Plo1 Kinase Recruitment to the Spindle Pole Body and Its Role in Cell Division in *Schizosaccharomyces pombe*

Daniel P. Mulvihill,*† Janni Petersen,* Hiroyuki Ohkura,†‡ David M. Glover,† and Iain M. Hagan*§

*School of Biological Sciences, University of Manchester, Manchester M13 9PT, United Kingdom; and †CRC Cell Cycle Genetics Group, Department of Anatomy and Physiology, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland

Polo kinases execute multiple roles during cell division. The fission yeast polo related kinase Plo1 is required to assemble the mitotic spindle, the prophase actin ring that predicts the site for cytokinesis and for septation after the completion of mitosis (Ohkura *et al.*, 1995; Bahler *et al.*, 1998). We show that Plo1 associates with the mitotic but not interphase spindle pole body (SPB). SPB association of Plo1 is the earliest fission yeast mitotic event recorded to date. SPB association is strong from mitotic commitment to early anaphase B, after which the Plo1 signal becomes very weak and finally disappears upon spindle breakdown. SPB association of Plo1 requires mitosis-promoting factor (MPF) activity, whereas its disassociation requires the activity of the anaphase-promoting complex. The *stf1.1* mutation bypasses the usual requirement for the MPF activator Cdc25 (Hudson *et al.*, 1990). Significantly, Plo1 associates inappropriately with the interphase SPB of *stf1.1* cells. These data are consistent with the emerging theme from many systems that polo kinases participate in the regulation of MPF to determine the timing of commitment to mitosis and may indicate that pole association is a key aspect of Plo1 function. Plo1 does not associate with the SPB when septation is inappropriately driven by deregulation of the Spg1 pathway and remains SPB associated if septation occurs in the presence of a spindle. Thus, neither Plo1 recruitment to nor its departure from the SPB are required for septation; however, overexpression of *plo1+* activates the Spg1 pathway and causes transient Cdc7 recruitment to the SPB and multiple rounds of septation.

INTRODUCTION

Mutation of the *polo* gene in *Drosophila melanogaster* confers a pleiotropic phenotype initially observed to include defects in spindle formation and chromosome segregation (Sunkel and Glover, 1988; Llamazares *et al.*, 1991) and more recently cytokinesis defects (Carmena *et al.*, 1998). The protein kinase encoded by *polo* has now been found to have a counterpart in a wide range of eukaryotes. Evidence is accumulating to suggest that these polo-like kinases (Plks) play roles in diverse mitotic functions, including spindle pole maturation, regulation of bipolar spindle formation, and the activity of anaphase-promoting complex (APC) ubiquitin protein ligase (Glover *et al.*, 1998; Nigg, 1998). Consistent with these multiple roles, Plks have been localized in animal cells to the spindle poles and centromeres early in mitosis and to the central spindle and midbody during telophase and cytokinesis (Golsteyn *et al.*, 1995; Lee *et al.*, 1995; Logarinho and Sunkel, 1998; Wianny *et al.*, 1998).

There is also growing evidence for a role for polo kinases in the commitment to and exit from mitosis. Mitosis-promoting factor (MPF) p34cdc2 cyclin B is regulated by a balance between the activities of inhibitory Wee1/Mik1 kinases and the opposing Cdc25 phosphatase. When a critical portion of the p34cdc2 becomes dephosphorylated, it triggers the activation of Cdc25 by at least one intermediary kinase, thus amplifying the initial MPF activity in a positive feedback loop (Hoffmann *et al.*, 1993; Dunphy, 1994; Izumi and Maller, 1995; Kuang *et al.*, 1994). The *Xenopus* Plk, Plx1, was suggested as a potential component of this amplification loop

because it was found to associate with and phosphorylate Cdc25 (Kumagai and Dunphy, 1996). This has been substantiated by studies of the G2/M transition in *Xenopus* oocytes or cell-free systems derived from activated eggs (Abrieu *et al.*, 1998; Qian *et al.*, 1998a). Immunodepletion or immunoinhibition of Plx1 blocks the conversion of p34^{cdc2} into its mitotic form. Conversely, microinjection of Plx1 into oocytes accelerates activation of both Cdc25 and MPF. The recent identification of a polo kinase kinase in *Xenopus*, xPlkk, suggests either that multiple protein kinases participate in the feedback loop or that polo kinases can be activated independently of MPF activation (Qian *et al.*, 1998b).

p34^{cdc2} is inactivated after the metaphase–anaphase transition by the proteolytic degradation of its regulatory subunit, a B-type cyclin. It has been proposed that Plks have a role in activating the APC, a ubiquitin protein ligase that is responsible for cyclin degradation. The budding yeast Plk, Cdc5p, is required to activate the APC and degrade the B type cyclin, Clb2, before itself becoming a substrate of the APC (Charles *et al.*, 1998; Cheng *et al.*, 1998; Shirayama *et al.*, 1998). Moreover, Descombes and Nigg (1998) have presented evidence that *Xenopus* Plx1 is required for the degradation of APC targets for cytostatic factor-arrested extracts to exit M phase, and mammalian Plk will phosphorylate and inactivate APC components in vitro (Kotani *et al.*, 1998).

Although the *Saccharomyces cerevisiae cdc5.1* mutant arrests in the late stages of nuclear division with an elongated spindle, deletion of the fission yeast gene *plo1*$^+$ leads to defects in two quite different stages of the division cycle: the formation of the spindle and execution of cytokinesis. Cells lacking the essential Plo1 protein accumulate in mitosis with a monopolar rather than a bipolar spindle and are neither able to form the prophase actin ring that is required to initiate cytokinesis nor to organize any septal material (Ohkura *et al.*, 1995). Recent studies have identified conditional *plo1* mutations that alter the selection of the site for prophase actin ring (Bahler *et al.*, 1998). Complementary experiments showed that strong overexpression of the *plo1*$^+$ gene resulted in both defects in spindle formation and inappropriate septation in interphase cells. Inappropriate septation during interphase can also be driven by manipulation of a regulatory network that involves the G protein Spg1 (Gould and Simanis, 1997; Schmidt *et al.*, 1997). The GTP-bound form of Spg1 recruits the Cdc7 protein kinase to the spindle pole bodies (SPBs), and by the time of cytokinesis the active form of the complex becomes restricted to one of the two SPBs (Fankhauser and Simanis, 1994; Sohrmann *et al.*, 1998). The position of Plo1 in this regulatory cascade is currently under study (Gould and Simanis, 1997).

In this work we describe a series of experiments that examine the role played by recruitment of Plo1 to the SPB during mitosis. We show that Plo1 localizes to the SPB upon commitment to mitosis and that this association diminishes after APC activation but still persists until the end of mitosis. Plo1 undergoes premature SPB association in the Cdc25 bypassing mutant *stf1.1* (Hudson *et al.*, 1990; Bridge *et al.*, 1998). These observations are discussed in the context of a potential role for Plo1 in the regulation of MPF at the commitment to mitosis.

MATERIALS AND METHODS

Cell Culture and Strains

Cell culture and maintenance were carried out according to Moreno *et al.* (1991). All Plo1 asynchronous localization experiments were carried out in rich medium (yeast extract [YES]). When promoter repression or derepression was required, cells were grown in appropriately supplemented EMM2. Repression of the *nmt1* promoter (Maundrell, 1993) was carried out by the addition of 4 μM thiamine to the minimal media. All synchronization experiments were conducted in appropriately supplemented EMM2. Matings were carried out on MSA plates (Egel *et al.*, 1994).

Synchronous cultures were generated by size selection either after centrifugation on a lactose gradient as described previously (Carr *et al.*, 1995) or by centrifugal elutriation (Mitchison and Carter, 1975). The strains used in this study are listed in Table 1.

Molecular Genetic Manipulations

An *Not*I site containing oligonucleotide, designed to keep the gene in frame, was inserted at the *Nru*I site located 40 bp from the 3′ end of the *plo1*$^+$ gene in pHN205. An *Not*I DNA fragment from pDMHA encoding three of the hemagglutinin (HA) epitopes that are recognized by the mAb 12CA5 was inserted at the novel *Not*I site to create p41plo1.CHA. An *Nde*I–*Sma*I fragment containing the *plo1*$^+$ gene was isolated from the plasmid pHN205 (Ohkura *et al.*, 1995) and ligated into pREP41HA (Craven *et al.*, 1998) to give plasmid p41plo1.NHA. Plasmid p41plo1.NHA was transformed into diploid IH295, in which one copy of the *plo1*$^+$ gene has been completely replaced with the *ura4*$^+$ gene. The resulting strain was sporulated, and spores were plated onto minimal media lacking uracil (to select for growth of cells containing the *plo1.d1* allele) and leucine (to select for those containing p41plo1.NHA). Many ura$^+$ leu$^+$ colonies were obtained, whereas none were obtained in control transformation in which pREP41HA was used. This showed that p41plo1.NHA is able to complement the *plo1.d1* deletion.

An HA-tagged version of *plo1*$^+$ for integration into the genome was made by excising the *Xho*I–*Sac*I plo1$^+$ fragment from p41plo1.NHA and cloning it into pINT541 (a gift from M. Sohrmann and V. Simanis, ISREC, Lausanne, Switzerland) to produce plasmid pDM14. Because pINT541 lacks a termination and polyA addition sequence, this was reintroduced by cutting a *Sac*I fragment from p41plo1.NHA (which contained the *nmt1*$^+$ polyA addition sequences) and inserting this fragment into pDM14 to generate pI41plo1.NHA. This DNA was integrated into the *S. pombe* genome as described previously (Fankhauser and Simanis, 1994).

A version of *plo1*$^+$ fused to both HA and green fluorescent protein (GFP) at its amino terminus was created by excising an *Nde*I fragment containing the GFP encoding DNA from pGEMT-GFP (Craven *et al.*, 1998), and inserting it into the *Nde*I site of pI41plo1.NHA. The resulting plasmid was called pI41plo1.NHAG. This DNA was integrated into the *Schizosaccharomyces pombe* genome at the *leu1* locus as described previously (Fankhauser and Simanis, 1994).

Immunological Techniques

Western Blotting. For each preparation, 2×10^7 cells were harvested from a log phase culture by centrifugation at $2000 \times g$ for 2 min. They were then washed with 1 ml of Stop buffer (Simanis and Nurse, 1986) and transferred to a 2-ml microfuge tube. This was then centrifuged at 13,000 rpm for 1 min, the buffer was removed, and the cells were then either processed directly or snap-frozen in liquid nitrogen and stored at −80°C. The cells were resuspended in 50 μl of HE buffer containing 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 mM PMSF (Simanis and Nurse, 1986). The cells were then disrupted with 1 ml of acid-washed glass beads by agitation for 3 s in a Hybaid Ribolyser at power setting 6. The cell extract was spun out through a hole in the bottom of the tube.

Table 1. Strains used in this study

Strain number	Genotype	Source
IH109	h⁻ cut4.533	Hirano et al., 1986
IH110	h⁻ nuc2.633 leu1.32	Hirano et al., 1986
IH111	h⁻ cdc14.118	Nurse et al., 1976
IH112	h⁻ cdc15.140	Nurse et al., 1976
IH113	h⁻ cut2.120	Hirano et al., 1986
IH119	h⁻ cut8.563	Hirano et al., 1986
IH132	h⁻ cdc25.22	Nurse et al., 1976
IH136	h⁻ cut7.24 leu1.32	Hagan and Yanagida, 1995
IH154	h⁻ cut9.665 leu1.32	Hirano et al., 1986
IH164	h⁻ cdc2.33	Nurse et al., 1976
IH183	h⁻ cdc12.112	Nurse et al., 1976
IH259	h⁻ cut1.RB5.leu1.32	Lab stock
IH280	h⁻ cut12.1 leu1.32	Bridge et al., 1998
IH295	h⁻/⁺ plo1::ura4/plo leu1.32/leu1.32ura4.d18.ura4.d18 ade6,N219.ade6,216 his2/HIS2	Ohkura et al., 1995
IH365	h⁻ ura4.d18 leu1.32	Lab stock
IH366	h⁺ ura4.d18 leu1.32 his2	Lab stock
IH376	h⁻ cdc10.v50	Lab stock
IH385	h⁻ cut11.1 leu1.32	Lab stock
IH572	h⁻/⁺ cut12::ura4/cut12 leu1.32/leu1.32 ura4.d18/ura4.d18 ade6.M210/ade6.216 his1/his1.102	Bridge et al., 1998
IH666	h⁺ stf1.1 leu1.32	Hudson et al., 1990
IH738	h⁺ cdc7.A20 ura4.d18	Schmidt et al., 1997
IH1292	h⁻ ura4.d18 pINT5 spg1	Schmidt et al., 1997
IH1297	h⁻ cdc16.116 ura4.d18	Nurse et al., 1976
IH1298	h⁻ cdc11.H1 ura4.d18 leu1.32	A. Poziemba and I. Hagan, and unpublished data
IH1308	h⁻ ura4.d18	This study
IH1313	h⁹⁰ ura4.d18 leu1::ura4+pl41plo1.NHA	This study
IH1314	h⁹⁰ ura4.d18 leu1::ura4+pl41Plo1.NHAG	This study
IH1319	h⁻ stf1.1 ura4.d18	This study
IH1353	h⁻ stf1.1 ura4.d18 leu1::ura4+pl41 Plo1.NHA.G	This study
IH1123	h⁻ leu1.32 ura4.d18 p41plo1.NHA	This study
IH1367	h⁺ cdc25.22 his2 ura4.d18 leu1::ura4+pl41plo1.NHA	This study
IH1366	h⁺ cdc25.22 his2 ura4.d18	This study
IH1319	h⁻ plo1::ura4 leu1.32 ura4.d18 ade6.M210 p41plo1.NHA	This study
IH1409	h⁻ ura4.d18 leu1.32 p41plo1	This study
IH1410	h⁻ ura4.d18 leu1.32 p41plo1.NHA	This study
IH1548	h⁻ cdc25.22 leu1.32 pHN 204	Ohkura et al., 1995

Boiling SDS-PAGE sample buffer was added to the protein extract, and the mix was incubated at 100°C for 3 min.

Western blot detection was carried out after equal amounts of protein samples were run on a 10% SDS-PAGE gel. Final protein loadings for cell cycle Western blot analysis were determined after developing one blot for TAT1 and adjusting the loading volumes accordingly. Detection was carried out using the Pierce Super Signal Substrate (Pierce, Rockford, IL) (see Figure 1) or alkaline phosphatase reagents (see Figures 3 and 7) and using standard immunoblotting protocols (Harlow and Lane, 1988). To quantitate the intensity of the signals in the cell cycle Western blots, the blots were scanned, and the signal density in the bands was measured using NIH image.

HN184 sera were used at a concentration of 1:100, and TAT1 anti-α tubulin antibodies were used at 1 in 100 (Woods et al., 1989).

Fluorescence Microscopy. For GFP autofluorescence, microscopy cells were fixed in 3.7% formalin for 10 min, and the DNA was stained using DAPI as described in Moreno et al. (1991). Calcofluor staining to score septation indices was also according to Moreno et al. (1991).

Immunofluorescence microscopy was performed according to Hagan and Hyams (1988), with the exception that for Plo1 immunofluorescence glutaraldehyde was omitted and fixation was limited to 10 min. Affinity-purified rabbit anti-Sad1 antibodies, AP9.2

(Hagan and Yanagida, 1995), were used at a dilution of 1:25, whereas sheep affinity-purified anti-Sad1 antibodies (K. S. Sheldrick and I. M. Hagan, unpublished data) were used at 1:50; the anti-tubulin antibody, TAT1 (Woods et al., 1989), was used at 1:80; anti-GFP antibodies (generous gift from Ken Sawin, ICRF, London) were used at 1:50; and the Plo1 antibodies (HN184) were used at a dilution of 1:20. Appropriate secondary antisera were from Sigma (Poole, Dorset, UK). For double-labeling, cells were incubated with both antibodies simultaneously. Images (250), which were captured with a Hamamatsu SIT camera (Hamamatsu, Bridgewater, NJ), were integrated into one final image with pixel pipeline frame grabber in NIH image. Merging different images to produce color overlays and the generation of final figures were both done in Adobe Photoshop v4.0. Cell-length measurements were determined using the appropriate NIH image tool.

RESULTS

### Reagents for the Analysis of Plo1 Protein

Localization of a protein to specific regions of the cell or to discrete organelles can play a key role in its participation in specific cellular events. We therefore wished to determine

the distribution of Plo1 protein kinase at all stages of cell cycle progression.

The generation of the anti-Plo1 antibody (HN184) has been described previously (Ohkura *et al.*, 1995). A number of additional reagents were generated to verify the validity of the results obtained with HN184. Expression of a gene in which the HA epitope tag was 24 amino acids before the end of the *plo1* coding sequence (*plo1.CHA*) from the Rep41 *nmt1* promoter was unable to support the growth of spores lacking *plo1⁺*. In contrast, the expression of either the wild-type gene (*plo1⁺*) or a version in which three HA epitopes were fused at the N terminus in frame with the *plo1* ATG (*plo1.NHA*) complemented the deletion, indicating that *plo1.NHA* encoded a functional protein. Western blot analysis using HN184 confirmed these results and demonstrated the specificity of this anti-Plo1 antisera (Figure 1A). A doublet was detected at 77 kDa in wild-type cells (Figure 1A, lane 1). The intensity of the upper band was enhanced relative to the lower band when the phosphatase inhibitor okadaic acid was included in the cell extraction buffer (Figure 1A, lane 5). The intensity of the lower band was markedly increased in extracts prepared from wild-type cells in which *plo1⁺* was expressed from the *nmt1* promoter of pREP41 (Figure 1A, lane 2). Both bands were absent from extracts of *plo1.d1* cells, which were kept alive by the expression of *plo1.NHA* (Figure 1A, lane 3). In these cells the 77-kDa doublet was replaced by a novel band at 80 kDa that corresponded to the expected increase in mass arising from fusion with the tag. In both cases, where Plo1 was overproduced a single rather than a double band was seen. The relative intensity of this Plo1NHA band was markedly reduced when expression was driven from a version of the gene that had been integrated at the *leu1* locus and regulated by the weaker *nmt41* promoter (Figure 1B, lane 2). To facilitate protein localization, the cDNA encoding GFP was fused to *plo1.NHA* in pINT541, and the construct was integrated into the genome. Induction of this gene resulted in the appearance of a novel band at 110 kDa (Figure 1B, lane 3). Together these data show that the HN184 sera specifically recognized the Plo1 protein.

## Plo1 Associates with the Mitotic but Not Interphase SPB

In a first approach we observed the autonomous fluorescence of a chimeric protein in which the coding sequence for GFP was fused in frame to the amino terminus of Plo1. This fusion protein was integrated into the genome at *leu1* locus and regulated by the *nmt81* promoter, which gives a very low level of expression. Illumination of living or formalin-fixed cells with the appropriate wavelength to excite GFP revealed one or two dots in cells that were in the constant length and division stages of the cell cycle (Figure 2A) (Mitchison and Nurse, 1985). No signal could be detected in interphase cells. Immunolocalization of the fusion protein with antibodies against GFP confirmed the pattern given by intrinsic GFP fluorescence and permitted counterstaining with antibodies to the SPB component Sad1. Early mitotic cells showed one or two dots of GFP–Plo1 that also stained with anti-Sad1 antibodies, confirming that they represented the SPB; however, although Sad1 was associated with interphase SPBs, Plo1 was not (Figure 2B).
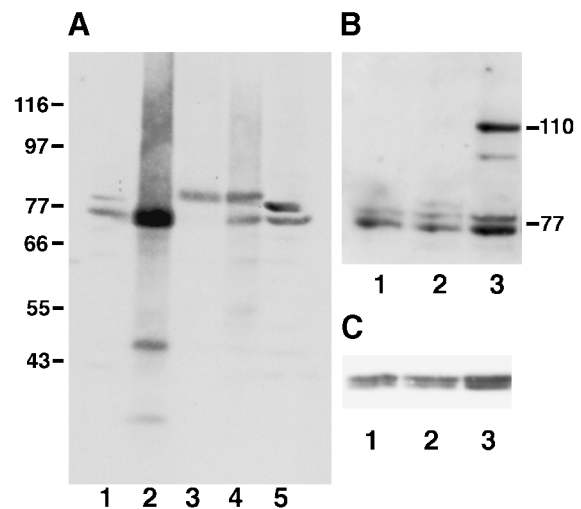
Figure 1. Characterization of HN184 antibody. Protein extracts from different *S. pombe* strains were analyzed by immunoblots and probed with HN184 antisera (A, B) or the anti-α tubulin antibody TAT1 as a loading control (C). (A) A wild-type cell (IH365) extract shows a Plo1 doublet of the expected size of ~77 kDa (lane 1). The intensity of the lower band increased in wild-type cells, which expressed *plo1⁺* from a multicopy plasmid under the control of the nmt41 promoter (IH1409) (lane 2). In a strain from which the genomic copy of *plo1⁺* has been deleted, and the cells were kept alive by the expression of an HA-tagged *plo1⁺* gene from the multicopy plasmid p41plo1.NHA, the two wild-type bands were replaced by a single band at 80 kDa (IH1391) (lane 3). As predicted from lanes 1–3, two bands were seen in a wild-type strain overexpressing the HA-tagged protein (IH1289), a bottom wild-type band and an upper HA-tagged band (lane 4). The intensity of the upper band in wild-type cells was increased in extracts prepared in the presence of 0.5 μM okadaic acid (lane 5). Blotting with TAT1 showed similar loading levels in each lane. (B) Lane 1, IH365 extract. Lane 2, extract of IH1313 showing the faint additional HA-tagged plo1 band at 80 kDa. Lane 3, extract of IH1314 showing an additional GFP- and HA-tagged band of Plo1 at 110 kDa. C shows the same filter as depicted in B, probed with TAT1 to demonstrate consistency of loading.

Direct detection of endogenous Plo1 with the HN184 antiserum gave similar patterns (Figure 2C). The first sign of Plo1 staining with these antibodies was a single dot on the side of the nucleus. The frequency of cells with single dots in asynchronous cultures (0.8% at 25°C, 1.2% at 36°C [n = 500]) suggests that Plo1 associates with the SPB for a significant period before the appearance of two distinct juxtaposed dots of Plo1 staining during spindle formation. SPB staining was bright and persisted until early anaphase B, whereupon its intensity diminished abruptly. SPB staining was still detected until spindle breakdown, however, but it was extremely faint and barely detectable above background levels (Figure 2C). We also detected association of Plo1 with the spindle in some cells. This association was seen from the beginning of spindle formation until the time in anaphase B when the SPB staining diminished radically. The specificity of HN184 immunostaining was indicated by the fact that the mitotic SPBs of germinating spores that lacked *plo1⁺* were not recognized by the antibodies (Figure 2F). We are therefore confident that the fluorescence signals seen using

Figure 2. Plo1 localizes to the SPBs and mitotic spindle in a cell cycle-specific manner. (A) GFP autofluorescence in formalin-fixed cells of the strain IH1314 grown at 25°C in EMM2 supplemented with leucine to fully induce the nmt41 promoter. Two dots were seen in mitotic cells up to early anaphase B (cell 1, 2, 3). (B) Immunofluorescence staining of these cells with anti-GFP antibodies (B, top left panel) and Sad1 (B, top right panel) antibodies. Sad1 (top right, and red in bottom right) and Plo1 (top left, and green in bottom right) colocalized to give a yellow signal in the mitotic cell in the merged image (bottom right panel). DAPI staining (blue, bottom right) along with phase contrast (B, bottom left panel) shows chromatin localization with respect to the outline of the cell. (C) Plo1 immunofluorescence staining of the wild-type strain IH365 using HN184 antisera (top panels) and the same cells stained with DAPI and coincidentally viewed with phase contrast (bottom panels) to reveal the location of chromatin relative to the cell periphery. The panels are arranged from left to right to show successive stages as mitosis progresses. The earliest localization of Plo1 was to a single SPB. It then localized as two bright and distinct dots as the SPBs separated, and as anaphase progressed the localization to the SPBs continued, but the intensity was greatly reduced (arrows in the final panels). The images in Figure 4C were processed to the same extent, to highlight the variations in Plo1 intensity rather than to give the best qualitative image. (D) HN184 stained short spindles of wild-type cells until the start of anaphase, when very briefly it was at its brightest after the chromatin had separated. After this the staining could no longer be seen. (E) Microtubules of IH365 cells were depolymerized by incubation in an ice bath for 20 min. Left-hand panels show HN184 staining; right-hand panels show DAPI phase-contrast images of the same cells. The top cell was stained before cooling, and the bottom cell was stained after cooling. Plo1 SPB association was unaffected by microtubule depolymerization. (F) HN184 staining of germinating spores from which *plo1*+ has been deleted. The top panel shows no HN184 signal, whereas the DAPI phase-contrast image in the bottom panel shows the condensed chromatin, which is characteristic of mitotically arrested cells.
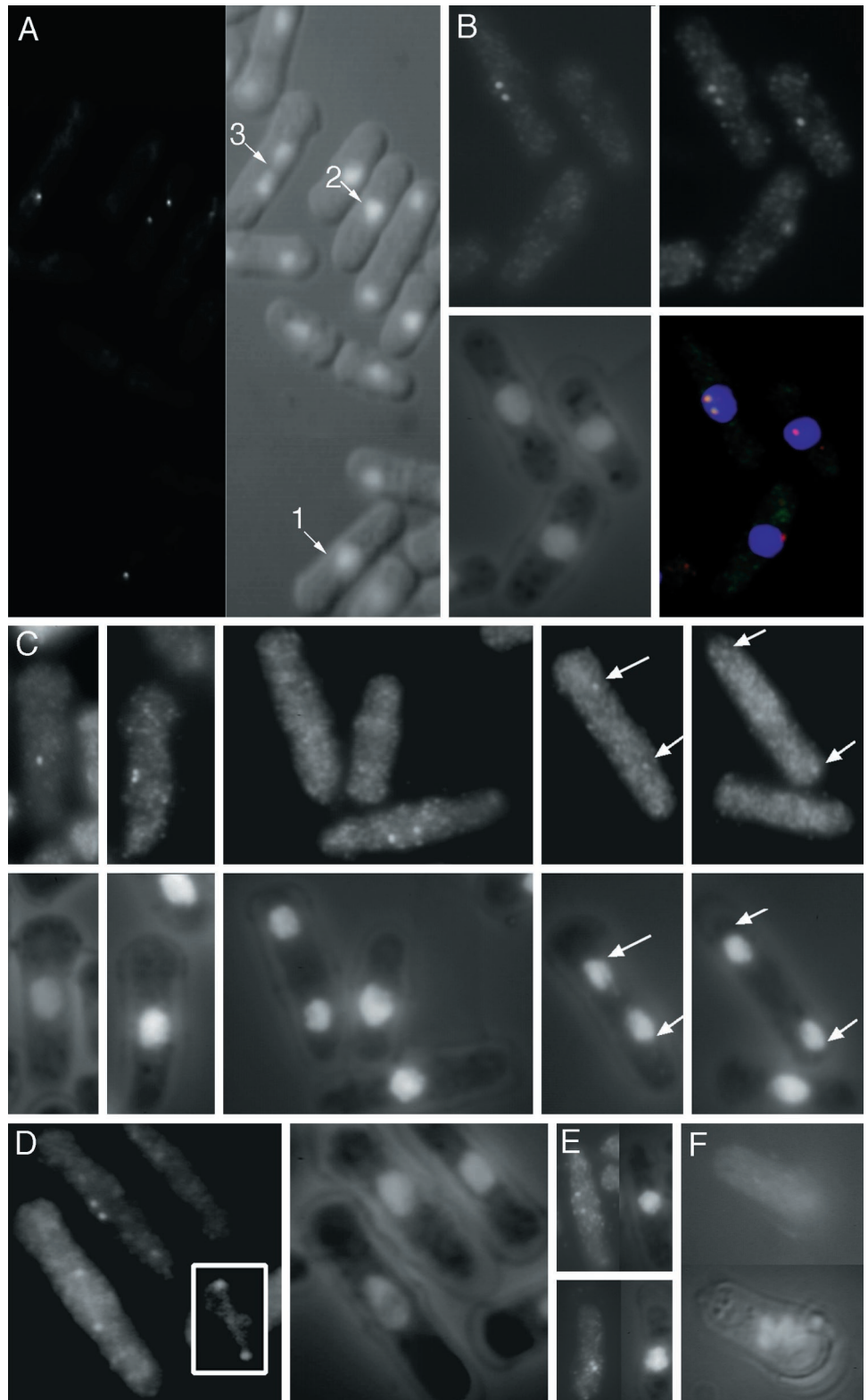
HN184 resulted from detection of Plo1 protein. Given that both the immunofluorescence and GFP fluorescence data independently showed a similar cell cycle variation in Plo1 signals at the SPB, we conclude that this variation was not due to processing artifacts, fluorescence quenching, or epitope masking but reflected a genuine cell cycle regulation of Plo1 association with the SPB.

Proteins may either associate directly with the SPB or be transported there by microtubule motor proteins. Given that polo kinases associate with motor proteins (Lee *et al.*, 1995; Adams *et al.*, 1998) and that we observed spindle association of Plo1 (Figure 2E), we determined whether Plo1 would associate with the SPB after microtubule depolymerization by cold shock (Hagan and Yanagida, 1995). Although microtubules were completely depolymerized by incubation on ice, Plo1 remained bound to the SPB (Figure 2E).

Plo1 Association with the SPB Is Not Determined by Fluctuations in the Level of the Protein

One possible explanation for the lack of any detectable association of the Plo1 with the SPB during the majority of the cell cycle is that the protein is present only during mitosis. We therefore monitored Plo1 protein levels in whole-cell extracts from a wild-type culture that had been synchronized with respect to cell cycle progression by elutrient centrifugation. Large fluctuations in Plo1 protein levels did not accompany cell cycle progression; rather, protein levels appeared constant (Figure 3A). Measurements of the ratio of the intensity of the Plo1 doublet to the α tubulin doublet recognized by TAT1 indicated that the ratio of Plo1 to α tubulin levels did not fluctuate. Most significantly, Plo1 was present throughout interphase. Because Plo1 associated only with the SPBs of mitotic cells in a similarly synchronized culture (Figure 3B), we conclude that the cell cycle variation in Plo1 association with the SPB is not generated by alteration of the stability of the protein.

Plo1 Association with the SPB and the Regulation of Septation

Loss of Plo1 protein blocks septation, whereas excessive overproduction of the protein drives septation during interphase (Ohkura *et al.*, 1995). Because several regulators of septation associate with the SPB early in mitosis (Sohrmann *et al.*, 1998), it is possible that the SPB association of Plo1 is an integral part of its role as a regulator of septation. To explore this possibility further we determined the subcellular distribution of Plo1 in strains that had been genetically manipulated to either block septation or drive septation at inappropriate stages.

Multiple septation events can be driven during interphase in single cells either by dramatically increasing the levels of the G protein Spg1 or by reducing the activity of the Cdc16/Byr4 complex that provides the GAP activity for Spg1. The latter can be achieved by shifting the temperature of a culture of *cdc16.116* cells to the restrictive temperature (Minet *et al.*, 1981; Schmidt *et al.*, 1997; Furge *et al.*, 1998). We observed that the frequency of Plo1 localization to the SPB decreased upon induction of Spg1 (Figure 4A). This reduction may arise indirectly from the block of mitotic commitment that accompanies the DNA damage or the reduction in cell volume resulting from septation in interphase (Nurse, 1990). To
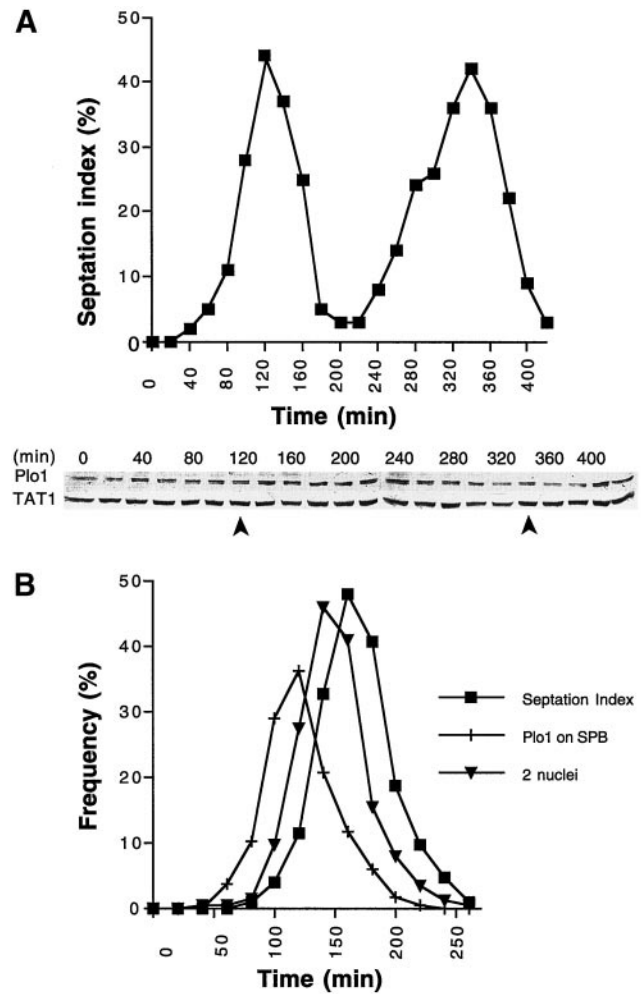
Figure 3. Fluctuations in Plo1 protein levels do not account for the cell cycle specificity of Plo1 localization to the SPB. (A) A cell cycle-synchronized population of wild-type cells (IH365) was generated by elutrient centrifugation at 25°C in appropriately supplemented EMM2. The small G2 cells were harvested at the beginning of the experiment, and samples were then taken every 20 min for Western blot analysis and scoring the septation index (n = 200 for each time point). The top panel shows the septation index, which indicates good cell cycle synchrony in the culture, and the bottom panel shows a Western blot of whole-cell extracts. The blot was cut into two, and the top portion was probed with antibodies to Plo1 and the bottom portion was probed with antibodies to a tubulin (TAT1). The tubulin blot acts as a loading control. The position of the peaks seen in the septation index are indicated by arrows beneath the blots. Quantitation of the ratio of the TAT1 to Plo1 signals for two independent blots for each of two independent experiments (each of which had two complete cell cycles) led to the conclusion that there is no apparent fluctuation in Plo1 levels as cells progress through the cell cycle. (B) Anti-Plo1 immunofluorescence analysis of an elutrient centrifugation synchronized population of wild-type cells growing in EMM2 at 25°C. Small G2 cells were harvested at the beginning of the time course at time point 0 and processed every 20 min for anti-Plo1 immunofluorescence microscopy. The septation index of each sample was determined by calcofluor staining before the cells were processed for immunofluorescence microscopy. Plo1 association with the SPB precedes anaphase B by ~20 min and septation by ~30 min. n = 200 for each data point. A repeat of this experiment gave similar profiles.
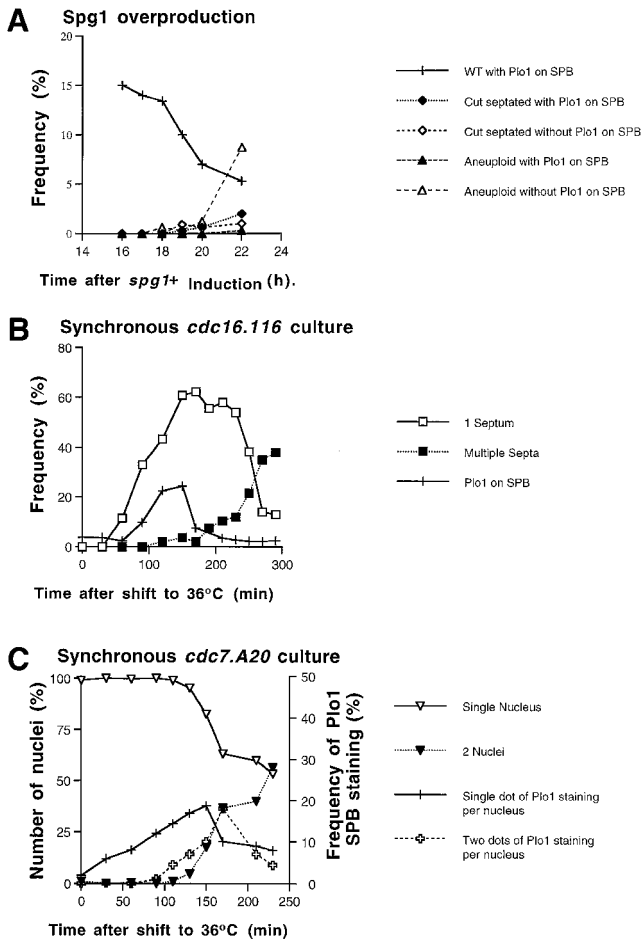
Figure 4. Plo1 localization to the SPB is not driven by the premature activation of Spg1 that drives inappropriate septation but is affected in *cdc7. A20* mutants. (A) Induction of cytokinesis by overexpressing *spg1*+ from an integrated version of the gene that was controlled by the *nmt1* promoter (IH1292). Samples were taken for immunofluorescence every hour after gene induction by removal of thiamine from the medium and stained with HN184 antibodies and DAPI. Overproduction of Spg1 led to a reduction in the number of cells with Plo1 on their SPB. (B) *cdc16.116* cells were synchronized with respect to cell size by elutrient centrifugation and immediately incubated at 36°C at time 0 on the graph in B. Correlative HN184 immunofluoresence, DAPI, and calcofluor staining showed that the septation started to occur within 1 h of the shift to the restrictive temperature. In contrast, Plo1 association with the SPB correlated with the normal timing of mitosis in such an experiment and did not rise once the second round of septation was initiated (filled squares). (C) *cdc7. A20* cells were synchronized with respect to cell size by elutrient centrifugation, and the isolated small G2 cells were immediately cultured at 36°C. Samples were taken for immunofluoresence and stained with HN184 and DAPI. Scoring for one or two dots of intense Plo1 staining showed one dot of Plo1 staining on the nuclei of G2 cells much earlier than in wild-type cells. The appearance of two dots per nucleus coincided with the normal timing of mitosis. n = 200 for each data point on the graphs. Each experiment gave similar results when repeated.

examine the relationship of SPB association of Plo1 to mitotic progression in cells destined to undergo repeated septation without an intervening mitosis, we first selected G2 cells from a *cdc16.116* culture by elutrient centrifugation. We then shifted the temperature of the culture of these G2 cells to the restrictive temperature and followed SPB association of Plo1, mitosis, and septation. Septation in some cells was induced within 30 min of the temperature shift (Minet *et al.*, 1981), but the association of Plo1 with the SPB occurred later in a single peak at the normal time expected for a mitotic event (Figure 4B). Plo1 did not associate with the SPBs during the generation of the second septum in the arrested cells. Thus, when septation is induced by inappropriate Spg1 activation, either through overexpression of Spg1 or inactivation of Cdc16, it occurs without previous association of Plo1 with the SPB.

Although these data show that septation does not require previous Plo1 association with the SPB, they do not clearly establish whether it is necessary for Plo1 to be lost from the mitotic SPB before septation can occur. Septation will take place in cells blocked in mitosis as a result of mutations in *cut4* and *cut9*, genes that encode components of the APC, in *cut7*, which encodes a kinesin-related protein needed for spindle formation, and in *cut8*, which encodes a protein required for sister chromatid separation (Hirano *et al.*, 1986; Hagan and Yanagida, 1990; Samejima and Yanagida, 1994a,b, Yamashita *et al.*, 1996). We therefore incubated *cut7.24*, *cut4.533*, *cut8.563*, and *cut9.665* mutant cells at the restrictive temperature for 3 h to induce cytokinesis in mitotically arrested cells and examined Plo1 localization. In each case Plo1 was clearly associated with the SPB in all septating mitotic cells. One such example of a *cut7.24*-arrested cell is shown in Figure 5A. These experiments showed that the disassociation of Plo1 from the SPB was not required for septation. Moreover, they also show that Plo1 disassociation from the SPB requires APC function.

Activation of Spg1 recruits Cdc7 kinase to the SPB (Sohrmann *et al.*, 1998). In the absence of Spg1 or Cdc7 function, the Dmf1 and F-actin rings form, but the subsequent events leading to septation do not occur. In addition to mutations in *spg1*+ and *cdc7*+ mutation in a number of other genes, including *cdc14*+ and *cdc11*+ have identical septation defects, and genetic data suggest that they function in conjunction with Spg1 and Cdc7 to regulate cytokinesis (Gould and Simanis, 1997; Schmidt *et al.*, 1997). Because the intimate relationship between Plo1 and septation suggests an interaction of Plo1 with the Spg1 pathway, we localized Plo1 in mixed cultures of these cytokinesis-regulating mutants containing wild-type cells as morphologically distinct processing controls. Plo1 was strongly present at the SPBs of both the wild-type processing control and the multinucleate cells of the mutants *cdc7.A20*, *cdc11.IH1*, and *cdc14.118* (Figure 5B–D). The HN184 antibody also stained the mitotic SPBs of *cdc15.140* cells, which lack a structural component of the actin ring and are blocked in cytokinesis at a similar stage (Figure 5E) (Nurse *et al.*, 1976; Fankhauser *et al.*, 1997). *cdc7.A20* and *cdc7.24* were unique among these mutants in that twice as many *cdc7*ts as *cdc7*+ cells had strong SPB staining at the restrictive temperature. In contrast, essentially the same proportion of mutant and wild-type cells had Plo1 on the SPB in mixed cultures of *cdc11.IH1/cdc11*+ and *cdc14.118/cdc14*+ at the restrictive temperature.
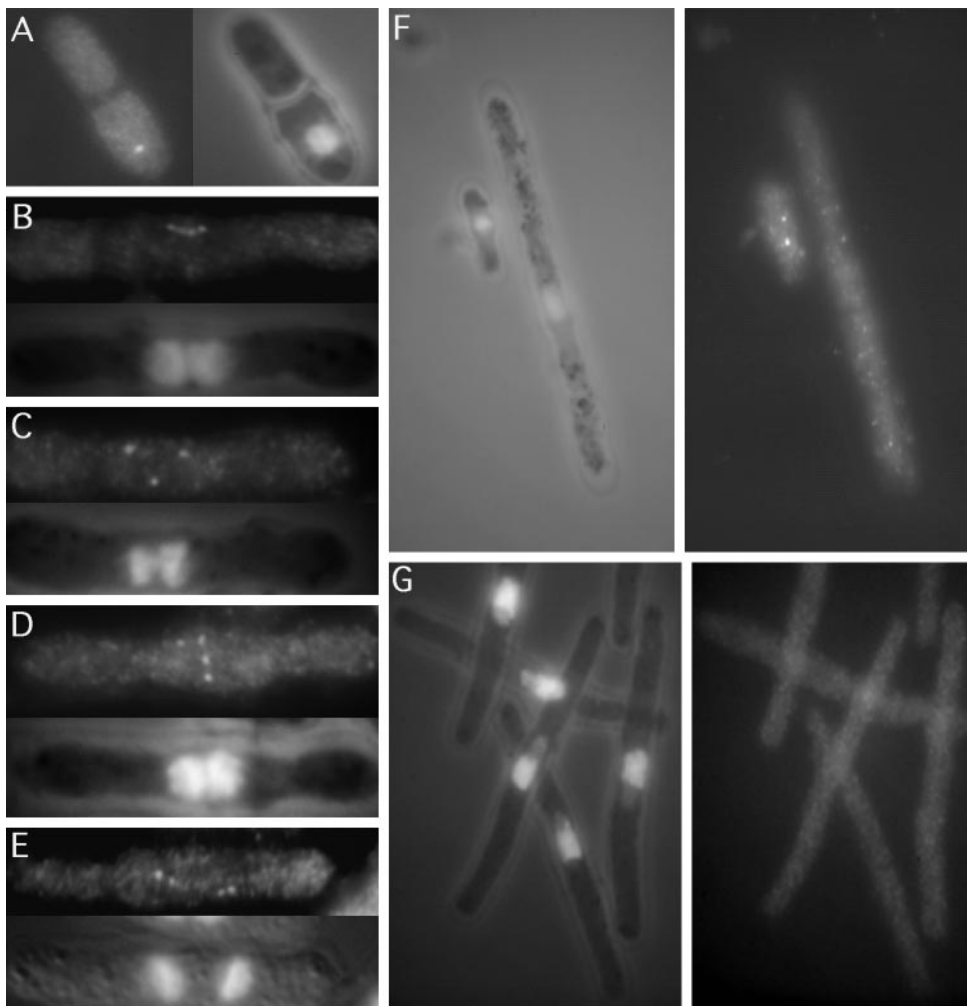
Figure 5. Anti-Plo1 immunofluorescence localization of Plo1 in *cdc* and *cut* mutants. (A) *Cut7.24* cells that arrest in mitosis but continue to execute cytokinesis regardless of the mitotic defect still had strong Plo1 staining at the time of this abnormal cytokinesis. Anti-Plo1 immunofluorescence microscopy with HN184 revealed that Plo1 shows strong association with the mitotic SPBs of the early septation mutants: (B) *cdc7. A20*, (C) *cdc11. H1*, (D) *cdc14.118*, (E) *cdc15.140*. Images of cells in the early stages of spindle formation in the first division after cytokinesis that had been inhibited by incubation at the restrictive temperature are shown. Thus, each of the two nuclei had two SPBs that stained with Plo1 antibodies. Plo1 did not localize in (F) *cdc25.22* (long cell in A) or (G) *cdc2.33* mutant cells after incubation of the temperature-sensitive mutants for 4 h at the restrictive temperature. Wild-type cells (small cell in *F*) were mixed with *cdc25.22* cells to show that the lack of Plo1 staining was not due to a technical problem. Because the smaller wild-type cells stained normally, we conclude that there was no association of Plo1 with the SPB.

To determine any cell cycle specificity of this additional SPB association in *cdc7*[ts] mutant backgrounds, Plo1 localization was monitored in a cell cycle-synchronized *cdc7.A20* culture that had been shifted to the restrictive temperature in early G2 phase. Plo1 associated with the SPB in ~10% of G2 cells after the temperature shift, rising to a maximum in anaphase (Figure 4C). Scoring cells with single or double dots showed a predominance of single-dot staining in interphase cells.

We conclude from these experiments that although there was some influence of mutations in the Cdc7 protein kinase upon the recruitment of Plo1 to the SPB, activation of the Spg1 cascade did not recruit Plo1 to the SPB.

Plo1 Overexpression Activates the Spg1 Cascade

Because *plo1*[+] overexpression drives septation, it is possible that it may do so by activating the Spg1 pathway (Ohkura *et al.* 1995). To test this possibility *plo1*[+] was overexpressed in temperature-sensitive *cdc25.22* cells, which are blocked in interphase at the restrictive temperature because of an inability to activate MPF. This results in septation without

mitosis (Ohkura *et al.* 1995). After a culture of *cdc25.22* cells was induced to overexpress *plo1*[+] from a multicopy plasmid, it was split into two, and two independent synchronous cultures were generated 13 and 15 h later and incubated at 36°C. Septation was induced with different kinetics in these two cultures (Figure 6A). Analysis of the culture in which Plo1 had been induced for 15 h showed that Plo1 overexpression resulted in Cdc7 recruitment to the SPBs (Figure 6, B and C). The kinetics of Cdc7 association suggest that the protein cycles on and off the SPB with every round of septation. Initially the proportion of cells with Cdc7 on the SPB exceeds the septation index, showing that it is recruited before septation. The proportion of cells with Cdc7 on the SPB in the later stages of the experiment did not reach the same level as the septation index, showing that once a septum is generated, in these conditions, Cdc7 leaves the SPB.

SPB Association of Plo1 Requires Cdc25 and Cdc2 Activation

Commitment to fission yeast mitosis is regulated by the activity of the MPF regulators Wee1 and Cdc25. Analysis of

Figure 6. Overproduction of Plo1 resulted in the association of Cdc7 with the SPB in G2-arrested *cdc25.22* cells. (A) Two independent synchronous cultures were generated by elutrient centrifugation 13 and 15 h after the removal of thiamine from IH1548 (h⁻ *cdc25.22 leu1.32* pHN 204) cells cultured in EMM2 medium at 25°C to induce overexpression of the *plo1⁺* gene. The temperature of each synchronous culture was immediately shifted to the restrictive temperature of 36°C after its generation. Although inappropriate septation was induced in each culture, many more septa were seen in the 15-h culture (filled squares) than the 13-h culture (open diamonds) as each was followed into the *cdc25.22* arrest. As a control the temperature of an uninduced asynchronous culture was shifted to 36°C (filled circles). Septation was inhibited in this culture for 4 h, after which some cells began to leak past the *cdc25.22* arrest point. (This is a common feature of an asynchronous culture. In contrast to the synchronized cells, some members of this population had been at the *cdc25.22* arrest point for the full 4-h time course of the experiment.) Staining of the 15-h and control culture showed that Spg1 was activated to recruit Cdc7 to the interphase SPBs as a consequence of Plo1 overproduction. (C) Cdc7 and DAPI/phase-contrast images of the same field of cells taken from the 210-min time point of the 15-h culture (indicated by a star in A). Cells 1–3 have Cdc7 staining of their SPBs in nuclei, which have been bisected by the inappropriately initiated septum.
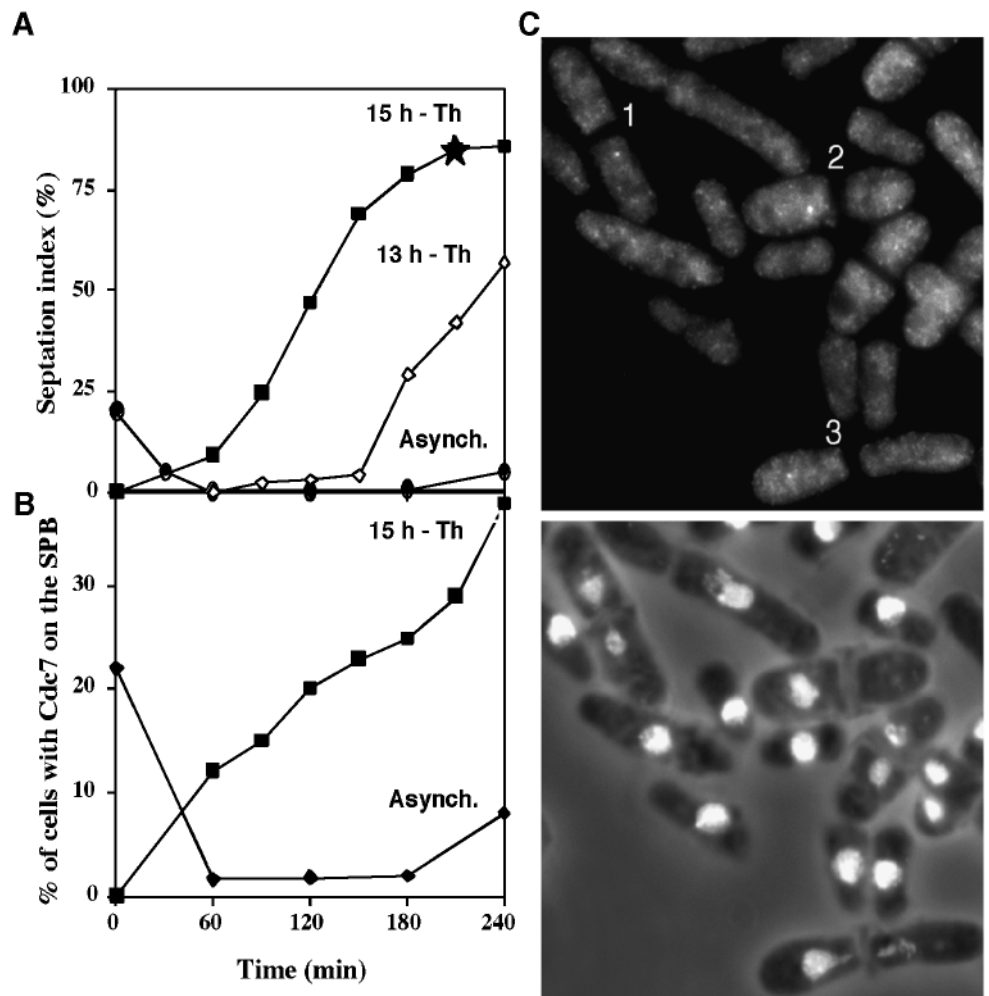
the *Xenopus* polo-like kinase Plx1 suggests that Plo1 may participate in the activation of MPF and that this role may involve regulation of Cdc25 activity. Because Plo1 association with the SPB appeared to be a very early event in mitosis, we were keen to determine whether it required Cdc25 or Cdc2 activity. We examined the ability of Plo1 to associate with the SPB in temperature-sensitive *cdc25* and *cdc2* mutants in which cell cycle progression was blocked because of incubation at the nonpermissive temperature. Plo1 did not associate with the SPBs *cdc25.22*, which had been arrested by incubation at 36°C for 4 h (Figure 5F). The fact that Plo1 was clearly visible on the SPBs of wild-type cells that had been mixed in with these *cdc25.22* mutants before fixation showed that the lack of staining was not an artifact of sample preparation in this particular experiment (Figure 5F). Thus, Plo1 association with the SPB required Cdc25 activity. Anti-Plo1 immunofluorescence microscopy of *cdc2.33*, which had been incubated at the restrictive temperature for 4 h to inactivate p34^cdc2, again showed that Plo1 was not associated with the SPB (Figure 5G). Thus, associa-

tion of Plo1 with the SPB is an early mitotic event that follows activation of p34^cdc2

Premature Recruitment of Plo1 to the SPB in the *cdc25* Bypassing Mutant *stf1.1*

Dominant mutations in the *stf1/cut12⁺* gene, which encodes an essential SPB component, can bypass the normally essential requirement for Cdc25 in the activation of MPF (Hudson *et al.*, 1990; Bridge *et al.*, 1998). Given the association of Plo1 with the SPB at the onset of mitosis and its potential role in MPF activation (see INTRODUCTION), it was of interest to examine the localization of Plo1 in the *stf1.1* mutant background.

In contrast to wild-type cells, the SPBs of small interphasic as well as mitotic *stf1.1* cells showed strong Plo1 staining (Figure 7A). Cell length is a measure of cell cycle progression in fission yeast. It is clear from the plot of the frequency of Plo1 association with the SPB against cell length in Figure 7B that Plo1 associated with the SPBs of small G2 cells in *stf1.1*
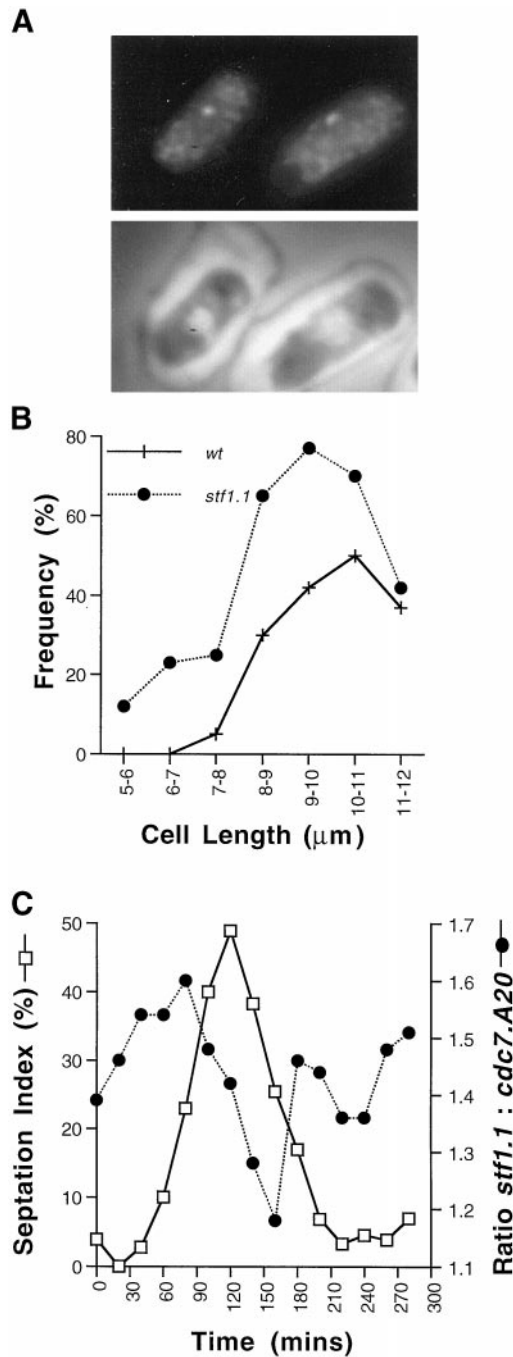
Figure 7. Premature recruitment of Plo1 to the SPBs in *stf1.1* mutant cells. (A) An asynchronous *stf1.1* culture was fixed and stained using HN184 antibodies and DAPI. Plo1 localized to the SPB of small interphase cells. The cells in A have shrunk after processing for immunofluorescence. This is because a very short fixation regime is used for Plo1 staining, and the cell structure has contracted in the 2 d before this image was captured. All statistical analysis throughout this article were conducted on freshly stained cells before shrinkage occurred. (B) Asynchronous wild-type IH365 and *stf1.1* cells were processed for immunofluoresence using HN184 antibodies and DAPI. The presence or absence of Plo1 staining was noted, and the frequency of a positive score was plotted as a function

but not wild-type cells. One possible explanation for the variation in the localization of Plo1 to the SPB in *stf1.1* mutants is a variation in the processing conditions between different experiments. To control for such possible variations in processing between experiments, asynchronous cultures of either wild-type or *stf1.1* cells were mixed with *cdc7.A20* arrested cells before immunofluorescence microscopy. After processing for immunofluorescence microscopy, it was trivial to distinguish the *cdc7.A20 stf1+* long multinucleate *cdc7.A20* cells from the shorter, mononucleate or binucleate *cdc7+ stf1.1* cells and to score the frequency of cells with Plo1 on the SPB of each strain independently. Using this data we established that Plo1 associated with the SPB in 2.9 times as many cells in a *stf1.1* culture as in the isogenic *stf1+* control. In parallel samples double-stained with antibodies to Plo1 and Sad1, Sad1 antibodies stained all SPBs, indicating that the difference in staining of the SPBs with the Plo1 antibodies did not reflect a general reduction in antibody accessibility to the SPB. To rule out the possibility that the variation in Plo1–SPB association reflected a variation in epitope exposure, the fluorescence emerging from the fusion protein in which Plo1 is tagged at its amino terminus with GFP was monitored in living *stf1+* control and *stf1.1* strains. Of the *stf1.1* cells, 9% had a single spot of SPB fluorescence, whereas only 1% were seen in wild-type cells (n = 400). Moreover, 10% of *stf1.1* cells in which cell cycle progression had been arrested by treatment with hydroxyurea for 3 h had strong SPB staining with Plo1 antibodies, whereas isogenic *stf1+* controls had no staining. We conclude that Plo1 associated prematurely with the SPB in a *stf1.1* mutant background.

To determine the kinetics of SPB association of Plo1 in *stf1.1* cells, a culture of *stf1.1* cells that had been synchronized with respect to cell cycle progression by elutriant centrifugation were processed for immunofluorescence microscopy. To control for variations in processing between different time points, asynchronous cell cycle-arrested *cdc7.A20* cells were mixed with the synchronized cells before processing for immunofluorescence. The ratio of Plo1 association with the SPBs of the small mononucleate or binucle-

Figure 7 (cont). of the cells' length. This was carried out for a minimum of 30 cells for each length category. Plo1 localizes to smaller cells and a higher proportion of *stf1.1* cells than wild-type. (C) A culture of *stf1.1* cells was synchronized by elutriant centrifugation. Samples were taken every 20 min and mixed with asynchronous *cdc7. A20* mutant cells that had been at the restrictive temperature for 4–6 h. These samples were stained with HN184 antibodies and DAPI. The inclusion of the septation mutant provided an internal control for the quality of sample preparation in the sample because the long multinucleate *cdc7. A20* cells were easily distinguishable from the shorter *stf1.1* cells. Thus the ratio of Plo1 SPB association in *stf1.1* relative to *cdc7. A20* staining gives a measure of Plo1 association with the *stf1.1* SPB in interphase. The level of SPB association in *cdc7. A20* remained constant throughout the experiment, but the relative staining on the SPBs of *stf1.1* cells varied. Two hundred cells of each strain were scored at each time point. The ratio of Plo1 association in *stf1.1* cells relative to *cdc7. A20* cells is presented (circles) alongside the septation index (open squares). Although this complex experiment was only performed once with a synchronous culture, data from the analysis of mixed asynchronous populations result in similar conclusions (see RESULTS).

ate *stf1.1* cells relative to that in the long multinucleate *cdc7.A20* cells varied with cell cycle progression. SPB association increased in G2 and was maximal just before maximal septation. It subsequently declined to its lowest level after septation before increasing once again early in the next cell cycle (Figure 7C). At all stages, more *cdc7+ stf1.1* cells had Plo1 associated with the SPB than the *cdc.A20 stf1+* cells.

A trivial explanation for the increased affinity for the SPB in *stf1.1* mutants could be that the interphase levels of Plo1 protein are elevated in *stf1.1* cells. We therefore analyzed Plo1 protein levels in a culture of *stf1.1* cells that had been synchronized with respect to cell cycle progression. Plo1 protein levels remained constant throughout the cell cycle (Figure 8). Therefore we conclude that Plo1 is prematurely recruited to the SPB in strains harboring the *stf1.1* mutation.

Plo1 and Stf1 Associate with the SPB Independently of the Presence of Each Other

One possible explanation for the premature recruitment of Plo1 to the SPB in *stf1.1* cells could be that Plo1 binds directly to Stf1/Cut12 and that the *stf1.1* mutation results in inappropriate promotion of this association in a proportion of cells. A prediction of this model is that Plo1 should not bind to the SPB in the absence of Stf1/Cut12 protein; however, Plo1 associated with both of the mitotic SPBs of cells containing the recessive *cut12.1* temperature-sensitive mutation at the restrictive temperature. Plo1 also localized to both mitotic SPBs of germinating spores in which the *stf1/cut12* gene had been replaced with *ura4+* (Bridge *et al.*, 1998) (Figure 9A–C). Conversely Stf1/Cut12 localized to the SPBs of germinating spores in which the *plo1+* gene had been replaced with *ura4+* (Ohkura *et al.*, 1995) (Figure 9D–H). Thus Plo1 does not appear to require functional Stf1/Cut12 protein to associate with the mitotic SPB and vice versa.

DISCUSSION

The original *Drosophila polo[1]* mutation resulted in highly pleiotropic phenotypes (Sunkel and Glover, 1988). This appears to be because the hypomorphic nature of the mutation reveals roles for the enzyme at more than one stage of cell division. This is accentuated by differences in cell cycle regulation in the embryo, in imaginal cells, and in meiosis (Carmena *et al.*, 1998). Recent studies of the function of Plks in budding yeast, *Xenopus*, and mammalian cells point to roles in the regulation of commitment to mitosis, spindle formation, pole maturation, the regulation of APC activity, and in cytokinesis (see INTRODUCTION) (for review, see Glover *et al.*, 1998; Nigg, 1998). Our present characterization of the localization of the fission yeast polo-like kinase Plo1 suggests that recruitment of Plo1 to the SPB may be of key importance in regulating the timing of mitosis and possibly spindle formation.

Plo1 Association with the Mitotic SPB

We have shown that native Plo1 and Plo1 GFP fusion proteins associate with the fission yeast SPB during the early stages of mitosis. Our results extend and are consistent with those of Bahler *et al.* (1998). They showed that the endogenous fluorescence of a fusion protein in which the coding
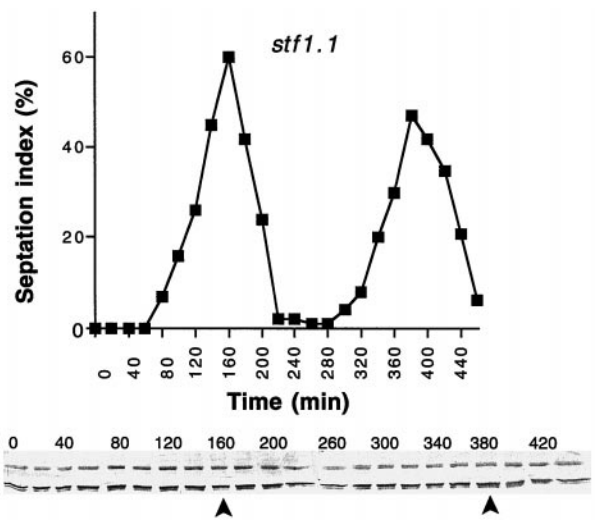
Figure 8. Plo1 protein levels remain constant as *stf1.1* cells progress through the cell cycle. A cell cycle-synchronized population of *stf1.1* cells (IH666) was generated by elutrient centrifugation at 25°C in supplemented EMM2. The small G2 cells were harvested at the beginning of the experiment, and samples were then taken every 20 min for Western blot analysis and scoring the septation index (n = 200 for each time point). The top panel shows the septation index, which indicates good cell cycle synchrony in the culture, whereas the bottom panel shows a Western blot of whole-cell extracts. The blot was cut into two, and the upper portion was probed with antibodies to Plo1 and the lower portion with antibodies to a tubulin (TAT1). The tubulin blot acts as a loading control. The position of the peaks seen in the septation index are indicated by arrows beneath the blots. The relative intensity of the TAT1 and Plo1 bands for each time point were determined for three independent Western blots of both this culture and another synchronous *stf1.1* culture. Plo1 levels showed no significant variation during the time course of either experiment (a total of four distinct cell cycles).

sequence for GFP had been fused to the 3′ end of the *plo1+* ORF resulted in the appearance of one or two nuclear associated dots and that a line was often seen connecting two dots. They found that the dots disappeared during anaphase B. It is most likely from our observations that these dots are the SPBs and that the line between them represents the spindle. Bahler *et al.* (1998) also recorded a ring of Plo1GFP fluorescence that is seen in the middle of the cell at the time when the prophase actin ring would be forming. Our failure to detect such a ring would be consistent with the demonstration that the signal of this ring was disrupted by the fixation protocols that we have used.

SPB association of Plo1 is strong from mitotic commitment until just after the initiation of anaphase B. A Plo1 signal is still seen at the SPB until spindle dissolution, although the signal is very faint. It remains to be determined whether all of the Plo1 in the cell is associated with the equatorial ring and SPB at these stages or whether there is an additional pool of Plo1 kinase executing additional functions at other cellular locations. If there is more Plo1 present in the cell, it is unclear whether the Plo1 that we detect on the SPB is anchored there from the time that the signal is first seen until it goes away again. The staining of the SPB with GFP Plo1 fusion proteins and with antibodies that recognize Plo1 may
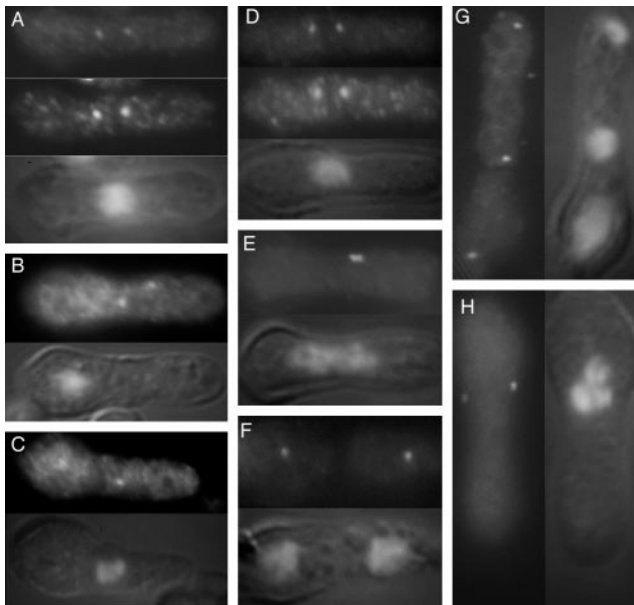
Figure 9. Plo1 associates with the SPB of *stf1/cut12* mutants and Stf1/Cut12 associates with the SPB of cells lacking Plo1. (A–C) Germinating spores from a homozygous *ura4.d18* diploid strain in which one copy of the *stf1/cut12+* gene had been replaced with the *ura4+* gene were inoculated into media lacking uracil and incubated at 30°C for 20 h before processing for immunofluorescence microscopy. The top panels show anti-Plo1 staining, whereas the middle panel shows the result of simultaneous staining with a sheep anti-Sad1 antibody. The bottom panel in each image is a combined DAPI/Nomarski image of the cell in the other panels. The cells in B and C were stained only with Plo1 antibodies. Plo1 localizes to each SPB in all cases, regardless of their lack of Stf1/Cut12 and the inherent inability to execute mitosis. (D–H) Germinating spores from a homozygous *ura4.d18* diploid strain in which one copy of the *plo1+* gene had been replaced with the *ura4+* gene were inoculated into media lacking uracil and incubated at 30°C for 14 h before processing for immunofluorescence microscopy. The top panels in D–F and the left-hand panels in G and H show anti-Stf1/Cut12 staining, whereas the bottom panel in D–F and the right-hand panels in G and H show DAPI DIC images. The middle panel in D shows the result of simultaneous staining with a sheep anti-Sad1 antibody. The cells in E–H were stained only with Stf1/Cut12 antibodies. Stf1/Cut12 localizes to each SPB in all cases, regardless of their lack of Plo1.

have identified Plo1 molecules transiently associating with the SPB. In this scenario Plo1 may require SPB association for activation before diffusing away to execute specific functions at distal sites. In such a situation the SPB would merely be a loading site for the formation of an active complex so that controlled biochemical events at the SPB would have global consequences. This speculation would be testable by photobleaching of the GFP-tagged Plo1 at the SPB to determine whether the SPB remains dim throughout the remainder of mitosis.

Stability of Plo1

In budding yeast, Cdc5p activates the APC and is degraded by APC at the end of anaphase (Charles *et al.*, 1998;

Shirayama *et al.*, 1998). Similarly, Plk protein levels fluctuate in mammalian cells, although it is unclear whether this is due to cell cycle-regulated degradation or to the constitutive turnover of proteins that are transcribed periodically; however, Fang and coworkers (1998) report that in *Xenopus* extracts Plk1 is an APC substrate. In contrast, in *S. pombe* Plo1 levels do not undergo drastic fluctuations at any cell cycle stage, suggesting that regulation is mediated by posttranslational modification, compartmentalization, or association with regulatory subunits; however, it is difficult to rule out the possibility that discrete subpopulations of Plo1 undergo destruction at specific locations.

Anaphase Redistribution as a Common Theme for Polo Kinases

The redistribution of Plo1 during anaphase and the need for APC function to accomplish this rearrangement is reminiscent of other systems. In *S. cerevisiae*, Cdc5p is associated with the SPB until APC activation (Shirayama *et al.*, 1998). In mammals and *Drosophila*, Plks redistribute from the spindle poles and the kinetochores to the interzone microtubules during anaphase to finally partition to the midbody during telophase (Golsteyn *et al.*, 1995; Lee *et al.*, 1995; Adams *et al.*, 1998; Logarinho and Sunkel, 1998). This redistribution is possibly mediated in part by the MKLP kinesin-related protein, which is the CHO1 antigen in mammalian systems and the product of the *pavarotti* gene in *Drosophila* (Nislow *et al.*, 1992; Lee *et al.*, 1995; Adams *et al.*, 1998). Polo associates with this kinesin-related protein in both systems, and mammalian Plk can phosphorylate MKLP1. Mutation of *pavarotti* results in a considerable reduction in the number of interzone microtubules and abolishes Polo relocation to the interzone. Conversely, *polo* mutants fail to relocate Pavarotti protein to the interzone of late anaphase and telophase spindles in male meiosis. Mutation of either gene leads to defects in cytokinesis (Adams *et al.*, 1998; Carmena *et al.*, 1998). These data suggest that Polo redistribution to the midzone on microtubules is required for it to correctly regulate cytokinesis in higher systems. Moreover, Polo may also regulate its own relocation by controlling the activity of the MKLP1 motor.

The maintenance of the central position of the septum when the interzone has wandered >10 μm from the centroid (Hagan and Hyams, 1988) indicates that cytokinesis in fission yeast is independent of the central overlap zone of the fission yeast mitotic spindle. Moreover, Plo1 is only detected at the site of cytokinesis much earlier during mitosis (Bahler *et al.*, 1998); however, despite these differences in the organization and execution of cytokinesis and Plo1 distribution, the fact that polo kinases are required for cytokinesis in such diverse systems suggests that although the spatial coordination of polo kinase function may be different in different systems, the downstream effectors in these diverse systems may be similar.

SPB Association and the Control of Septation

The localization of the septation regulator Spg1 to the SPBs raises the possibility that Plo1 recruitment to the SPB may be a crucial event in the regulation of septation. Activation of Spg1 results in a physical interaction with the protein kinase Cdc7 and the recruitment of Cdc7 to the SPB (Sohrmann *et*

al., 1998). Thus, Plo1 association with the SPB at the beginning of mitosis may enable the Spg1 pathway.

If SPB-associated Plo1 does indeed regulate the commitment to septation by activating the SPB-bound Spg1 pathway, it should be possible to bypass this requirement by activating the downstream Spg1 cascade independently of SPB-bound Plo1. We show that this does in fact happen. Spg1 overexpression or Cdc16 inactivation drives inappropriate septation in interphase in the absence of previous SPB association of Plo1. The ability of Plo1 to both drive septation and recruit Cdc7 to the SPB upon overexpression would also put Plo1 upstream of Spg1; however, whether this is a direct or indirect effect is currently still unclear. There must be some feedback, however, from the Spg1 pathway because Plo1 was inappropriately recruited to the interphase SPB by inactivation of Cdc7 protein kinase. Thus, loss of the function of the Spg1 effector increases the affinity of Plo1 for the SPB in interphase; however, there are some problems with such a straightforward interpretation because it is unclear why the *cdc11* mutant does not have an equivalent phenotype. Genetic data suggest that *cdc11*[+] acts antagonistically to *cdc16*[+] (Marks *et al.*, 1992). Thus, loss of Cdc11 function might be expected to have the same consequences as loss of Cdc7. A simple explanation is that the influence of Cdc7 on Plo1 recruitment to the pole is a functionally distinct role compared with its previously characterized role in Spg1 association.

It is equally possible that association of Plo1 with the SPB may be entirely independent of its function in septation. In this case, its role at the SPB may only be required until chromosome segregation is initiated, and consequently it is lost from the SPB after activation of the APC, as we have observed. The reduction of the affinity of Plo1 for the SPB could also require a specific function of Cdc7, which also undergoes reorganization at the SPBs at this time. Whichever model emerges as reflecting the in vivo status, it is significant that Plo1 can remain at the SPB in cells in which mitosis is blocked as a result of a number of mutations and yet cytokinesis still occurs. This indicates that disassociation of Plo1 from the SPB is not a requirement for cytokinesis.

Localization of Plo1 to the SPB and Commitment to Mitosis

It has been established previously that the formation of the fission yeast mitotic spindle occurs in the presence of an intact cytoplasmic microtubule cytoskeleton (Hagan and Yanagida, 1995). Because this array persists throughout interphase, it suggests that full commitment to mitosis is not an instantaneous process in fission yeast. It is therefore significant that Plo1 associates with the SPB to give a single dot of fluorescence for a considerable time before the presence of two distinct SPBs signal the formation of the prophase spindle. Thus, there is an intact interphase cytoskeleton when there is a single spot of Plo1 on the SPB. Because association of Plo1 with the SPB is dependent on previous activation of MPF, it is the earliest biochemical or cytological mitotic event that has been determined for fission yeast to date.

Commitment to mitosis involves the activation of a positive feedback loop whereby a trigger level of MPF is amplified by a downstream kinase through the activation of p34[cdc2] phosphatase Cdc25 (Hoffmann *et al.*, 1993; Kuang *et*

al., 1994; Izumi and Maller, 1995; Kovelman and Russell, 1996). Data from the analysis of Plx1 support the notion that polo kinases play a key role in this feedback loop (Kumagai and Dunphy, 1996; Abrieu *et al.*, 1998; Qian *et al.*, 1998a). The timing of Plo1 association with the SPB would be coincident with the activation of this feedback loop. It has been reported previously that semidominant mutants in the SPB component encoded by the *stf1/cut12*[+] gene bypass the requirement for Cdc25 activation of MPF (Hudson *et al.*, 1990; Bridge *et al.*, 1998). If Wee1 is inhibited at the same time that Cdc25 is activated during the amplification loop, it is entirely possible that cells harboring the *stf1.1* mutation no longer require Cdc25 function because they are inappropriately triggering the feedback loop to inhibit Wee1 in the absence of previous stimulation by MPF. If this were the case, premature mitotic commitment would be inhibited by a dominant size control that probably acts through the regulation of Wee1 (Nurse, 1975; Fantes, 1977; Fantes and Nurse, 1977). Once the restraint of this size control is removed in *stf1.1* mutants, the inappropriate activation of the feedback loop would drive cells into mitosis.

The ability of Plo1 to associate with the SPBs of cells in which the *stf1/cut12*[+] gene has been mutated recessively to abolish its function suggests that the effect of Stf1/Cut12 on Plo1 recruitment is unlikely to be mediated by a direct interaction between the two proteins, or if it is, that Plo1 interacts with multiple SPB components. The recently identified polo kinase kinase that is distinct from p34[cdc2] in *Xenopus* could be a candidate for the kind of molecule that could bridge an indirect link between Stf1/Cut12 and Plo1 function (Qian *et al.*, 1998b). Whatever the molecular basis underlying this phenomenon, the premature localization of Plo1 to the SPB in *stf1.1* mutants reveals a close relationship between Plo1 recruitment to the SPB and mitotic commitment. Together the data that we present support the notion that association with the spindle poles could be a key mechanism through which Plks regulate mitosis and cytokinesis.

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