Chemical genetic analysis of the regulatory role of Cdc2p in the *S. pombe* septation initiation network

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Summary

The protein kinase Cdc2p is the master regulator of cell cycle progression in the fission yeast *Schizosaccharomyces pombe*. It is required both for entry into mitosis and for onset of DNA replication. Cdc2p must be inactivated to permit exit from mitosis, licensing of replication origins and cytokinesis. To study the role of Cdc2p in greater detail, we generated a *cdc2* allele that is sensitive to an inhibitory ATP analogue. We show that the inhibitor-induced cell cycle arrest is reversible and examine the effect of inhibiting Cdc2p on the regulation of the septation initiation network (SIN), which controls the initiation of cytokinesis in *S. pombe*. We found that specific inactivation of Cdc2p in a mitotically arrested cell promotes the asymmetrical recruitment of SIN proteins to the spindle poles and the recruitment of the most downstream SIN components and β-(1,3) glucan synthase to the contractile ring. Thus, we conclude that inactivation of Cdc2p is sufficient to activate the SIN and promote cytokinesis.

Key words: Chemical genetics, Cell cycle, cdc2, Cytokinesis, Yeast

Introduction

The activity of the cyclin-dependent kinase (CDK) Cdc2p is pivotal for cell cycle progression in *S. pombe* and functional homologues of Cdc2p, commonly referred to as CDK1, are found throughout the eukaryote kingdom (Berthet and Kaldis, 2007; Kaldis and Aleem, 2005; Lee and Nurse, 1987; Malumbres and Barbacid, 2005). Cdc2p is required both for the onset of S phase and entry into mitosis (Nurse and Bissett, 1981), and must be inactivated to permit exit from mitosis, licensing of replication origins and cytokinesis. Cdc2p is also important for establishing the dependency of mitosis on S-phase completion and to prevent re-replication (Broek et al., 1991; Wuarin et al., 2002).

Cdc2p activity is regulated by association with cyclins and by phosphorylation (Gould, 2004). Cdc2p is phosphorylated on Thr167 (Gould et al., 1991) by CDK-activating kinase (Hermand et al., 1998; Lee et al., 1999), which promotes association of Cdc2p with the cyclin regulatory subunits, Cdc13p, Cig1p, Cig2p and Puc1p, during vegetative growth. Assembly of the Cdc2p-cyclin complexes is also facilitated by chaperones (Munoz and Jimenez, 1999; Turnbull et al., 2006). The cyclin Cig2p promotes S phase (Fisher and Nurse, 1996; Martin-Castellanos and Moreno, 1996; Mondesert et al., 1996), and Puc1p is thought to participate in the G1 size control (Martin-Castellanos et al., 2000). The B-type cyclin Cdc13p (Booher and Beach, 1988; Hagan et al., 1988) is the sole fission yeast cyclin able to promote G2-M transition and can also initiate S-phase (Fisher and Nurse, 1995; Fisher and Nurse, 1996). Cdc2p is also phosphorylated on Tyr15 (Gould and Nurse, 1989) by wee1p (Russell and Nurse, 1987), which inhibits the Cdc2p-Cdc13p complex during interphase. Mitosis is initiated through dephosphorylation of Tyr15 by the phosphoprotein phosphatase Cdc25p (Russell and Nurse, 1986). Active Cdc2p-Cdc13p is localised in the nucleus, and also associates with the spindle and spindle poles during mitosis (Alfa et al., 1989; Alfa et al., 1990; Decottignies et al., 2001; Yanagida et al., 1999).

The use of thermosensitive mutants to study the roles of Cdc2p in the cell cycle is subject to several limitations. The temperature change provokes a heat-shock stress response; thermosensitive mutant alleles may not be inactivated rapidly, complicating the interpretation of experiments conducted over a short time interval. Finally, the range of permissive temperatures of the available collection of thermosensitive mutants may not permit certain processes to be examined. We have therefore used a chemical genetics strategy (Bishop et al., 2000) to study Cdc2p function, in particular its role as a regulator of SIN proteins, which regulate cytokinesis.

The *S. pombe* SIN is a signal transduction network that regulates the initiation of septum formation (reviewed by Krapp et al., 2004; Wolfe and Gould, 2005). The core of the signalling module comprises three protein kinases and their associated subunits: Cdc7p-Spg1p (Fankhauser and Simanis, 1994; Schmidt et al., 1997; Sohrmann et al., 1998), Sid1p-Cdc14p (Fankhauser and Simanis, 1993; Guertin et al., 2000) and Sid2p-Mob1p (Hou et al., 2000; Salimova et al., 2000; Sparks et al., 1999). The SIN is activated by the polo-like kinase Plo1p (Tanaka et al., 2001), which regulates many aspects of mitosis in addition to cytokinesis. These proteins associate with the spindle pole body (SPB), where they bind to a scaffold comprised of the coiled-coil proteins Cdc11p (Krapp et al., 2001; Tomlin et al., 2002), Sid4p (Chang and Gould, 2000) and Ppc89p (Rosenberg et al., 2006). Signalling through the pathway is mediated by the nucleotide status of the GTPase Spg1p, which is regulated by the GTPase-activating protein (GAP) Byr4p-Cdc16p (Fankhauser and Simanis, 1993; Furge et al., 1998; Song et al., 1996). The core kinases only associate with the SPB during mitosis (reviewed by Simanis, 2003). Cdc7p binds to Spg1p-GTP on the SPBs from mitotic entry (Sohrmann et al., 1998), and becomes asymmetrical after the anaphase A-B transition, localising only to the new SPB (Grallert et al., 2004). By contrast, Sid1p and Cdc14p

associate only with the new SPB and only after the onset of anaphase B (Guertin et al., 2000). Sid2p and Mob1p are located on both spindle poles throughout mitosis, and also associate with the medially placed contractile ring just prior to septum formation (Hou et al., 2000; Salimova et al., 2000; Sparks et al., 1999). Arresting cells in early mitosis using a β-tubulin mutant prevents Sid1p binding to the SPB (Guertin et al., 2000) and Sid2p-Mob1p localisation to the contractile ring (Hou et al., 2000; Salimova et al., 2000; Sparks et al., 1999). It is not clear whether the anaphase-specific localisation of SIN proteins is regulated by the anaphase-promoting complex (APC/C), inactivation of mitotic CDK, or both (Chang et al., 2001).

The data presented here indicate that inactivation of Cdc2p using an inhibitory ATP analogue is sufficient to promote (1) association of Sid1p with one SPB in early mitotic cells, (2) the transition from a symmetrical to an asymmetrical distribution of Cdc7p on the SPBs, and (3) recruitment of Mob1p to the contractile ring, thereby creating what is presumed to be the active configuration of the SIN. Consistent with this, the enzyme Bgs1p, which is required for synthesis of the division septum, is also recruited to the contractile ring, and cells form a division septum without completing mitosis. Thus, we conclude that inactivation of Cdc2p in mitotic cells is sufficient to activate the SIN and promote septum formation.

Results

Creation and characterisation of the *cdc2-as* mutant

We have created an 'analogue-sensitive' mutant allele of *cdc2*, which has a modified ATP-binding pocket to accommodate an ATP analogue that inhibits the modified kinase specifically (Bishop et al., 2000). Previous studies (Bishop et al., 2000; Liu, Y. et al., 1999) have demonstrated that mutation of the active site of *S. cerevisiae* Cdc28p renders it sensitive to the inhibitor 4-amino-1-*tert*-butyl-3-(1′-naphthylmethyl)pyrazolo [3,4-d] pyrimidine (hereafter referred to as analogue). Since *S. pombe cdc2*$^+$ and *S. cerevisiae CDC28* are functionally equivalent and will cross-complement (Beach et al., 1982; Booher and Beach, 1986), we made the mutation F84G in *cdc2*$^+$, which we refer to hereafter as *cdc2-as*. This was integrated into the *cdc2*$^+$ locus or into the *leu1* locus followed by crossing into a *cdc2::ura4*$^+$ background (see Materials and Methods).

A range of analogue concentrations was added to exponentially growing *cdc2-as* and *cdc2*$^+$ cells; cell number was determined, and fixed cells were examined after staining with DAPI and Calcofluor. Analogue concentrations up to 15 μM, or an equivalent volume of its solvent DMSO, had no effect upon the division of wild-type cells (Fig. 1A and not shown). Addition of analogue to 1 μM blocked the division of *cdc2-as* cells at 25°C within one cell cycle (Fig. 1A). Determination of the execution point from the residual cell number increase after addition of analogue yielded a value of 0.7, consistent with those determined for *cdc2* alleles previously (Nasmyth and Nurse, 1981; Nurse et al., 1976). Examination of fixed, DAPI-Calcofluor stained c*dc2-as* cells showed that they became elongated and contained a single nucleus upon treatment with 1 μM analogue (Fig. 1B); no effect was observed in a *cdc2*$^+$ control. FACS analysis showed that cells arrested with a 2C DNA content (Fig. 1C). As described previously, the FACS profile shifts to the right as cells elongate, owing to an increase in mitochondrial DNA content (Sazer and Sherwood, 1990).

To confirm that the cells were arrested in G2, we stained them with Rhodamine-conjugated phalloidin. This revealed the presence of F-actin patches at both tips of the cell (98% two-end staining, 2% one-end staining and no medial rings; 3.5h after adding analogue), indicating a post-NETO G2 arrest (Fig. 1D). Taken

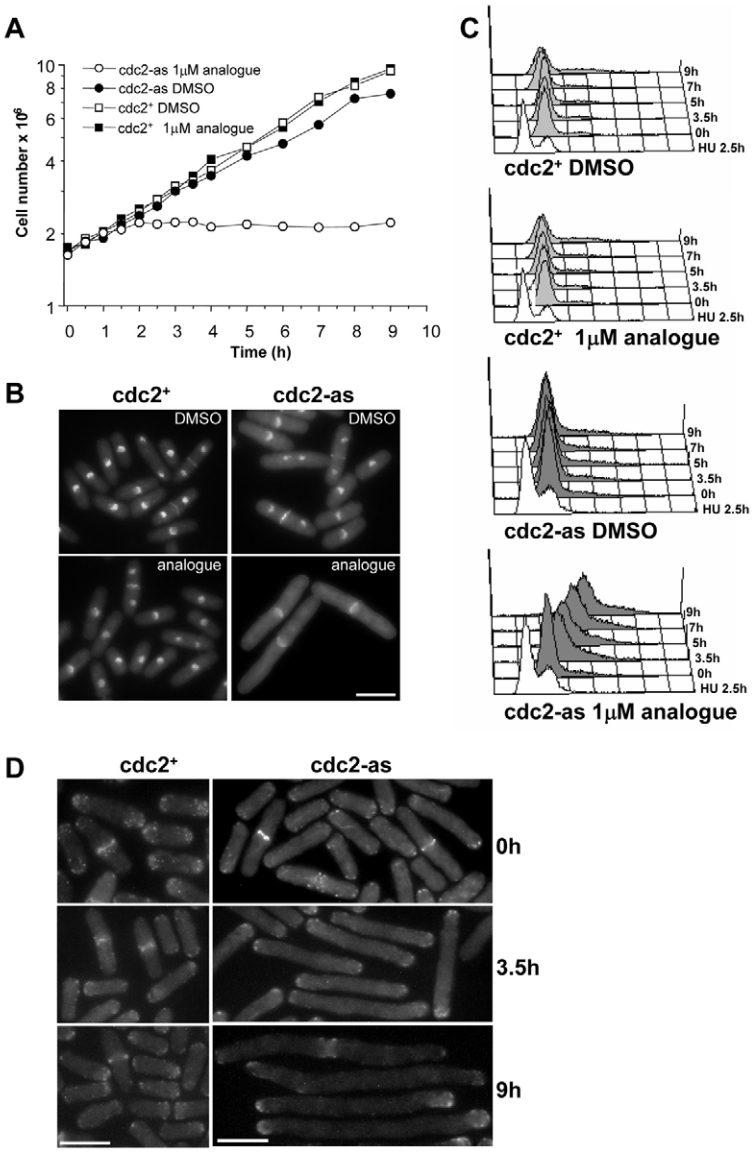


Fig. 1. The *cdc2-as* mutant is sensitive to analogue. (A) Wild-type and *cdc2-as* cells were grown in YE medium to early exponential phase and analogue was added to a concentration of 1 μM; the controls received an equal volume of DMSO. Cell number was determined at intervals. (B) Micrographs of the cells described in panel A, stained with DAPI and Calcofluor. Cells were incubated for 3.5 hours at 25°C after addition of analogue. (C) FACS analysis of the cells described in (A). HU 2.5h are cells treated for 2.5 hours with 12 mM hydroxyurea, to generate G1 and G2 peaks as 1C and 2C references. Note that in exponential growth, an *S. pombe* FACS profile shows only a single peak, corresponding to 2C DNA content. This is because G1 is very short and cells have completed S-phase before the daughters separate. (D) Samples from the experiment shown in panel A were fixed and stained with Rhodamine-conjugated phalloidin to reveal F-actin. Scale bars: 10 μm.

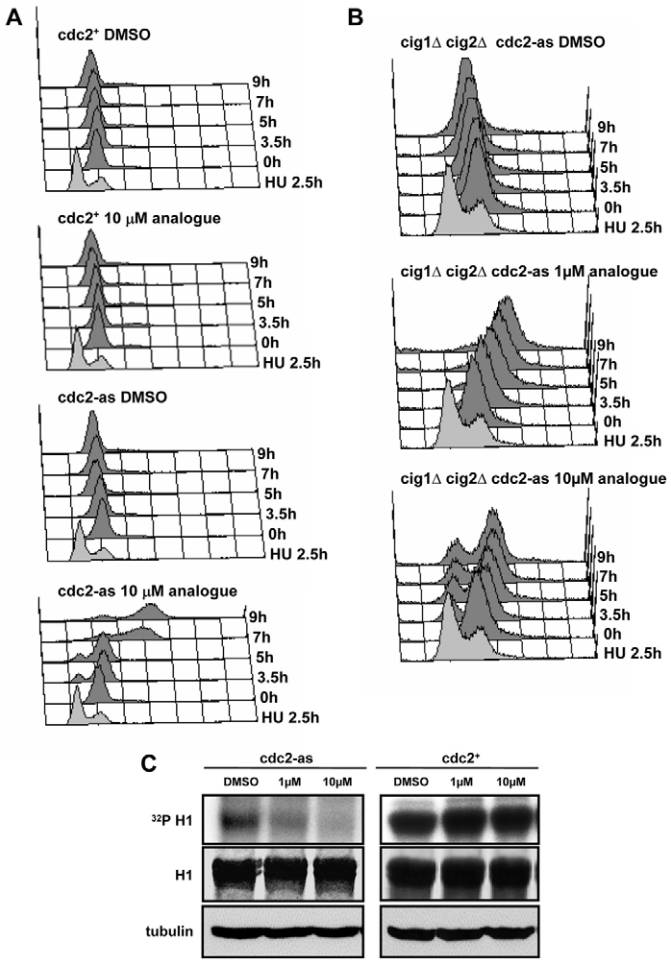


Fig. 2. The effects of analogue on *cdc2-as* division and kinase activity. (A,B) The indicated cells were grown in YE medium to early exponential phase and analogue was added to the indicated final concentrations; the controls received an equal volume of DMSO. Samples were removed at the indicated times and analysed by FACS. (C) A protein extract was prepared from *cdc2-as* 10 minutes after release from the analogue arrest. As a control, protein extracts were prepared from $cdc2^+$ *cdc25-22* arrest-release-synchronised cells (20 minute time point, when cells are in early mitosis and Cdc2p activity is high) and assayed. Analogue, or an equivalent volume of solvent, was added to the extracts at the indicated concentrations, and an H1 kinase assay was performed. The upper panel shows a scanned autoradiograph of the phosphorylated H1. The middle panels show the Coomassie-Blue-stained histone H1 and the bottom panels show a western blot of the extracts used for the assay, probed with TAT-1 antibody as loading control. Note that the kinase activity is reduced by addition of analogue to the *cdc2-as* extract, but not the $cdc2^+$ extract.

together, these data indicate that *cdc2-as* arrests predominantly in G2 in the presence of 1 μM analogue.

The stage in the cell cycle at which the *S. cerevisiae CDC28-as* mutant arrests depends upon the concentration of analogue used (Bishop et al., 2000). Therefore, we tested whether higher concentrations of analogue would arrest *cdc2-as* in both G1 and G2. Treatment of *cdc2-as* with 10 μM analogue arrested cells in both G1 and G2, with the former corresponding to approximately 15% of cells (Fig. 2A; 3.5h and 5h). This is similar to *cdc2* mutants, which arrest both before START and mitosis (MacNeill et al., 1991; MacNeill and Nurse, 1993; Nurse and Bissett, 1981). As before, no effects were observed on the control $cdc2^+$ cells (Fig. 2A).

Table 1. The *cdc2-as* mutant diploids in stationary phase

Cell density ($\times 10^{-6}$ per ml)	$cdc2^+$ (% diploids)	*cdc2-as* (% diploids)
1	<0.2	<0.2
3	<0.2	<0.2
10	<0.2	<0.2
Stationary 24 hours	<0.2	3
Stationary 48 hours	<0.2	23
Stationary 72 hours	<0.2	44

A haploid $cdc2^+$ or *cdc2-as* colony was inoculated into YE medium at 25°C in the absence of analogue and the culture was sampled at various time points. Diploids were identified by their darker colour after replica-plating colonies to YE medium containing Phloxin B (Moreno et al., 1991). Samples were taken at the indicated cell densities, and after 1, 2 and 3 days in stationary phase (taken to be $>2\times10^7$ cells/ml^{-1}). At least 500 colonies were counted for each point.

Prolonged incubation resulted in an abrupt doubling of DNA content between 5 and 7 hours (Fig. 2A). This is not due to cell elongation, which produces a gradual shift of FACS peaks to the right (Sazer and Sherwood, 1990); for examples, see Fig. 1C and Fig. 2B. The abrupt increase in DNA content is consistent with the diploidisation due to re-replication of DNA without mitosis that is observed in some *cdc2* mutants following nitrogen starvation and heat-shock in G2 (Broek et al., 1991), as well as the *cdc13* null mutant (Fisher and Nurse, 1996).

Re-replication of the genome induced by a variety of treatments requires the cyclins Cig1p and Cig2p (Connolly and Beach, 1994; Snaith and Forsburg, 1999). Therefore, we treated the triple mutant *cdc2-as cig1Δ cig2Δ* with the analogue. Like the *cdc2-as* cells, the triple mutant arrested in G2 when treated with 1 μM analogue (Fig. 2B), and in both G1 and G2 when treated with 10 μM analogue. The gradual shift caused by cell elongation is still seen, as in Fig. 1C and Fig. 2A. However, in contrast to Fig. 2A, no increase in ploidy was observed at later time points, indicating that the re-replication requires Cig1p and Cig2p (Fig. 2B).

We also noted a significant tendency for the *cdc2-as* mutant to become diploid in the stationary phase (Table 1), because 44% of *cdc2-as* cells were diploid after three days in stationary phase. This suggests that *cdc2-as* cells may be less competent than $cdc2^+$ in making an orderly exit from the cell cycle into stationary phase even in the absence of analogue.

To test whether the kinase activity of Cdc2p was inhibited by the analogue, a kinase assay was performed on protein extracts from either *cdc2-as* or $cdc2^+$ cells. Addition of analogue at either 1 μM or 10 μM inhibited the kinase activity in *cdc2-as* extracts, but not $cdc2^+$ extracts (Fig. 2C), consistent with the lack of effect of the analogue on $cdc2^+$ cells in vivo (see above).

In summary, we have constructed a *cdc2-as* mutant that is specifically inhibited by an ATP analogue. In vitro, addition of analogue inhibits Cdc2p activity, whereas in vivo, *cdc2-as* cells arrest either in G2, or at both major commitment points in the cell cycle, depending upon the concentration of analogue.

Effects of *cdc2-as* upon meiosis

Since previous studies had demonstrated that some *cdc2* mutants block progression through meiosis II, producing dyads, rather than tetrads (Hayles et al., 1986; Nakaseko et al., 1984), we examined whether the *cdc2-as* mutant had any effects upon meiosis. Crosses between *cdc2-as* h^+ and h^- were performed in the absence of analogue at 25°C. After two days, the majority of spore-containing

cells (65%) were dyads and only 2% of zygotes in which spores were seen gave rise to tetrads (Fig. 3A). Other phenotypes were also observed, including spores that contained no DAPI-stained material, and nuclei that had failed to become encapsulated into spores (Fig. 3A); the variety of phenotypes probably reflects multiple roles for Cdc2p in meiosis. Control crosses between *cdc2*$^{+}$ strains produced >99% tetrads (not shown). To investigate which meiotic division had failed in the dyads, crosses were performed using the centromere-linked *lys1-131* mutant in one of the mating partners, followed by dissection of dyads; 94% of dyads in *cdc2-as* crosses showed a 1:1 lys^{+}:lys^{-} phenotype. This was similar to the result obtained in a similar cross using *cdc2-N22* (Nakaseko et al., 1984), where 96% of dyads showed a 1:1 lys^{+}:lys^{-} phenotype. We conclude that the compromised activity of *cdc2-as* interferes mainly with meiosis II.

Genetic interactions of *cdc2-as*

In addition to its analogue-sensitive phenotype, the *cdc2-as* mutant is thermosensitive. To analyse the phenotype, *cdc2-as* cells were synchronised by elutriation at 25°C and shifted to 36°C in the presence of analogue, or DMSO. After 3 hours at 36°C, cells treated with DMSO were elongated; however, 23% of cells had a single septum between two nuclei, indicative of continued division, and 11% of cells had a septum in a mononucleate cell (Fig. 3B). Addition of analogue to 10 μM at the time of shift to 36°C gave rise to >97% elongated mononucleated cells after 3 hours (Fig. 3B). Similar data were obtained for 1 μM analogue (not shown). These data indicate that although it is thermosensitive, the *cdc2-as* mutant retains significant activity at 36°C in the absence of analogue. Mutation of the Cdc2p-associated protein Suc1p (Hayles et al., 1986), can rescue some alleles of *cdc2*. However, crosses to either *suc1-210* or *suc1-449* showed that *cdc2-as* thermosensitivity could not be suppressed by mutation of *suc1* (data not shown). At 19°C, *cdc2-as* cells are slightly elongated compared with cells incubated at 25°C, suggesting the mutant may also be cold sensitive; consistent with this, a *cdc2-as nda3-KM311* mutant showed a significant delay in entry into mitosis at 19°C (not shown), compared with the cold-sensitive β-tubulin mutant *nda3-KM311* alone (Umesono et al., 1983).

Studies in budding yeast have shown that increased expression of mitotic cyclins can rescue heat-sensitive mutants of Cdc28p (Surana et al., 1991). Therefore, we tested whether the introduction of a plasmid containing a genomic clone of the mitotic cyclin *cdc13*, expressed from its own promoter, could rescue the thermosensitivity and analogue sensitivity of *cdc2-as*. We observed that the heat sensitivity of *cdc2-as* was rescued by *cdc13* (Fig. 3C). Treatment of *cdc2-as* cells expressing *cdc13* with 1 μM analogue at 25°C no longer resulted in a cell cycle arrest (Fig. 3D). However, addition of analogue to 10 μM arrested cells both in G1 and G2 (Fig. 3D).

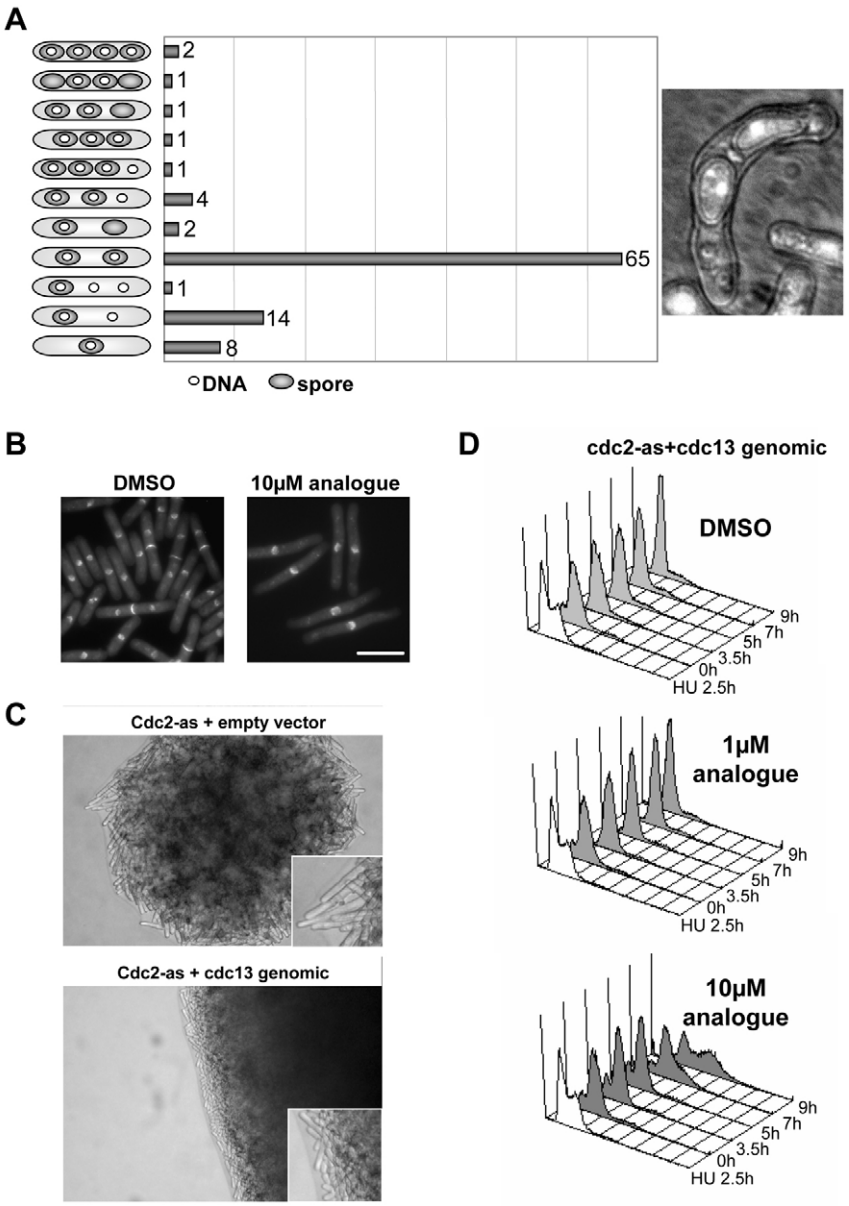


Fig. 3. The effects of *cdc2-as* on meiosis, and rescue by increased expression of *cdc13*. (A) A cross of *h*$^{+}$ *cdc2-as* with *h*$^{-}$ *cdc2-as lys1-131* was performed at 25°C. After 2 days, the mating mixture was suspended in PBS, fixed, and stained with DAPI and Calcofluor. The outcomes of the mating are indicated on the histogram. The figures indicate the percentage of each outcome class; more than 300 spore-containing cells were scored. The combined DAPI and transmission image of a dyad is shown. (B) Small cells were selected from an exponentially growing *cdc2-as* culture by centrifugal elutriation, and inoculated into fresh medium in the presence of 10 μM analogue or DMSO and incubated for 3 hours at 36°C. Cells were fixed and stained with DAPI and Calcofluor. (C) *cdc2-as* cells were transformed with a plasmid containing the genomic copy of *cdc13* or empty vector. Colonies were allowed to form on EMM2 medium at 25°C, replicated to 36°C and incubated at 36°C overnight. Colonies were photographed. The inset shows a magnification of the edge of the colony. (D) The cells described in panel C were treated with the indicated concentrations of analogue at 25°C and samples were analysed by FACS. Scale bars: 10 μm.

Genetic analysis revealed that the *cdc13-117 cdc2-as* mutant was inviable at any temperature; the double mutants germinated and died as elongated cells, mostly without dividing (data not shown). This was also true for the tagged *cdc13-myc*$_{13}$ allele (Cueille et al.,

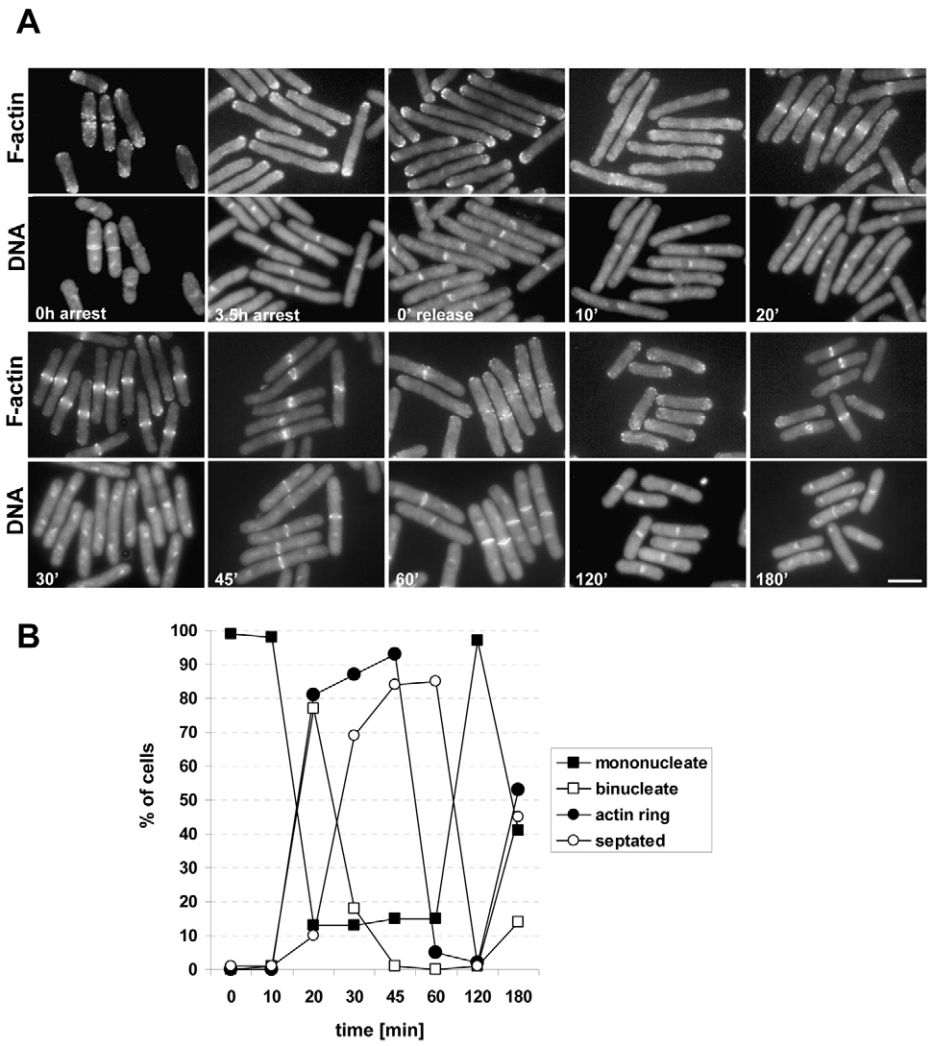


Fig. 4. The analogue-induced cell cycle arrest is reversible. (A) Exponentially growing *cdc2-as* cells were treated with 1 μM analogue for 3.5 hours at 25°C. Cells were washed and resuspended in fresh medium. Samples were removed and stained with DAPI and Calcofluor (DNA) or Rhodamine-conjugated Phalloidin (F-Actin). (B) The progression of the cells described in panel A through mitosis was plotted. Cells with two nuclei and no division septum were scored as binucleate. Scale bars: 10 μm.

2001). Taken together with the synthetic-lethal interaction between *cdc13-117* and *cdc2-as*, these results are consistent with the view that the interaction of Cdc2p with Cdc13p may be compromised in the *cdc2-as* mutant.

Double mutants between *cdc2-as* and mutants in other genes involved in mitotic control were analysed by tetrad dissection and replica plating. The double mutant with *cdc25-22 cdc2-as* did not show any additive effects compared with *cdc25-22*, and *wee1-50* did not rescue the thermosensitivity of *cdc2-as* (data not shown). To examine whether *cdc2-as* is dominant to wild-type $cdc2^+$, analogue was added to a $cdc2^+$ strain with *cdc2-as* inserted at *leu1*. Cells were indistinguishable from the wild type at all concentrations tested (not shown), demonstrating that *cdc2-as* is recessive to wild-type $cdc2^+$.

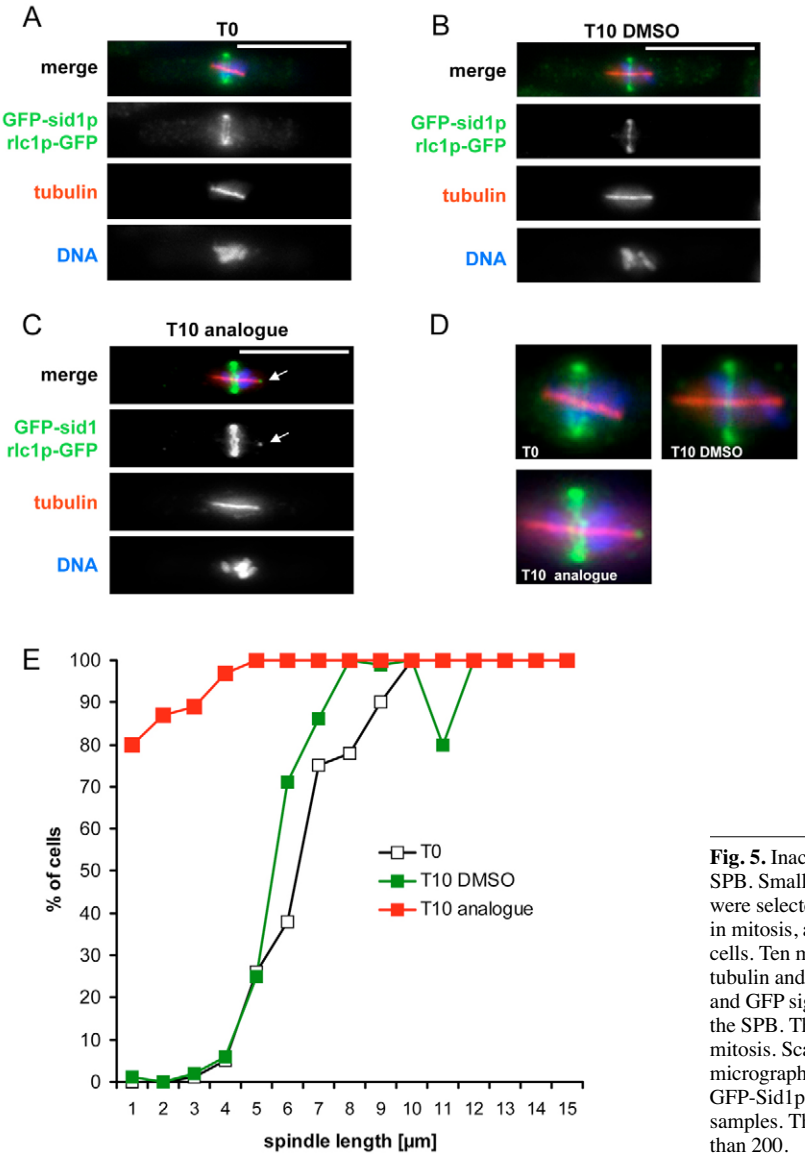
Analogue-induced cell cycle arrest is reversible

To test whether treatment with the analogue was reversible, *cdc2-as* cells were incubated in medium containing 1 μM analogue for 3.5 hours at 25°C, which corresponds to just over one cell cycle. Cells were then washed to remove the analogue, and resuspended in fresh medium. Samples were fixed and stained with DAPI, Calcofluor and Rhodamine-conjugated phalloidin to follow mitotic progression. The results, shown in Fig. 4A,B, indicate that the arrest is reversible and produces a highly synchronous progression through mitosis, comparable with that obtained by *cdc25-22* arrest-release (Moreno et al., 1989). Analogue-induced arrest release therefore provides a method to synchronise cells without the stress of a thermal shock.

Does Cdc2p inhibit the SIN in early mitosis?

SIN proteins display a major change in their localisation that is associated with the anaphase A-B transition. For example, arrest-release experiments using *nda3-KM311* have shown that the SIN regulator Sid1p associates with the SPB only when Cdc13p is no longer detectable and Cdc2p kinase has been inactivated (Guertin et al., 2000). To test whether inactivation of *cdc2-as* in mitotically arrested cells would promote recruitment of Sid1p to the SPB, GFP-Sid1p *cdc2-as* cells were transformed with a plasmid expressing *mad2*, which delays anaphase onset and CDK inactivation by interfering with APC/C function (He et al., 1997). This allowed us to investigate the effect of inactivating Cdc2p in cells where APC/C activity is reduced without the stress of a temperature shift; note that the delay in entry into mitosis of the *nda3-KM311 cdc2-as* mutant precludes its use for generating a mitotically arrested population of cells. The *mad2* overexpression-induced cell cycle arrest is leaky, therefore cells were synchronised by centrifugal elutriation after induction, to maximise the number of cells in early

mitosis (see Materials and Methods). As cells entered mitosis, a sample was removed from the culture and fixed, and then either analogue or DMSO was added to half the remaining culture. After 10 minutes, cells were fixed and the samples were stained with antibodies to α-tubulin and GFP. Since the arrest is leaky, a range of spindle lengths was observed. Before addition of analogue, no association of GFP-Sid1p with spindles ⩽3 μm was observed, and only 5% of 4 μm spindles had GFP-Sid1p on one SPB (Fig. 5A,D,E). By contrast, almost all spindles longer than 6 μm had GFP-Sid1p associated with one SPB (Fig. 5A,D,E). At 10 minutes, the DMSO control showed a similar distribution of GFP-Sid1p (Fig. 5B,D,E). This is consistent with the expectation that GFP-Sid1p only associates with the SPB after the anaphase A-B transition. By contrast, the culture treated with analogue for 10 minutes had GFP-Sid1p associated with more than 80% of spindles that were ⩽3 μm long and 96% of spindles of 3-5 μm length (Fig. 5C,D,E). The distribution of spindle lengths was similar before and after addition of the analogue (data not shown). We therefore conclude that inactivation of Cdc2p-as is sufficient to promote GFP-Sid1p recruitment to the SPB in a cell that has entered mitosis.



A similar experimental approach was used to examine the effect upon other SIN and medial ring proteins. The results are summarised in Table 2. Early in mitosis, the protein kinase Cdc7p associates with both SPBs, becoming asymmetrical during anaphase B (Sohrmann et al., 1998), when it remains associated with the new SPB (Grallert et al., 2004). After addition of analogue, only 12% of cells with spindles ⩽3 μm had Cdc7p-GFP associated with both SPBs, compared with 75% in the solvent control (Table 2). We also examined Mob1p-GFP, which associates with the medial ring just prior to its contraction, and with the SPBs throughout mitosis. We found that inactivation of Cdc2p-as promoted association of Mob1p-GFP with the medial ring in 80% of cells with short spindles (Table 2; Fig. 6A), compared with 22% in the control. Moreover, we observed that in the cells where Mob1p-GFP associated with the medial ring after addition of analogue, 25% of the Mob1p-GFP signals were split into a double ring, which is usually seen only during septation (Hou et al., 2000; Salimova et al., 2000).

Next, we investigated whether the APC/C^{Cdc20p} substrates Cdc13p and Cut2p were affected by addition of analogue. First, *cdc2-as cdc13-GFP* overexpressing *mad2* was arrested in mitosis, and analogue was added to 1 μM. Indirect immunofluorescence revealed that Cdc13p-GFP was present in the nuclei of analogue-treated cells, indicating that the altered localisation of SIN proteins did not result from degradation of Cdc13p (Fig. 6B). To analyse the behaviour of Cut2p, we used the strain *cdc2-as cut2-GFP* overexpressing *mad2*. Technical reasons prevented us from generating cultures of well-synchronised cells (see Materials and Methods), so this strain was examined after induction of *mad2* for 22 hours at 25°C, without elutriation. Live cells were examined after the addition of analogue to 1 μM, or DMSO as control. Cut2p-GFP showed strong staining of the early mitotic spindle in living cells (Kumada et al., 1998). A sample examined just before treatment with analogue revealed that 13% of cells displayed a short spindle strongly stained with Cut2p-GFP (*n*>500 cells). This percentage did not change significantly 10 minutes after the addition of analogue (13%) or DMSO (12%). Staining of fixed cells with antibody to α-tubulin revealed that the percentage of cells with short spindles (⩽4 μm) in the population was similar to the number of cells showing spindle-associated Cut2p-GFP. Thus, inactivation of Cdc2p does not promote degradation of Cut2p-GFP in cells overexpressing *mad2*.

We conclude that inactivation of Cdc2p-as by treatment with 1 μM analogue early in mitosis

Fig. 5. Inactivation of Cdc2p-as promotes recruitment of GFP-Sid1p to the SPB. Small cells of the strain *cdc2-as GFP-sid1 rlc1-GFP* pREP3X-*mad2* were selected by centrifugal elutriation. A sample was taken when cells were in mitosis, and analogue or DMSO was added to aliquots of the remaining cells. Ten minutes later, cells were harvested, fixed and stained to examine α-tubulin and GFP by indirect immunofluorescence. (A,B,C) DAPI, α-tubulin and GFP signals, and a merge. The arrows point to the GFP-Sid1p signal on the SPB. The ring signal is Rlc1-GFP, which serves as a marker for entry into mitosis. Scale bars: 10 μm. (D) Magnifications of the nuclear region of the micrographs shown in panels A-C. (E) Graph of the percentage of cells with GFP-Sid1p associated with the SPB, for a given spindle length, in each of the samples. The number of cells with spindles scored in each sample was more than 200.

Table 2. Inactivation of Cdc2p-as promotes the late-anaphase configuration of the SIN

Strain	Location	Percentage
Mob1p-GFP: T0	Medial ring	11
Mob1p-GFP: T10 DMSO	Medial ring	22
Mob1p-GFP: T10 analogue	Medial ring	80
Bgs1p-GFP: T0	Medial ring	18
Bgs1p-GFP: T10 DMSO	Medial ring	18
Bgs1p-GFP: T10 analogue	Medial ring	78
Cdc7p-GFP: T0	2 SPB	83
Cdc7p-GFP: T10 DMSO	2 SPB	75
Cdc7p-GFP: T10 analogue	2 SPB	12
Cdc7p-GFP flp1Δ: T0	2 SPB	79
Cdc7p-GFP flp1Δ: T10 DMSO	2 SPB	89
Cdc7p-GFP flp1Δ: T10 analogue	2 SPB	36
Plo1p-GFP: T0	On SPBs	86
Plo1p-GFP: T10 DMSO	On SPBs	82
Plo1p-GFP: T10 analogue	On SPBs	7
Plo1p-GFP flp1Δ: T0	On SPBs	85
Plo1p-GFP flp1Δ: T10 DMSO	On SPBs	89
Plo1p-GFP flp1Δ: T10 analogue	On SPBs	28

Cultures of *cdc2-as leu1-32* and the markers indicated in the table, were transformed with pREP1 expressing *mad2* from the full-strength *nmt1* promoter (He et al., 1997). Expression was induced as described in Materials and Methods and synchronous cultures were generated by centrifugal elutriation. Cells were fixed as described in Materials and Methods and indirect immunofluorescence was used to detect GFP and tubulin. The table lists the percentage of cells with the antigen at the location noted in column two, for cells with spindles of ⩽3 μm. The results of a representative experiment are given in each case. 150 to 200 cells with spindles were counted for each sample.

promotes the configuration of SIN proteins that is seen in late mitosis, which is presumed to correlate with activation of the SIN, the initiation of ring contraction and the onset of septum formation.

The protein kinase Plo1p associates with both SPBs early in mitosis, and the signal then diminishes during anaphase B (Mulvihill et al., 1999); we observed that only 7% of cells with spindles ⩽3 μm displayed Plo1p-GFP at the SPBs after inactivation of Cdc2p-as, compared with 82% in the solvent control (Table 2). Previous studies have shown that in fission yeast, Plo1p localisation to the SPB at G2-M does not occur in a *cdc2* mutant (Mulvihill et al., 1999). Our data indicate that CDK activity is also required to maintain Plo1p at the SPB during mitosis.

One of the final steps in assembly of the division apparatus is association of the β-(1,3) glucan synthase Bgs1p with the medial ring. This is essential for formation of the division septum, and does not occur until late anaphase (Cortes et al., 2007; Liu, J. et al., 1999). Moreover, its localisation depends upon an active SIN (Liu et al., 2002). Examination of the localisation of Bgs1p revealed that a Bgs1p-GFP ring was present in 78% of cells with spindles ⩽3 μm, compared with 22% in the control (Table 2). Thus, in addition to an active configuration of the SIN, late proteins that are needed for septum synthesis are also recruited to the contractile ring in response to inactivation of Cdc2p. Consistent with this, examination of cells at 20 minutes after addition of analogue showed that 18% of cells had formed a septum, yet they still contained condensed chromosomes in close proximity to the septum, indicating that septation had occurred without completion of mitosis (Fig. 6C). Examination of the solvent control revealed <0.05% of cells with this phenotype. The uncoupling of septum formation from chromosome separation indicates that, not only is the SIN in the active configuration, but also that the cytokinesis machinery has been activated. In other words, inactivation of Cdc2p is sufficient to induce cytokinesis even in the absence of chromatid separation.

Does Flp1p play a role in regulating the localisation of SIN proteins?

CDC14 family phosphoprotein phosphatases have been implicated in mitotic exit, in part by reversal of CDK-mediated phosphorylation earlier in mitosis (Bardin and Amon, 2001; Seshan and Amon, 2004; Stegmeier and Amon, 2004; Trautmann and McCollum, 2002). The *S. pombe* member of this protein family is called Flp1p/Clp1p (referred to hereafter as Flp1p). It is not essential, but is required for the function of the cytokinesis checkpoint (Cueille et al., 2001; Mishra et al., 2004; Trautmann and McCollum, 2005; Trautmann et al., 2001), SIN signalling (Cueille et al., 2001; Trautmann et al., 2001), timely elimination of the mitotic inducer Cdc25p (Esteban et al., 2004; Wolfe and Gould, 2004) and chromosome segregation (Trautmann et al., 2004). To determine whether Flp1p was required for the transition of the SIN from the early to late mitotic configuration after inactivation of Cdc2p, we examined the localisation of Cdc7p-GFP and Plo1p-GFP after inactivation of *cdc2-as* in a *flp1* null background. We observed that a significantly higher number of cells retained Cdc7p-GFP on both SPBs (36% of ⩽3 μm spindles, compared with 12% in the *flp1*$^{+}$ control; Table 2). This trend was also shown by Plo1p-GFP, which was associated with 28% of SPBs spindle ⩽3 μm, compared with only 7% in the control; Table 2). Thus, we conclude that Flp1p plays a role in promoting the transition of the SIN to the late-anaphase configuration.

Discussion

We have constructed a *cdc2-as* allele in *S. pombe* that is specifically inhibited by an ATP analogue. Our data demonstrate that upon addition of the analogue to exponentially growing cultures, *cdc2-as* cells arrest either in G2, or at both major commitment points in the cell cycle, depending upon the concentration of analogue. Our data also demonstrate that the analogue inhibits the kinase activity of Cdc2p in vitro.

Treatment of *cdc2-as* cells with 10 μM analogue initially causes a G1-G2 mixed arrest, followed by an increase in DNA content and ploidy. This is reminiscent of the consequences of eliminating Cdc13p from the cell, which allows re-replication of DNA without an intervening mitosis (Broek et al., 1991; Fisher and Nurse, 1996). The fact that 1 μM analogue produces a predominantly G2 arrest without an increase in ploidy suggests that at 10 μM the analogue may inhibit Cdc2p-Cdc13p in vivo to a sufficiently great extent that whatever activity remains is no longer sufficient to prevent resetting of origins of replication (Wuarin et al., 2002). The finding that deletion of *cig1* and *cig2* prevents re-replication suggests that either Cdc2p-Cig2p, which promotes S-phase, is less sensitive to inhibition by the analogue than Cdc2p-Cdc13p, or that the residual kinase activity of Cdc2p is sufficient to promote S-phase after a delay.

Results in a variety of systems suggest that inactivation of CDK1 (Cdc2p) is sufficient to induce the transition from mitosis to G1 phase (reviewed by Paulson, 2007). For example, inactivation of Cdc28p in *S. cerevisiae* arrested by expression of a non-degradable *CLB2* promotes mitotic exit and cytokinesis (Ghiara et al., 1991). Inactivation of CDK1 is also required for localisation of the vesicle fusion machinery to the bud neck during mitotic exit and cytokinesis

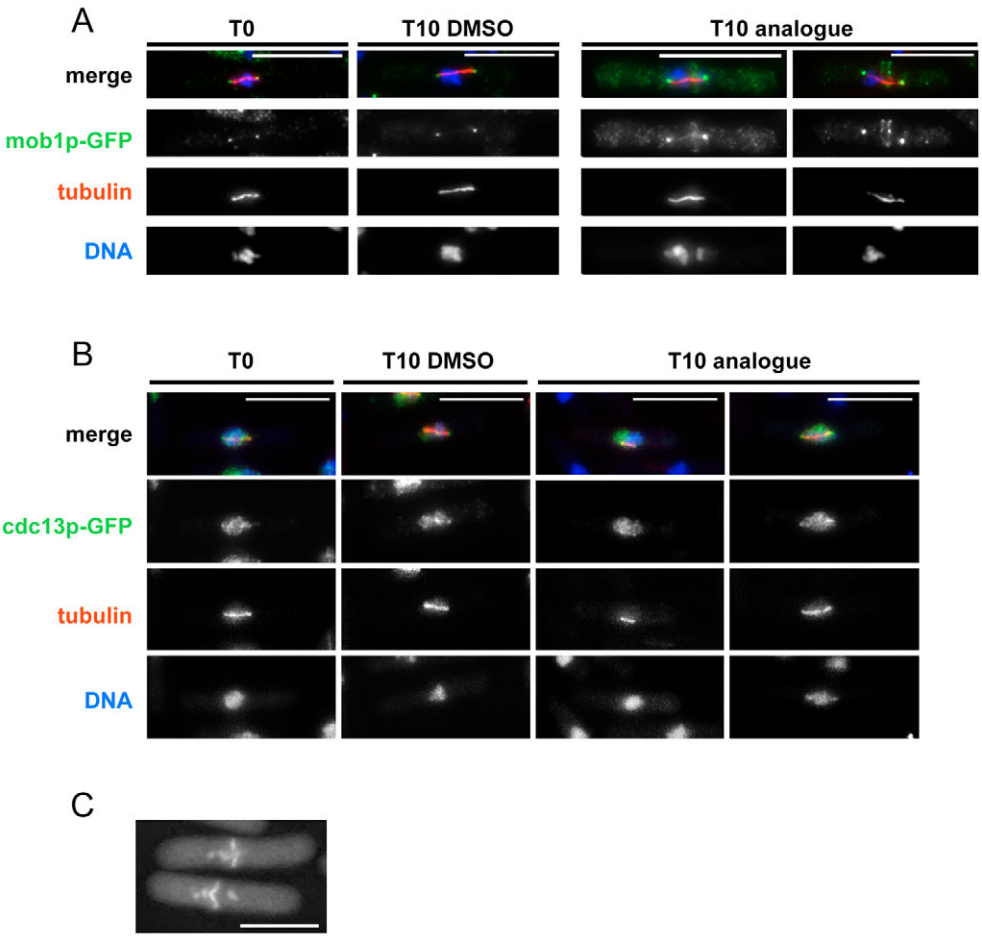


Fig. 6. Analysis of the effects of Cdc2p-as inactivation upon the regulation of cytokinesis. (A) Small cells of the strain *cdc2-as mob1-GFP* pREP3X-*mad2* were selected by centrifugal elutriation. A sample was taken when cells were in mitosis, and analogue or DMSO was added aliquots of the remaining cells. Ten minutes later, cells were harvested, fixed and stained to examine α-tubulin and GFP by indirect immunofluorescence. The images show representative DAPI staining, α-tubulin and GFP indirect immunofluorescence in the three samples. For the T10 analogue panels, the left-hand image shows a cell with a single Mob1p-GFP ring, whereas the right hand images show a cell with a double ring. Note that Mob1p-GFP is present on the SPBs throughout mitosis. Quantification is presented in Table 2. (B) *cdc2-as cdc13-GFP* pREP3X-*mad2* cells were treated as described in the legend for panel A. (C) Cells of the strain *cdc2-as cdc7p-GFP* pREP3X-*mad2* were treated as described in the legend for panel A. A sample was examined 20 minutes after addition of analogue. Cells were fixed and stained with DAPI and Calcofluor. Scale bars: 10 μm.

(VerPlank and Li, 2005). In *S. pombe*, increased expression of nondegradable Cdc13p blocks septation (Yamano et al., 1996) and inactivation of Cdc2p – using a thermosensitive mutant in cells arrested prior to the metaphase-anaphase transition – promotes mitotic exit and septum formation (He et al., 1997). Studies of cultured vertebrate cells have shown that when nocodazole-arrested mouse FT210 cells, which carry a temperature-sensitive mutation in CDK1, are shifted to their nonpermissive temperature, they exit mitosis (without chromatid segregation or cytokinesis) and are capable of completing another cell cycle (Paulson, 2007). Furthermore, treatment of mitotically arrested cells with CDK1 inhibitors will induce mitotic exit and cytokinesis. Formation of the cleavage furrow upon CDK1 inhibition occurs even if cells are treated with proteasome inhibitors or express nondegradable cyclin B (Potapova et al., 2006; Skoufias et al., 2007). Together, these data from a variety of model systems – ranging from yeast to vertebrate cells – point to a universal requirement for inactivation of CDK1 to promote cytokinesis.

The results presented here demonstrate that inactivation of fission yeast Cdc2p in early mitotic cells by addition of analogue is sufficient to bring about the transition of the SIN, which regulates the onset of septum formation from its early mitotic configuration to that normally found after the anaphase A-B transition. Moreover, proteins such as the β-(1,3) glucan synthase Bgs1p, whose association with the contractile ring depends upon SIN activation, are also recruited, strongly suggesting that the SIN proteins do not simply localise asymmetrically to the SPBs and the contractile ring, but are also activated. Consistently with this, we observed that 20 minutes after addition of analogue, many cells had formed a septum bisecting the unsegregated, condensed chromosomes.

Mitotic exit involves, in part, reversal of CDK-dependent mitotic phosphorylation events. Cdc14 family phosphoprotein phosphatases have been implicated in this process, particularly in budding yeast. Our data suggest that Flp1p, the *S. pombe* orthologue of *S. cerevisiae* Cdc14p, plays a role in regulating relocalisation of SIN proteins following inactivation of Cdc2p. Since *flp1* is not an essential gene, it is likely that Flp1p co-operates with one or more phosphatases to regulate the SIN. Potential candidates for this would be PP2A-Par1/Pab1, which has been implicated in regulation of the SIN (Jiang and Hallberg, 2000; Jiang and Hallberg, 2001; Le Goff et al., 2001), or the functionally redundant type1 phosphatases Dis2p and Sds21p (Kinoshita et al., 1990; Ohkura and Yanagida, 1991). Future studies will investigate the role played by these phosphatases in regulating the localisation of SIN proteins after inactivation of Cdc2p. Another possibility which we have considered is that APC/C-dependent protein ubiquitylation and degradation brings about the relocalisation of SIN proteins we have studied in this paper. However, since APC function is reduced in cells arrested by *mad2* overexpression, and Cdc13p-GFP and Cut2p-GFP are still present in *cdc2-as* cells treated with analogue, we favour the explanation outlined above. The relevant substrate(s) phosphorylated by Cdc2p to prevent activation of the SIN in early mitosis are unknown. By analogy with *S. cerevisiae*, Cdc7p, the orthologue of Cdc15p (Jaspersen and Morgan, 2000) and Byr4p, the orthologue of Bfa1p

(Hu et al., 2001; Pereira et al., 2002) would both be potential candidates. Some of the SIN scaffold proteins, most notably Cdc11p, are multiply phosphorylated during mitosis; however, in a previous study (Morrell et al., 2004), all the identified Cdc2p sites in Cdc11p were mutagenised without affecting its biological function. This finding makes it less likely that Cdc11p is the key substrate for Cdc2p in SIN regulation. Nonetheless, it is noteworthy that Cdc13p interacts with Cdc11p (Morrell et al., 2004). It is therefore possible that Cdc11p acts as a scaffold to position Cdc2p-Cdc13p to regulate SIN proteins.

In summary, the *cdc2-as* mutant has allowed us to explore the effect of rapid, chemically mediated inactivation of Cdc2p upon the localisation and activation of the SIN. Use of this chemical genetic approach should also allow the role of Cdc2p in regulating other cellular events to be evaluated.

Materials and Methods

Analogue synthesis

The ATP analogue 4-amino-1-tert-butyl-3-(1′-naphthylmethyl)pyrazolo[3,4-d]pyrimidine was synthesised as described (Hanefeld et al., 1996) with some modifications. The identity of the product, a white solid that gave a single spot in TLC analysis, was confirmed by ^{1}H NMR, HH COSY and high-resolution mass spectrometry. ^{1}H NMR (27 MHz, CDCl3) gave peaks at 1.84 (singlet, 9H), 4.75 (singlet, 2H), 4.85 (broad singlet, 2H), 7.18 (doublet, 1H), 7.38 (triplet, 1H), 7.54 (multiplet, 2H), 7.79-7.92 (multiplet, 2H), 8.22 (doublet, 1 H) and 8.24 (singlet, 1H), in close agreement with published data (Bishop et al., 1999). High resolution mass spectrometry gave a mass of 331.181712 for the molecular ion (calculated mass for C20H21N5, 331.179696). Aliquots of the product were dissolved at 15 mM in DMSO and stored at –70°C.

Fission yeast techniques and reagents

GFP-tagged strains used in this study have been described previously (Bahler et al., 1998a; Guertin et al., 2000; Liu et al., 2002; Salimova et al., 2000; Sohrmann et al., 1998). In experiments with GFP-Sid1p, the medial ring marker Rlc1p-GFP (Le Goff et al., 2000) was also included to facilitate identification of cells in mitosis. Standard techniques were used for the growth and manipulation of fission yeast (Moreno et al., 1991).

Cell synchronisation

Cdc2::ura4$^+$ *cdc2-as GFP-SIN* cells were transformed with pRep3X-*mad2* and selection synchrony was additionally performed as described (Schmidt et al., 1997) using a Beckman JS 5.0 elutriation system with a 40 ml separation chamber. For experiments involving overexpression of *mad2*, cells were grown under inducing conditions for 16 hours at 25°C before size selection by centrifugal elutriation, which took approximately 90 minutes for loading and elution. Cells were concentrated by vacuum filtration and resuspended at 3×10^6 per ml in supplemented EMM2 medium.

Cell biology and image capture

For visualisation of GFP and α-tubulin by indirect immunofluorescence, cells were processed as described (Hagan and Hyams, 1988). Briefly, cells were fixed by addition of freshly made paraformaldehyde (Sigma) to 3.7% (w/v). Thereafter cells were washed three times with PEM (0.1 M PIPES pH 6.9 1 mM EGTA 10 mM $MgSO_4$) and then digested for 40 minutes at 37°C in PEMS (PEM plus 1.2 M sorbitol) containing 1 mg/ml zymolyase 20T (Seikagaku Corporation, Japan). Cells were washed once in PEMS plus 1% Triton X-100, three times with PEM, resuspended in PEM plus 1% BSA (Sigma), 100 mM lysine hydrochloride, 0.1% sodium azide (PEMBAL) and incubated for 30 minutes on a rotating wheel. Cells were resuspended in PEMBAL containing the primary antibodies and incubated overnight at room temperature. The following primary antibodies were used: α-tubulin was detected using TAT-1 (1:50) (Woods et al., 1989). GFP was detected by rabbit anti-GFP antibody (1:500) (Krapp et al., 2001). After three washes in PEMBAL, cells were incubated with PEMBAL containing secondary antibodies [Goat-anti-mouse Cy3 (1:500, Jackson) and goat-anti-rabbit Alexa Fluor 488 (1:500, Molecular Probes)] with end-over-end agitation for 4 hours at 37°C. Cells were then washed once in PEM, once in PBS (pH 8.0) and then resuspended in PBS (pH 8.0) containing 1 μg/ml DAPI. Images were captured using a TILL Olympus IX70 microscope equipped with a SensiCam 12 Bit cooled CCD camera (PCO) and Metamorph 6.3r software. The images shown in Figs 5 and 6 are maximum intensity projections of Z-stacks (11 sections at 0.5 μm). Image J software (v1.37) was used for image processing and measurement of spindle lengths. Contrast and levels adjustments were made using Adobe® Photoshop CS.

Cells were fixed and processed to detect F-actin using Rhodamine-conjugated phalloidin as described (Marks et al., 1986). DAPI staining was performed as described (Balasubramanian et al., 1997). Cells were viewed using an Axiophot microscope (Zeiss) with a 100χ NA 1.4 lens and images were captured on a Nikon Coolpix 990 camera.

The *cut2-GFP* allele was created by oligonucleotide-mediated tagging (Bahler et al., 1998b), using VS425, 5′-GTCGGCGCCCTTTATCTCGTTCTATTCATTCGTT-ATCATCTTCACGAATTGATTTTTCATCTTTGGATACAGGATTGTTACGGAT-CCCCGGGTTAATTAA-3′ and VS426, 5′-CAAATTAAACAACAAGGGAAAT-CAAAGCCATCGGAAGAATCATACTTACAATCGTAGAAGCGGAAGCCAGT-ACGCACGAATTCGAGCTCGTTTAAAC-3′ . Colonies resistant to G418 were selected and correct insertion of the tag was verified by PCR. The *Cut2-GFP* allele is thermosensitive and unable to form colonies at 36°C. The localisation of Cut2p-GFP *in vivo* recapitulates the published localisation (Kumada et al., 1998). We were obliged to analyse the Cut2p-GFP signal in living cells, as we were unable to preserve any Cut2p-GFP signal on the mitotic spindle that could be detected by antibodies to GFP after fixation with either paraformaldehyde or paraformaldehyde and glutaraldehyde. The double mutant *cdc2-as cut2-GFP* shows a mild negative genetic interaction; cells are more elongated than *cdc2-as* and cannot easily be separated by centrifugal elutriation. For this reason, the localisation of Cut2p-GFP in cells overexpressing *mad2* was examined in cells that had not been presynchronised by elutriation. To examine the localisation of Cut2p-GFP, expression of *mad2* was induced for 22 hours at 25°C. Fields of cells were photographed using a Zeiss axiovert 200 microscope equipped with a confocal scanner unit model CSU10 (Yokogawa Electric Corporation), a coolSNAPHQ camera (Photometrics) and a 63× NA plan-apo objective. Images were collected using Metamorph software (Universal Imaging). Cells were treated with 1 μM analogue or an equivalent volume of DMSO for 8 minutes before mounting for observation. The total time from the addition of analogue to completion of image capture was 10-15 minutes.

Molecular biology

Standard techniques were used for molecular biology (Sambrook et al., 1989). The *cdc2-as* mutant was created by site-directed mutagenesis of Phe84 to Gly by PCR using the following oligonucleotides: forward primer VS 551, 5′-GTTGTAT-CTTGTTGGTGAGTTTTTAGAC-3′ and the reverse primer VS552, 5′-GTCTA-AAAACTCACCAACAAGATACAAC-3′ . A 3 kb *PstI* fragment containing the *cdc2-as* mutation was cloned into the pINTA [pINT5 (Fankhauser and Simanis, 1994), from which the *nmt1* promoter has been removed; a gift from J. Petersen, University of Manchester, Manchester, UK] vector that targets integration at the *leu1* gene. Integration was performed in a *cdc2*$^+$ strain, and the *cdc2-as* was introduced into a *cdc2::ura4*$^+$ background by crossing to a *cdc2::ura4*$^+$ strain that was rescued by human *CDC2/CDK1* (Lee and Nurse, 1987). Integration at the *cdc2*$^+$ locus was also obtained during this transformation, presumably by gene conversion. The phenotypes of the *cdc2::ura4*$^+$ *leu1*::*cdc2-as* and *cdc2-as* strains were indistinguishable in either complete (YE) or minimal (EMM2) medium; most experiments described here use *cdc2-as*. The existence of the as mutation at the sites of integration was verified by sequencing using VS 551/552 primer. DNA-sequencing reactions and oligonucleotide primer synthesis were performed at Microsynth laboratory (Balgach, CH).

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