc25.22 ura4.d18 leu1.32 his2</i>	Nurse <i>et al.</i> (1976)
IH635	<i>h⁻ cdc25.22 ura4.d18</i>	Nurse <i>et al.</i> (1976)
IH1297	<i>h⁻ cdc16.116 ura4.d18</i>	Nurse <i>et al.</i> (1976)
IH1314	<i>h⁻ leu1::pint5(ura4⁺)- plo1.NHA ura4.d18</i>	Mulvihill <i>et al.</i> (1999)
IH1354	<i>h⁻ leu1::pint5(ura4⁺)- plo1.NHAK69R ura4.d18</i>	this study
IH1558	<i>h⁻ cdc25.22 leu1::pint5(ura4⁺)- plo1.NHA ura4.d18</i>	this study
IH1559	<i>h⁻ cdc25.22 leu1::pint5(ura4⁺)- plo1.NHAK69R ura4.d18</i>	this study
IH1720	<i>h⁺ leu1::pint5(ura4⁺)- plo1.NHAT14A ade6.M216 ura4.d18</i>	this study
IH1724	<i>h⁺ cdc25.22 leu1::pint5(ura4⁺)- plo1.NHAT14A ura4.d18</i>	this study
IH1740	<i>h⁻ cdc7.A20 spg1.B8 leu1.32 ura4.d18</i>	this study
IH1750	<i>h⁻ leu1.32 ura4.d18 pHN204</i>	this study
IH1751	<i>h⁻ cdc7.A20 spg1.B8 leu1.32 ura4.d18 pHN204</i>	this study
IH1755	<i>h⁻ plo1.GFP^{S65T}</i>	Bähler <i>et al.</i> (1999)
IH1829	<i>h⁻ cdc16.116 sid2.250 ura4.d18 ade6-M216</i>	this study
IH1830	<i>h⁺ cdc16.116 ura4.d18 leu1.32</i>	this study
IH1831	<i>h⁻ sid2.250 ura4.d18</i>	Balasubramanian <i>et al.</i> (1998)
IH1845	<i>h⁻ ade6-M216 leu1.32 plo1.GFP^{S65T} ura4.d18</i>	this study
IH1846	<i>h⁻ ade6-M210 leu1.32 plo1.d1 ura4.d18 p41plo1.NHA</i>	this study
IH1847	<i>h⁺/h⁺ ade6-M210/ade6-M216 plo1.d1/plo1.GFP^{S65T} leu1.32/leu1.32 ura4.d18/ura4.d18</i>	this study
IH1857	<i>h⁺/h⁺ ade6-M210/ade6-M216 plo1.d1/plo1.GFP^{S65T} leu1.32/leu1.32 ura4.d18/ura4.d18 p41plo1.NPK</i>	this study
IH1858	<i>h⁺/h⁺ ade6-M210/ade6-M216 plo1.d1/plo1.GFP^{S65T} leu1.32/leu1.32 ura4.d18/ura4.d18 p41plo1.NPKK69R</i>	this study
IH1859	<i>h⁺/h⁺ ade6-M210/ade6-M216 plo1.d1/plo1.GFP^{S65T} leu1.32/leu1.32 ura4.d18/ura4.d18 pREP81</i>	this study

et al., 1997). *Plo1* is also required for septum formation and can similarly drive septation in interphase cells when overproduced (Ohkura *et al.*, 1995).

A number of observations suggested that the major role for *Plo1* in regulating septation is executed before the SIN. First, inappropriate SIN activation in interphase cells did not stimulate *Plo1*-associated kinase activity and mitotic activation of the kinase was not affected by blocking SIN function. Thus, *Plo1*'s role in regulating septation is unlikely to lie downstream of the SIN. Secondly, *Spg1* activation could induce septation in cells containing a *plo1.ts* mutation that compromised the use of the F-actin ring for septation. Finally, a functional SIN was required for the prolific septation following *Plo1* overproduction. These data could suggest that *Plo1* acts in concert with the SIN to induce septation, but that SIN activation can override the *Plo1* requirement. Alternatively, *Plo1* could induce septation by acting before the SIN and so be required for SIN activation. The recruitment of *Cdc7* to the interphase SPB upon *Plo1* overproduction (Mulvihill *et al.*, 1999) favours the latter model and places *Plo1* above the SIN in the regulation of septation (Figure 10B). Positioning *Plo1* before the SIN also explains why no *plo1* mutants were found among 514 *sin⁻* mutants in a screen for suppressors of a *cdc16Δ* strain and why a screen for mutants that required elevated levels of *Plo1* for survival identified multiple *sin* genes (V.Simanis, personal communication; Cullen *et al.*, 2000). It is now important to establish whether *Plo1* directly phosphorylates a component of the *Spg1* switch, or whether the effect is indirect, for example, by forcing the APC to prematurely target the destruction of a SIN inhibitor. It will also be important to study the feedback between the SIN and *Plo1*.

Inactivation of *Plo1*-associated kinase activity during mitotic exit was less dramatic in *spg1.B8 cdc7.A20* than in wild-type cells. Similarly, the affinity of *Plo1* for the post-mitotic SPB is elevated in *cdc7.A20* mutants (Mulvihill *et al.*, 1999). A simple explanation for these observations is that the SIN may down-regulate *Plo1* during mitotic exit (Figure 10).

Materials and methods

Cell culture and genetic manipulations

Schizosaccharomyces pombe cells were grown in EMM2 (Moreno *et al.*, 1991). EMM2 was supplemented with 4 μM thiamine to repress genes regulated by the *nm1* promoter (Basi *et al.*, 1993; Maundrell, 1993). Synchronous cultures were generated by centrifugal elutriation (Mulvihill *et al.*, 1999).

Strains used are listed in Table I. The QuikChange site-directed mutagenesis kit (Stratagene) was used with the appropriate oligonucleotides and p41plo1.NHA (Mulvihill *et al.*, 1999) as the substrate to mutate *Lys69* to arginine (*plo1.NHAK69R*) and so create p41plo1.NHAK69R. *plo1.NHAK69R* was cloned into pINT541 and the construct was integrated in the genome at the *leu1* locus to create IH1354. The same approach was used to generate IH1720 in which the *plo1.NHAT14A* mutant gene was expressed from the *leu1* locus under the control of the same, intermediate strength *nm1* promoter. p41plo1.NPK and p41plo1.NPKK69R were created by replacing the *AgeI-NdeI* fragment of p41plo1.NHA and p41plo1.NHAK69R, respectively, with the *AgeI-NdeI* fragment from pREP41PkN (Craven *et al.*, 1998). IH1847 was mated with IH1845 to generate haploid *plo1.d1* spores, which harbored the p41plo1.NPKK69R plasmid. These spores were treated with helicase, 30% ethanol and washed before being germinated in EMM2 media containing adenine and histidine.

Biochemical manipulations

Soluble cell extracts were prepared and blotted as described previously (Mulvihill *et al.*, 1999). Affinity-purified IH3 antibodies (Takeuchi and Yanagida, 1993) were used to detect *Cdc13*. For one kinase reaction, 4 × 10⁸ cells were washed with 1 ml of STOP buffer and snap-frozen as

pellets in liquid nitrogen to be stored at -80°C until use. Pellets were resuspended in 100 μl of IP buffer [50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin, 1 $\mu\text{g/ml}$ leupeptin, 20 mM β -glycerophosphate, 0.1 mM Na_3VO_4 , 50 mM NaF] and disrupted by agitation with 1 ml of acid-washed glass beads in a Hybaid Ribolyser for 3 s at speed 6.5. Cell debris was removed from the cell extract by a 10 min spin in a microcentrifuge. Protein A-Sepharose beads (Amersham-Pharmacia) were washed with IP buffer. Ten microlitres of packed beads were loaded either with 5 μl of anti-Plo1 antisera (HN184; Ohkura *et al.*, 1995) or 50 μl of anti-HA monoclonal antibody (12CA5; Boehringer Mannheim) in 100 μl of IP buffer + 0.1 M lysine, 10 $\mu\text{g/ml}$ bovine serum albumin at 4°C for 1 h. After four washes with IP buffer, 10 μl of beads were mixed with 100 μl of extract for 1 h at 4°C , washed twice with wash buffer A (50 mM HEPES pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100), once with wash buffer B (50 mM HEPES pH 7.5, 1 mM EDTA) and once with CK buffer [20 mM HEPES pH 7.5, 150 mM KCl, 10 mM MgCl_2 , 2 mM dithiothreitol (DTT), 1 mM EGTA] and resuspended in 6.3 μl of CK buffer + 3 μCi of [γ - ^{32}P]ATP (5000 Ci/mmol; Amersham), 160 μM ATP, 0.5 $\mu\text{g/ml}$ heparin, 7.5 μg casein. After incubation for 20 min at 30°C , 5 μl of sample buffer were added, the mix run on 10% SDS-PAGE and blotted to a nitrocellulose membrane. The membranes were probed with HN184 and developed with alkaline phosphatase-conjugated secondary antibodies. The relative level of Plo1 (*Plo1L*) in each lane was calculated by measuring the pixel density of a scanned membrane image with NIH image software. The radioactivity of casein (*Plo1P*) was quantified with a Fujix BAS2000 phosphorimager. Kinase activity per unit Plo1 in each reaction was calculated as *Plo1P* \div *Plo1L*. Histone H1 kinase assays were as described in Creanor and Mitchison (1996). Phosphatase treatment of HN184 immunocomplexes was initiated by two washes with pre-AP buffer [PAP; 50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 5 mM DTT, 1 mM PMSF, 15 mM *p*-nitrophenyl phosphate (pNPP)] or pre-API buffer (PAP + 10 mM Na_3VO_4 , 50 mM NaF, 60 mM β -glycerophosphate). The beads were then resuspended in either AP buffer [50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij 35, 2 mM MnCl_2 , 15 mM pNPP, 100 U of λ phosphatase (New England Biolabs)] or API buffer (AP + 10 mM Na_3VO_4 , 50 mM NaF, 50 mM EDTA, 60 mM β -glycerophosphate) and incubated at 30°C for 30 min. The beads were then washed four times with CKI (CK buffer + 10 mM Na_3VO_4 , 50 mM NaF, 50 mM EDTA) and once in CK buffer. After resuspension in CK, the sample was split in two and half used for kinase assay while 15 mM pNPP was added to the other half. The lack of a pNPP reaction showed that phosphatase activity had not been carried through into the kinase reaction.

Fluorescence microscopy

DAPI staining of nuclei and calcofluor staining to score septation indices were as described in Moreno *et al.* (1991). Cells were fixed with 2.5% glutaraldehyde to score condensed chromosomes (Toda *et al.*, 1981). Plo1 (with HN184), PK/GFP [with MAB336 (Serotec) and antibodies cited in Sawin *et al.*, 1999] and tubulin [with TAT1 (Woods *et al.*, 1989)] immunofluorescence and phalloidin staining were carried out according to Marks and Hyams (1985) and Mulvihill *et al.* (1999).

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