Activity of Cdc2 and its interaction with the cyclin Cdc13 depend on the molecular chaperone Cdc37 in *Schizosaccharomyces pombe*

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Summary

Cdc37 is a molecular chaperone whose clients are predominantly protein kinases, many of which are important in cell-cycle progression. Temperature-sensitive mutants of *cdc37* in *Schizosaccharomyces pombe* are lethal at the restrictive temperature, arresting cell division within a single cell cycle. These mutant cells elongate during incubation at the restrictive temperature, consistent with a cell-cycle defect. The cell-cycle arrest arises from defective function of the mutant Cdc37 proteins rather than a reduction in Cdc37 protein levels. Around 80% of the arrested, elongated cells contain a single nucleus and replicated (2C) DNA content, indicating that these mutants arrest the cell cycle in G2 or mitosis (M). Cytological observations show that the majority of cells arrest in G2. In fission yeast, a G2 cell-cycle arrest can arise by inactivation of the cyclin-dependent kinase (Cdk) Cdc2 that regulates entry into mitosis. Studies of the *cdc37* temperature-sensitive mutants show a genetic interaction with some *cdc2* alleles and overexpression of *cdc2* rescues the lethality of some *cdc37* alleles at the restrictive temperature, suggesting that Cdc2 is a likely client for the Cdc37 molecular chaperone. In *cdc37* temperature-sensitive mutants at the restrictive temperature, the level of Cdc2 protein remains constant but Cdc2 protein kinase activity is greatly reduced. Inactivation of Cdc2 appears to result from the inability to form complexes with its mitotic cyclin partner Cdc13. Further evidence for Cdc2 being a client of Cdc37 in *S. pombe* comes from the identification of genetic and biochemical interactions between these proteins.

Key words: Cdc37, Heat-shock protein (Hsp90), Cdc2, Cell cycle, Fission yeast, Molecular chaperone

Introduction

Cdc37 is a molecular chaperone identified simultaneously as the product of the *cdc37* gene, isolated during a screen for *Saccharomyces cerevisiae* mutants that arrest at Start within the cell division cycle (Reed, 1980a; Reed, 1980b) and as the 50 kDa Cdc37 protein (p50) associated with the client kinase Src from chick cells (Hunter and Sefton, 1980). Cdc37 client proteins are involved in a range of cellular processes including signal transduction, DNA and protein synthesis and cell-cycle regulation (see MacLean and Picard, 2003 for review). In *S. cerevisiae*, spores deleted for *CDC37* undergo outgrowth to form microcolonies of 4-60 cells which are heterogeneous in phenotype (Gerber et al., 1995), indicating that Cdc37 function affects a variety of processes within the cell. Loss of Cdc37 function in *S. cerevisiae* temperature-sensitive mutants at the restrictive temperature arrests the cell cycle in Start (Reed, 1980a; Reed, 1980b; Valay et al., 1995) or with a population split between G1 and G2-M phase arrest (Dey et al., 1996), suggesting that Cdc37 client proteins have crucial roles in regulating progression through the cell cycle.

Cdc37 clients are predominantly protein kinases and include cyclin-dependent kinases (Cdks) such as Cdk4 in mammalian cells (Dai et al., 1996; Lamphere et al., 1997; Stepanova et al., 1996) which regulates cell proliferation in G1, and Cdc28 in *S. cerevisiae* which is important for the G1-S and G2-M transitions (Farrell and Morgan, 2000; Gerber et al., 1995; Mort-Bontemps-Soret et al., 2002). Cdc37 also associates with non-kinase clients such as the androgen receptor (Rao et al., 2001). Cdc37 binds the catalytic domains of client protein kinases such as Lck (Prince and Matts, 2004), Raf (Silverstein et al., 1998) and LKB1 (Boudeau et al., 2003). In a well-studied example, the heme-regulated eIF2R kinase (HRI) binds the N-terminal domain of Cdc37 (Shao et al., 2003). Many of these protein clients rely on the molecular chaperone Cdc37 for activation, folding or protection from degradation. Cdc37 has been found to display a range of chaperone activities towards different client proteins. Cdc37 chaperone function is required for the protein stability of a number of client protein kinases including Ste11 (Abbas-Terki et al., 2000) and LKB1 (Boudeau et al., 2003) and is essential for preserving the enzymatic activity of the client proteins Aurora B (Lange et al., 2002), IKK (Chen et al., 2002) and Raf-1 (Grammatikakis et al., 1999). Cdc37 does not appear to activate clients itself, but delivers clients to co-chaperones with ATPase activity such as Hsp70 and Hdj-1, which fold and activate them (Kimura et al., 1997). Cdc37 also binds client proteins such as Cdk4 and facilitates their assembly with cyclin partners (Lamphere et al., 1997; Stepanova et al., 1996).

In *S. cerevisiae* both the protein levels and kinase activity of Cdc28, the major cell-cycle regulatory Cdk, are reduced in the

temperature-sensitive mutant *cdc37-1* (Gerber et al., 1995). In fission yeast, the equivalent Cdk is Cdc2, which regulates the G1-S phase and G2-M transitions. Cdc2 protein levels remain constant throughout the cell cycle (Alfa et al., 1989). Regulation of cell-cycle progression by Cdc2 is controlled by modulating its activity at different stages by associating with specific cyclin partners and both negative and positive phosphorylation. Prior to the G2-M transition, Cdc2 is phosphorylated on Thr167 (Gould et al., 1991) by Cdk-activating kinases (CAKs) (Lee et al., 1999). This promotes the association of Cdc2 with Cdc13, a B-type cyclin (Booher and Beach, 1988; Booher et al., 1989; Hagan et al., 1988). Cdc2 is also phosphorylated on Tyr15, primarily by Wee1 (Gould and Nurse, 1989), which keeps the Cdc2-Cdc13 complex inactive during interphase. The Cdc13 protein shows periodic changes in abundance (Alfa et al., 1989), accumulating through interphase and then being actively degraded at the metaphase-anaphase transition. To initiate mitosis, Cdc2 bound to Cdc13 is activated by dephosphorylation on tyrosine 15 by the phosphatase Cdc25 (Nurse, 1997). Active Cdc2 is then localised to the nucleus by cyclin Cdc13 and the active complex promotes mitosis (Alfa et al., 1989).

There is little known about Cdc37, its clients or its cell-cycle role in the fission yeast *S. pombe*. The *cdc37* gene is essential for viability (Tatebe and Shiozaki, 2003; Westwood et al., 2004), and depletion of the Cdc37 protein in shut-off experiments leads to a range of cell phenotypes, indicating an involvement in several cellular functions that have not been elucidated (Westwood et al., 2004). One temperature-sensitive lethal *cdc37* mutant, *cdc37-681*, was isolated as a suppressor of hyperactivation of the stress-activated MAP kinase pathway, and direct interaction was demonstrated between Cdc37 and the client kinase Spc1/Sty1 (Tatebe and Shiozaki, 2003). In the present study, we generated three fission yeast temperature-sensitive (*cdc37*^{ts}) mutants of *cdc37* and analysed them alongside *cdc37-681*. Characterisation of these *cdc37*^{ts} mutants reveals that at the restrictive temperature, Cdc37 function is lost within a single cell cycle, arresting the cell cycle in G2 phase. Investigations into the cause of the G2 arrest show that Cdc2 activity is reduced within the first cell cycle and that Cdc2 shows reduced ability to bind its mitotic cyclin partner Cdc13. In this work we have also identified both biochemical and genetic interactions between Cdc37 and Cdc2, supporting the idea that Cdc2 is a client of Cdc37 in *S. pombe*.

RESULTS

Fission yeast *cdc37* temperature-sensitive (*cdc37*^{ts}) mutants arrest cell division within a single cell cycle at the restrictive temperature

Cdc37 was previously identified as an essential gene in *S. pombe* (Tatebe and Shiozaki, 2003; Westwood et al., 2004). Transcriptional shut-off experiments to deplete Cdc37 protein allows division to continue for up to 36 hours resulting in a phenotypically heterogeneous cell population (Westwood et al., 2004). To gain further insight into the mode of action of Cdc37 in fission yeast, we generated *cdc37*^{ts} mutants so that Cdc37 function could be rapidly switched off by the shift to the restrictive temperature. Temperature-sensitive mutants of *cdc37* were integrated into the *cdc37* locus of the haploid strain ED1090 (see Materials and Methods). Directed mutagenesis was used to introduce a mutation equivalent to the S. cerevisiae temperature-sensitive mutant *cdc37-184* (Valay et al., 1995) into *S. pombe*. Two *cdc37*^{ts} mutants were isolated by random mutagenic PCR amplification, named *cdc37-J* and *cdc37-13*. Analysis of these three fission yeast *cdc37*^{ts} mutants was carried out in parallel with a fourth, *cdc37-681* (Tatebe and Shiozaki, 2003). Sequencing of all four *cdc37*^{ts} mutant alleles identified mutations located in close proximity in the Cdc37 protein (Fig. 1A) and found that *cdc37-13* contained two nucleotide changes and *cdc37-J* had three.

To define the permissive and restrictive temperature ranges

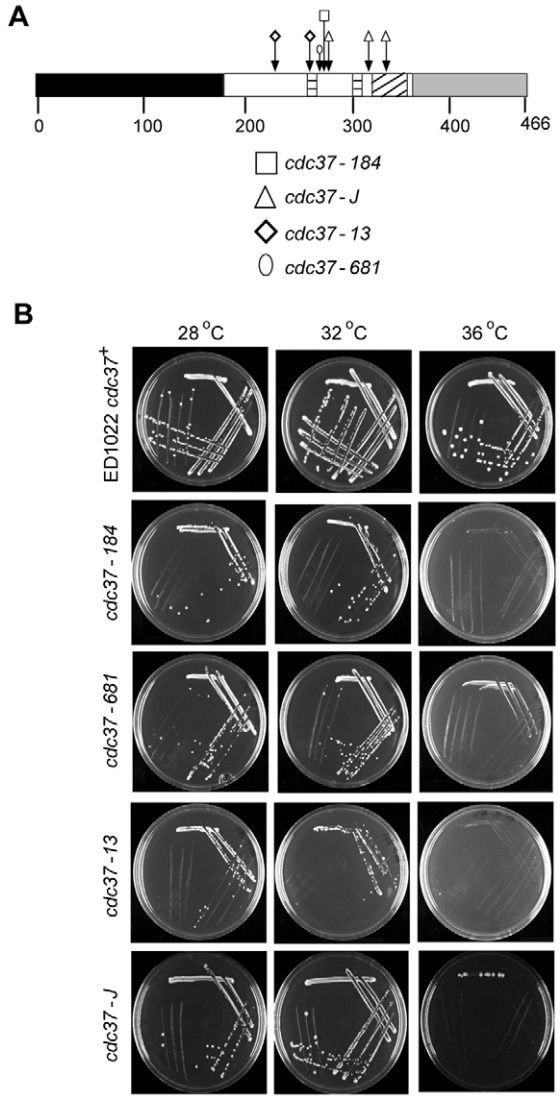

Fig. 1. (A) A schematic diagram indicating the location of mutations within the *cdc37*^{ts} mutant alleles *cdc37-681* (Leu285 to Pro), *cdc37-184* (Ala287 to Asp), *cdc37-13* (Glu237 to Lys and Tyr261 to His) and *cdc37-J* (Leu286 to Met, His305 to Leu and Arg314 to Gly). Alignment of the human and *S. pombe* Cdc37 protein sequences enabled the Hsp90-binding domains (Roe et al., 2004) (horizontal stripes) and the homodimerisation domain (Roe et al., 2004) (diagonal stripes) to be mapped from the human to the *S. pombe* protein. (B) The *cdc37*^{ts} mutants and the *cdc37*$^{+}$ strain ED1022 were streaked on YE plates and incubated at 28, 32 and 36°C for 4 days to examine the ability of each strain to form single colonies at different temperatures

of the *cdc37*^{ts} mutants, they were streaked onto YE plates and incubated for 4 days at a range of temperatures between 28 and 36°C. All four *cdc37*^{ts} mutants were able to grow and form single colonies at 28 and 32°C, but not at 36°C (Fig. 1B).

To determine how quickly cell proliferation stops in *cdc37*^{ts} mutants after the shift to the restrictive temperature, the increase in cell number in liquid cultures after the shift from 28 to 36°C was investigated. Cultures of strains carrying the mutant alleles *cdc37-681*, *cdc37-184*, *cdc37-J*, *cdc37-13* or the *cdc37*$^{+}$ strain ED1022 were grown at 28°C, then split and kept at 28°C or 36°C. Samples were taken every 2 hours and processed for cell number determination. The number of cells for *cdc37*$^{+}$ and *cdc37*^{ts} mutant strains cultured at 28°C increased over the time course; results for *cdc37*$^{+}$, *cdc37-184* and *cdc37-681* are shown in Fig. 2A; *cdc37-13* and *cdc37-J* behaved in a similar way (data not shown). By contrast, at 36°C the rate of cell division of the *cdc37*^{ts} mutants was greatly reduced within 2 hours of the temperature shift (Fig. 2A and data not shown). In contrast to the reduction in cell division rate, the accumulation of cell mass (OD_{600}) continued after the shift to 36°C, as discussed in more detail below.

Cdc37 protein levels in *cdc37*^{ts} mutants are unaffected by a shift to the restrictive temperature

In principle, the inability of *cdc37*^{ts} mutants to proliferate at the restrictive temperature might be due to a reduction in the level of mutant protein, as reported for the *S. cerevisiae* mutants *cdc37-1* (Gerber et al., 1995) and *cdc37-34* (Fliss et al., 1997) or due to defective function of the mutant proteins. To test this, the *cdc37*^{ts} mutant and *cdc37*$^{+}$ strains were

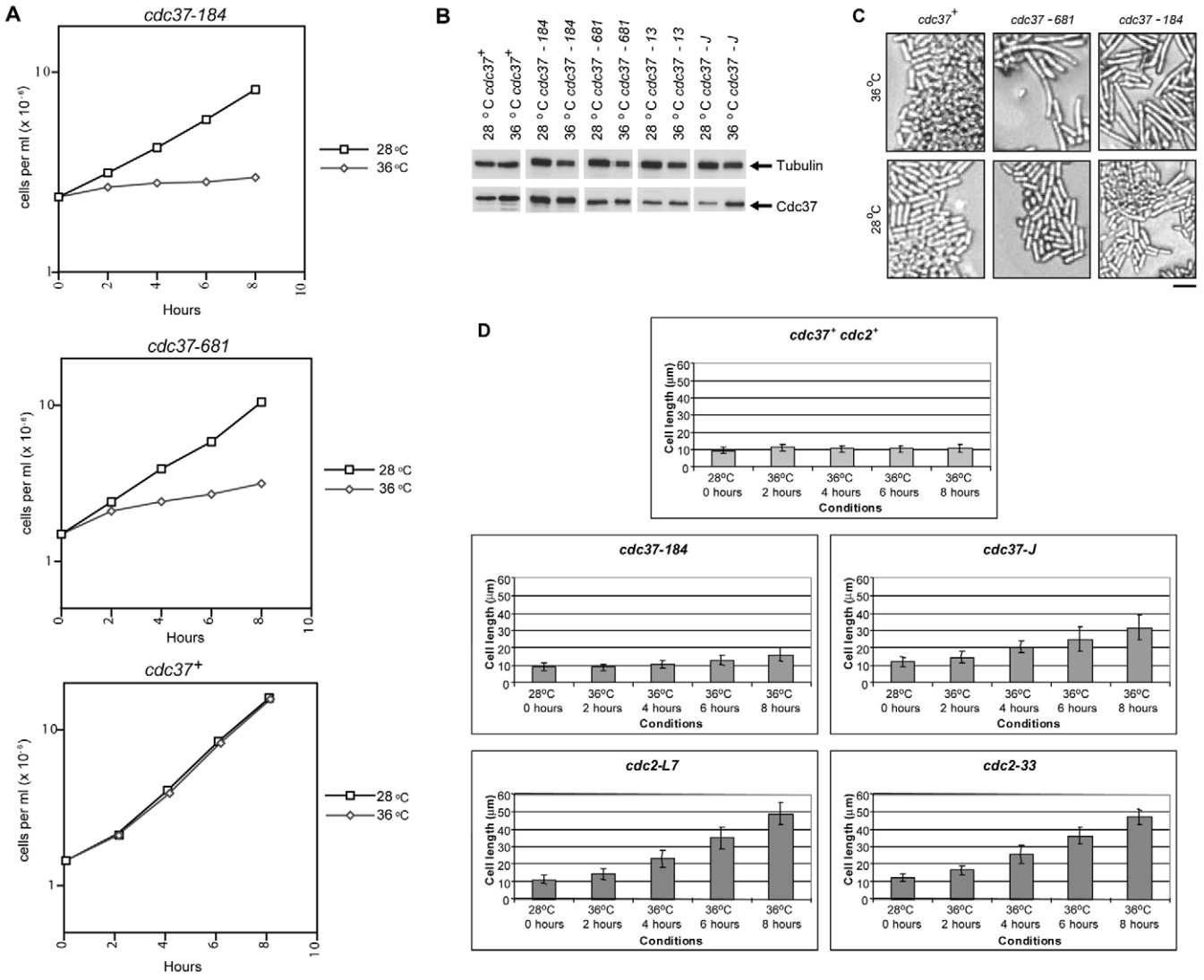

Fig. 2. (A) Analysis of cell number of *cdc37-184*, *cdc37-681* and *cdc37*$^{+}$ ED1022 strains. Strains were cultured at 28 and 36°C over an 8 hour time course, and samples taken at 2 hour intervals for determination of cell number using a Coulter electronic particle counter. (B) Comparison of Cdc37 protein levels in *cdc37*^{ts} mutants and the *cdc37*$^{+}$ strain ED1022 after 8 hours at 28 and 36°C. Western blot analysis was carried out on whole-cell protein extracts using the anti-*S. pombe* Cdc37 antibody. β-tubulin was detected by TAT1 antibody and used as a loading control. (C) Cell morphology of *cdc37*^{ts} mutants *cdc37-184* and *cdc37-681* and the *cdc37*$^{+}$ strain ED1022 on YE plates incubated at 28 and 36°C for 24 hours. Bar, 10 μm. (D) Mean cell length of *cdc37-184*, *cdc37-J*, *cdc2-33* and *cdc2-L7* and *cdc37*$^{+}$ cells (with s.d. bars). Strains were cultured in liquid YE at 28 and 36°C over an 8 hour time course and the lengths of 200 cells measured for each sample.

cultured as previously described and denatured protein extracts were prepared from each strain after incubation at 28 and 36°C for 8 hours. The level of Cdc37 protein was assayed by SDS-PAGE and western blot. Cdc37 protein levels in the *cdc37*^{ts} mutants did not differ greatly between cultures incubated at 28 and 36°C (Fig. 2B). The level of mutant Cdc37 protein in *cdc37-13* and *cdc37-J* was slightly reduced compared with other strains but this was not temperature related (Fig. 2B). These data indicate that the cessation of cell division at 36°C in all four *cdc37*^{ts} mutants is not due to reduced protein levels but presumably to impaired Cdc37 function.

cdc37^{ts} mutants display a *cdc* phenotype at the restrictive temperature

The morphology of *cdc37*^{ts} mutant and *cdc37*$^{+}$ cells was examined in cells grown on YE plates at 25, 28, 32 and 36°C for 24 hours. At the permissive temperatures of 25, 28 and 32°C, *cdc37*^{ts} mutant cells resembled *cdc37*$^{+}$ cells in appearance. The morphologies of *cdc37-184* and *cdc37-681* are shown in Fig. 2C. However, at 36°C *cdc37*^{ts} mutant cells were elongated (Fig. 2C) for *cdc37-184* and *cdc37-J* cells, whereas *cdc37-681* and *cdc37-13* displayed similar phenotypes (not shown). This is characteristic of the *cdc* phenotype, consistent with the inability of the cell to continue division while continuing to grow during arrest. To quantify the elongation, cell length was measured for *cdc37*^{ts} and *cdc37*$^{+}$ strains grown in liquid culture at 28 and 36°C. Samples of cells were taken every 2 hours, fixed and stained with Calcofluor. The lengths of 200 cells were measured for each sample and the average cell lengths calculated (Fig. 2D). At 28°C, the average cell length was similar for all strains (Fig. 2D), except for *cdc37-J* whose average cell length at the permissive temperature was 12.3 μm, slightly longer than observed for the *cdc37*$^{+}$ strain. By contrast, at 36°C the average cell length increased for all *cdc37*^{ts} mutants over the time course as shown for *cdc37*$^{+}$, *cdc37-184* and *cdc37-J* (Fig. 2D), confirming that these mutants arrest with a *cdc* phenotype. The greatest cell elongation was observed for *cdc37-J*, increasing in length by approximately threefold over 8 hours at the restrictive temperature. The other *cdc37* mutant strains showed increases in length intermediate between *cdc37-184* and *cdc37-J* under these conditions. Some *cdc* mutant strains such as *cdc2* mutants show far greater elongation than this over similar time periods (Nurse et al., 1976).

We carried out temperature-shift experiments with *cdc2-33* and *cdc2-L7* mutants in parallel with *cdc37* mutants and they did indeed show greater elongation (Fig. 2D). The reduced elongation of *cdc37* strains might in principle be due to 'leakiness' of the division arrest, or a reduced rate of biomass or length accumulation. OD_{600} measurements were carried out on *cdc2* and *cdc37* cultures (data not shown) and the doubling time calculated. At 28°C the OD doubling times for the strains examined were all between 2.8 and 3.6 hours. At 36°C the OD doubling time for the *cdc*$^{+}$ strain was 2.4 hours, and those for the *cdc2ts* strains were similar (*cdc2-33*, 3.0 hours; *cdc2-L7*, 2.9 hours). However the *cdc37ts* strains grew substantially more slowly: *cdc37-J* had a OD doubling time of 3.4 hours whereas *cdc37-184* grew much more slowly with a doubling time of 4.3 hours. This last result is consistent with the modest increase in cell length observed for *cdc37-184* during a tight division block (Fig. 2A,D). The rate of growth and cell elongation in *cdc37*^{ts} mutants may differ from *cdc2*^{ts} mutants because loss of Cdc37 function affects a range of clients, potentially including those involved in growth and elongation. As previously observed, depletion of Cdc37 results in a range of phenotypes not restricted to elongated cells (Westwood et al., 2004).

cdc37^{ts} mutants arrest in the G2 phase of the cell cycle

To identify the cell-cycle stage at which the *cdc37*^{ts} mutants arrest, flow cytometry analysis was carried out, which determines whether the DNA content of cells is 1C (unreplicated) or 2C (replicated). In a wild-type *S. pombe* population very few cells contain a 1C DNA content so we used the temperature-sensitive mutant *cdc10-129* to identify the position of the 1C peak. This mutant is defective in DNA synthesis at 36°C and cells therefore arrest with a 1C DNA content (Sazer and Sherwood, 1990). The *cdc37*^{ts} mutants, *cdc10-129* and the *cdc37*$^{+}$ strain ED1022 were cultured at 28 and 36°C over an 8 hour time course. Samples were taken every 2 hours, processed for flow cytometry and analysed in a FACScan instrument.

At 28°C essentially all cells of the *cdc37*$^{+}$ strain ED1022, *cdc10-129* and *cdc37*^{ts} mutant cells showed a 2C DNA content. Results for *cdc37-184* and *cdc37-J* are shown in Fig. 3A; *cdc37-681* and *cdc37-13* behaved in a similar manner. After 2 hours at 36°C, the cell population of *cdc10-129* was split between 1C and 2C DNA content, establishing the distribution for 1C and 2C DNA content peaks for other strains in this experiment. At 36°C *cdc37*$^{+}$ and all *cdc37*^{ts} mutant cells showed a very predominant 2C DNA content (Fig. 3A). This indicates that *cdc37*^{ts} mutant cells arrest with a replicated genome having passed through S phase. It is interesting to note that after 2 hours at 36°C, *cdc37-184* produces a minor but distinct peak of 1C cells, which completely disappears by the 4 hour time point (Fig. 3A). This suggests that *cdc37-184* cells undergo a delay in G1 at 36°C, producing a transient 1C peak. In some later time samples from the *cdc37*^{ts} cultures, a small peak probably representing 4C cells is seen: its possible significance is discussed below. For all strains, the position of the peaks determined by flow cytometry shifted to the right at

Fig. 3. (A) Flow cytometry of *cdc37-184*, *cdc37-J*, *cdc37*$^{+}$ ED1022 and *cdc10-129* to determine the DNA content of cells. Strains were cultured in YE at 28 and 36°C over an 8 hour time course. Samples of cells were taken every 2 hours and processed for flow cytometry. (B) Frequency of phenotypes observed by DAPI staining of *cdc37-184*, *cdc37-681* and *cdc37*$^{+}$ cells. Samples of cells were taken every 2 hours, fixed in formaldehyde and stained with DAPI. Phenotypes 1,2 and 3 are shown in C. Phenotype 4 is a cell with a single nucleus and a septum, phenotype 5 is a cell with a septum cutting through a single nucleus and phenotype 6 is a cell with multiple septa. (C) Cellular phenotypes 1, 2 and 3 observed with DAPI staining of *cdc37*+ and mutants at both 28 and 36°C. (D,E,F) Immunofluorescence of microtubules, using the TAT1 antibody, of *cdc37*$^{+}$ interphase microtubules (D), and arrested *cdc37-184* (E) and *cdc37-J* (F) cells. Strains were cultured at 28 and 36°C for 8 hours. (G) Immunofluorescence of *cdc37-184* and *cdc37*$^{+}$ ED1022 cells with the anti-*S. pombe* Cdc37 antibody. Samples of cells were processed for immunofluorescence with anti-*S. pombe* Cdc37 antibody or depleted antibody (see Materials and Methods). Bars, 10 μm.

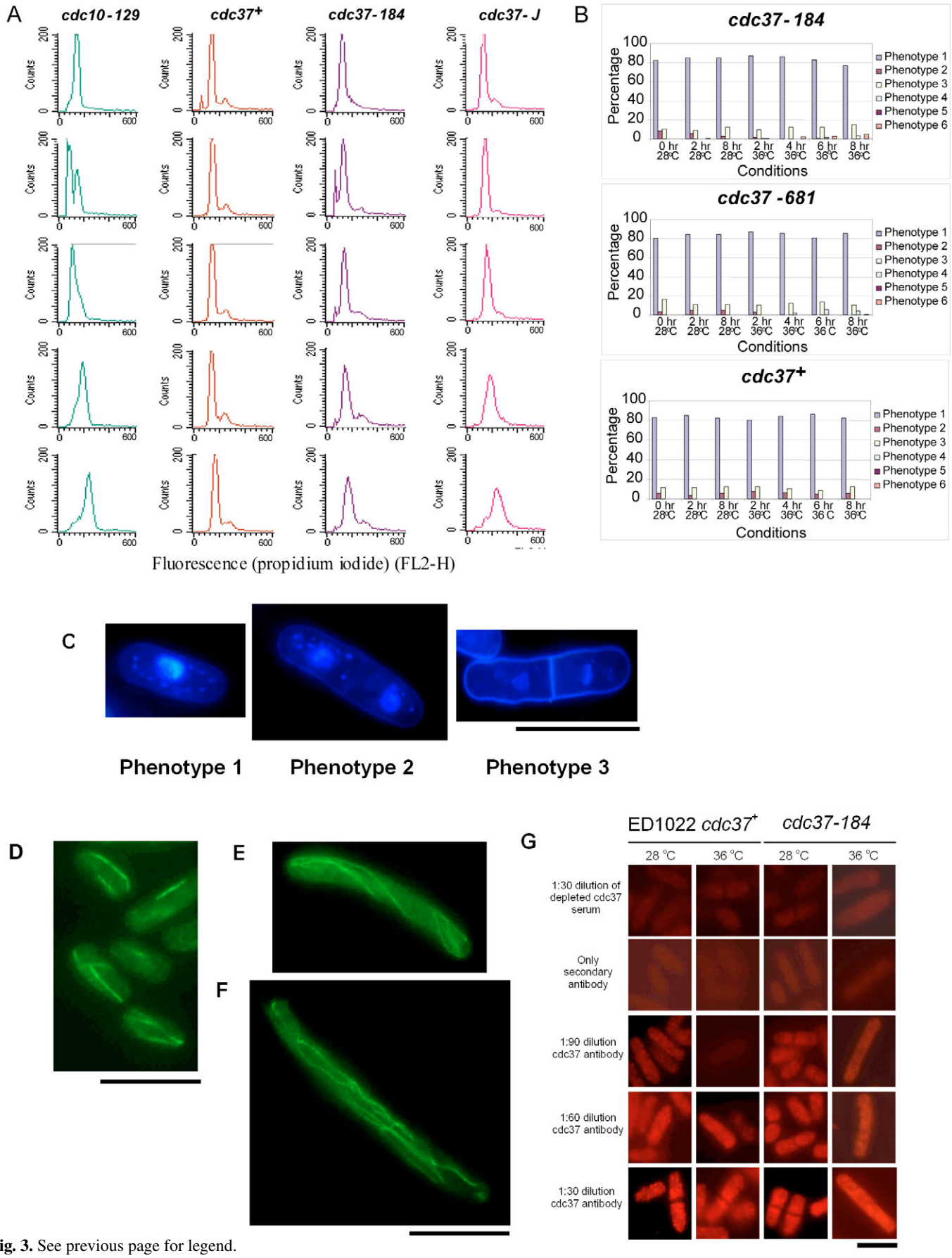

Fig. 3. See previous page for legend.

the later time points, as previously reported for other *cdc* mutants (Sazer and Sherwood, 1990).

The 2C DNA content of arrested *cdc37*^{ts} mutants indicates an arrest in either G2 or mitosis. To distinguish between these possibilities, the nuclear morphology of *cdc37*^{ts} mutants was examined. Cells were fixed and stained with DAPI to observe DNA morphology by microscopy. For all strains at 28 and 36°C approximately 80% of cells contained a single nucleus: results for *cdc37-184* and *cdc37-681* are shown in Fig. 3B,C, and *cdc37-J* and *cdc37-13* behaved similarly. The chromatin within cells containing a single nucleus showed no sign of condensation, indicating a G2 cell-cycle arrest. Furthermore, immunofluorescence with the TAT1 antibody for visualisation of cytoplasmic microtubules showed that the arrested *cdc37*^{ts} mutant cells did not display mitotic spindles, supporting the conclusion that cells are arresting in G2. The microtubule structures for *cdc37*$^{+}$ ED1022 cells, *cdc37-184* and *cdc37-J* are shown in Fig. 3D,E,F respectively and the other *cdc37*^{ts} mutant cells displayed similar features.

In the cultures grown at 28°C and in ED1022 at 36°C, the ~20% of cells containing more than a single nucleus were nearly all distributed between those containing two nuclei with no septum and those with a septum separating two nuclei (Fig. 3B,C; phenotypes 2 and 3 respectively). This is indicative of normal progress through the cell cycle beyond G2, through mitosis, followed by septum formation and cleavage. By contrast, the *cdc37-184* and *cdc37-681* mutant strains after the shift to 36°C showed a fall to zero in the proportion of binucleate cells lacking a septum (Fig. 3C, phenotype 2). This is consistent with cells in mitosis at the time of shift completing the process, whereas the G2 arrest prevents any further cells from entering mitosis. However the proportion of cells with two nuclei separated by a septum (Fig. 3C, phenotype 3) increased and remained at about 16% throughout most of the time course. This suggests that cells which complete mitosis after the shift form a septum which they are unable to cleave, which in turn suggests that Cdc37 function is required for a late (post-mitosis) stage in the cell cycle. Since preventing septum cleavage does not prevent DNA replication in the nuclei, this might account for the minority of *cdc37*^{ts} cells with apparent 4C DNA content at later time points in Fig. 3A. The pattern of behaviour shown by *cdc37-681* and *cdc37-184* (Fig. 3B,C) was also shown by the other *cdc37*^{ts} mutants (data not shown).

Cdc37 localisation is diffuse throughout the cells and does not change in *cdc37*^{ts} mutants.

The cellular distribution of the Cdc37 protein was investigated by immunofluorescence in *cdc37*$^{+}$ and *cdc37*^{ts} mutant cells at 28°C and after the shift to 36°C. Samples were taken every 2 hours and processed for immunofluorescence with the affinity-purified *S. pombe* Cdc37 antibody. The Cdc37 protein was distributed throughout the cell, forming punctate spots with no specific localised pattern in both *cdc37*^{ts} mutants and *cdc37*$^{+}$ cells at 28 and 36°C. Immunofluorescence of Cdc37 for *cdc37-184* and the *cdc37*$^{+}$ strain are shown in Fig. 3G, and *cdc37-681*, *cdc37-J* and *cdc37-13* displayed similar Cdc37 localisation. When fission yeast Cdc37 was tagged with GFP, it was also found to localise throughout the cell, although distinct localisation was observed in the chromatin region of the nucleus (Tatebe and Shiozaki, 2003). Differences in the techniques used to observe Cdc37 localisation, such as using a GFP tag compared with staining with a specific antibody and the treatment of cells to visualise Cdc37-GFP or Cdc37 antibody staining, could be responsible for the discrepancies between these findings and our observations. We did not observe bright spots around the chromatin region using a variety of immunofluorescence techniques with the *S. pombe* antibody.

To determine whether immunofluorescence associated with the Cdc37 antibody was specifically detecting the Cdc37 protein, a number of different conditions were tested. Cells from the strains *cdc37-184* and *cdc37*$^{+}$ ED1022 were incubated at 28 and 36°C for 8 hours and treated with 1:90, 1:60 and 1:30 dilutions of the *S. pombe cdc37* antibody, secondary antibody only or a 1:30 dilution of antiserum that had been previously depleted of anti-Cdc37 antibodies by adsorption onto GST-Cdc37 beads. Fluorescence of Cdc37 was clearly observed with 1:30 and 1:60 dilutions of the anti-*S. pombe cdc37* antibody, but not with secondary antibody only or Cdc37-depleted serum (Fig. 3G), indicating that the pattern of immunofluorescence shown in the bottom two rows of Fig. 3G for *cdc37-184* and *cdc37*$^{+}$ strain ED1022 genuinely reflects the distribution of Cdc37 within the cell.

cdc2 and *cdc37* interact genetically

The transition between G2 and mitosis is controlled in fission yeast by the cyclin-dependent kinase (Cdk) Cdc2, where entry into mitosis requires an active Cdc2 complexed with the cyclin Cdc13. Inactivation of Cdc2 in *S. pombe* causes cells to arrest at the G2-M boundary with a single nucleus, but they continue grow becoming morphologically elongated (MacNeill et al., 1991). The phenotype we have observed with *cdc37*^{ts} mutants is similar to this and we therefore investigated the relationship between Cdc2 and Cdc37. Initially the level of Cdc2 expression in all four *cdc37*^{ts} mutants was increased by introducing a genomic copy of *cdc2* on a plasmid, *pIRT-cdc2*. Increased expression of Cdc2 rescued the lethality of *cdc37-13* at 36°C (Fig. 4), resulting in morphologically wild-type cells; *cdc37-J* behaved similarly (data not shown). Increased expression of Cdc2 at 36°C partially rescued the lethality of *cdc37-184* (Fig. 4) permitting a low level of growth and *cdc37-681* behaved similarly (data not shown). However these cells were morphologically heterogeneous in appearance consisting of round, wild-type and elongated cells.

Further evidence connecting Cdc37 and Cdc2 comes from

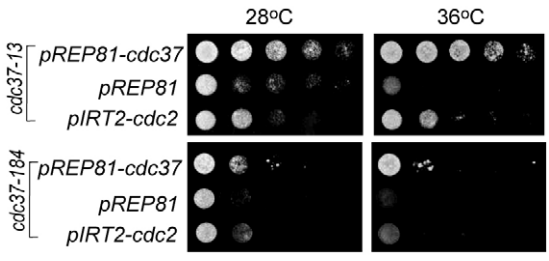

Fig. 4. Effect of increased expression of Cdc2 in *cdc37*^{ts} mutant strains *cdc37-13* and *cdc37-184*. The multicopy plasmid pIRT2-cdc2 which carries a genomic *cdc2* fragment was introduced into each strain and the resulting transformants tested for growth at 28 and 36°C by serial dilution.

the identification of synthetic genetic interactions. Crosses were carried out between *cdc37-684* and three *cdc2ts* mutants: *cdc2-33*, *cdc2-L7* and *cdc2-18*. Tetrads were dissected, and segregation consistent with the inviability of *cdc37 cdc2* double mutants was obtained. Specifically, we examined tetrads with three or four viable spores as being the most informative. Of these, four-spored tetrads (5 out of 17 scored) were invariably parental ditype consisting of four temperature-sensitive spores, and in each case two progeny showed much greater cell elongation at 36°C than the other two, indicative of *cdc2* and *cdc37* phenotype respectively. Of the 12 tetrads with three viable spores, 11 contained one wild-type and two temperature-sensitive spores, one of which was a *cdc2* type and one a *cdc37* type, suggesting tetratype segregation with the double mutant inviable. The remaining three-spored tetrad appeared to be parental ditype with one inviable spore. To test for the possible presence of double mutants, the progeny of eight clear tetratype tetrads from crosses between *cdc37-684* and *cdc2-33* or *cdc2-L7* were analysed further. The *cdc2*-like progeny were backcrossed to *cdc37-684*, and the *cdc37*-like progeny were backcrossed to the respective *cdc2* parent. In each case, wild-type (cdc$^+$) backcross progeny were obtained, indicating that each temperature-sensitive progeny spore harboured only a single temperature-sensitive mutation, and confirming that none of these tetrads contained any viable double mutant spores. The simplest explanation is that the combination of *cdc37-684* with any of the three *cdc2ts* alleles tested results in inviability. We crossed the Cdc13 temperature-sensitive mutant *cdc13-117* with *cdc37-681* and found the double mutant was not synthetically lethal at temperatures permissive for the single mutants (data not shown).

Cdc2 kinase activity is dramatically reduced in *cdc37*^{ts} mutants at the restrictive temperature

We studied the role of Cdc2 in the G2 cell-cycle arrest of the *cdc37*^{ts} mutants further to gain a better understanding of the relationship between Cdc2 and Cdc37. First, we examined Cdc2 activity in *cdc37*^{ts} mutants, as Cdc2 is required in an active state to promote mitosis. Cdc2 activity in *cdc37-184*, *cdc37-681*and *cdc37*$^+$ strains was assayed by its ability to phosphorylate histone H1 in vitro (Stern and Nurse, 1997). The *cdc37*$^+$ and *cdc37*^{ts} mutant strains were cultured at 28 and 36°C over a 3 hour time course. Samples were taken hourly and native protein extracts were prepared. Cdc2 was affinity-precipitated using p13^{suc1} beads and the H1 kinase activity of each sample was assayed. The ability of Cdc2 to phosphorylate histone H1 was greatly reduced in *cdc37*^{ts} mutants grown at 36°C, indicating a decrease in Cdc2 activity, whereas in *cdc37*$^+$ cells the level of activity remained constant (Fig. 5A).

A series of experiments were carried out to investigate the reason underlying the reduction of Cdc2 activity in *cdc37-184* and *cdc37-681* at 36°C. First, the level of Cdc2 protein in the mutants was determined. Native protein extracts were prepared and analysed by SDS-PAGE and western blotting with anti-PSTAIR antibodies against Cdc2. The Cdc2 protein levels did not change over the time course in any strain at 28 or 36°C (Fig. 5A). A similar experiment was also carried out with denatured protein extracts for all four *cdc37*^{ts} mutants and the *cdc37*$^+$ strain, and gave the same result (data not shown). This shows that reduced Cdc2 function is not due to lower Cdc2 protein levels. Our observations contrast with those made on

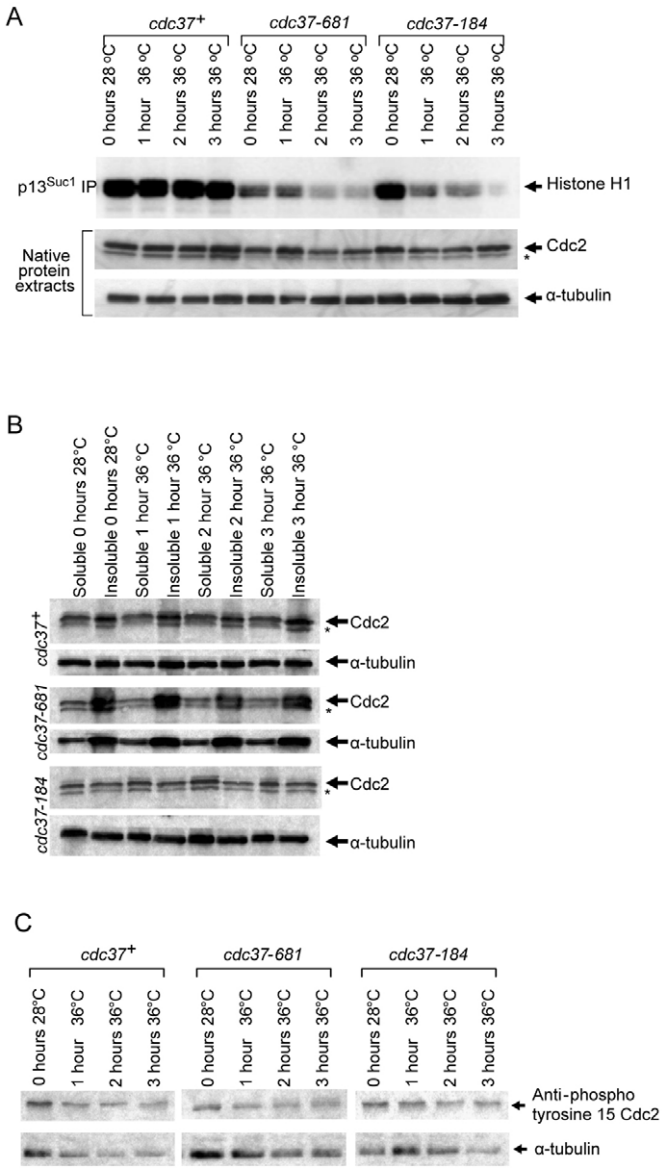

Fig. 5. (A) Cdc2 kinase activity and protein levels were assayed in *cdc37-681*, *cdc37-184* and *cdc37*$^+$ cells after growth at 28 and 36°C. Strains were cultured at 28 and 36°C over a 3 hour time course. Samples of cells were taken hourly and Cdc2 was affinity-precipitated on p13^{Suc1} beads. The kinase activity of Cdc2 was determined by its ability to phosphorylate histone H1. Cdc2 protein levels were determined by western blot with the anti-PSTAIR antibody and β-tubulin detected by TAT1 antibodies as a loading control. The asterisk indicates the position of p31 which is also recognised by the anti-PSTAIR antibody (see text). (B) The level of Cdc2 protein in soluble and insoluble fractions of extracts of *cdc37-184*, *cdc37-681* and *cdc37*$^+$ cells grown at 28°C or incubated at 36°C for 3 hours was analysed. Native protein extracts were prepared and the soluble and insoluble fractions separated by centrifugation at 20,000 ***g*** for 5 minutes at 4°C. Western blot analysis with anti-PSTAIR antibody against Cdc2 and TAT1 antibody as a loading control. (C) Level of phosphorylation on Tyr15 of Cdc2 in *cdc37-184*, *cdc37-681* and *cdc37*$^+$ strains incubated at 28 and 36°C over a 3 hour time course. Samples of cells were taken hourly and denatured *S. pombe* proteins extracted and western blotted with antibody specific for Cdc2 phosphotyrosine 15.

the *S. cerevisiae* temperature-sensitive mutant *cdc37-1*, where the level of Cdc28 (the Cdc2 homologue) was several-fold lower than the wild type (Gerber et al., 1995). Note that the antibody used recognises a band of higher mobility in addition to Cdc2 (asterisks in Fig. 5A,B). The antibody used is specific for the conserved PSTAIR motif and the extra band is presumably the Cdk p31 (Tournier et al., 1997).

In the *S. cerevisiae* mutant *cdc37-1*, the activity of the Cdc2 protein is reduced in part by aggregation of the Cdk into insoluble complexes (Farrell and Morgan, 2000). We tested whether this was happening in fission yeast *cdc37*^{ts} mutants as it would not affect overall cellular Cdc2 protein levels but would presumably reduce enzymatic activity. Native protein extracts were prepared from *cdc37-184*, *cdc37-681* and *cdc37*$^{+}$ cells grown at 28 and 36°C, and soluble and insoluble protein fractions for each sample were isolated by centrifugation at 20,000 ***g*** for 5 minutes at 4°C. Cdc2 was found in both the soluble and insoluble fractions of all strains, and the proportions did not change with increasing time at the restrictive temperature (Fig. 5B). These data show that reduced Cdc2 activity in these *cdc37*^{ts} mutants is not due to Cdc2 forming insoluble aggregates at 36°C.

Cdc2 is negatively regulated during G2 by phosphorylation on Tyr15 by Wee1 (Gould and Nurse, 1989; Russell and Nurse, 1987). To determine whether tyrosine phosphorylation was the cause of Cdc2 inactivation in *cdc37*^{ts} mutants, denatured protein extracts were prepared from *cdc37-184*, *cdc37-681* mutant and *cdc37*$^{+}$ strains cultured at 28 and 36°C over a 4 hour time course. Western blot analysis with a specific anti-phospho-Cdc2 (Tyr15) antibody was carried out. This revealed that the level of phosphorylation on Tyr15 of Cdc2 in *cdc37*^{ts} mutants did not increase over the time course at 36°C (Fig. 5C). This makes it unlikely that defective Cdc37 function in *cdc37*^{ts} mutants acts to prevent mitosis by increasing the level of Cdc2 tyrosine phosphorylation.

Another possible explanation for the reduced activity of Cdc2 in *cdc37*^{ts} mutants at 36°C is that the ability of Cdc2 to form a stable complex with the mitotic Cdc13 might be impaired. Formation of a complex between Cdc13 and Cdc2 is essential for Cdc2 activity and entry into mitosis (Nurse, 1997). Native protein extracts were prepared from *cdc37-184*, *cdc37-681* and *cdc37*$^{+}$ strains cultured at 28 and 36°C. For comparison, a *cdc25-22* strain was treated in the same way. Immunoprecipitations were carried out with the anti-Cdc13 antibody. Western blot analysis with the anti-PSTAIR antibody revealed that the level of Cdc2 that co-precipitated with Cdc13 was reduced in *cdc37*^{ts} mutants incubated at 36°C (Fig. 6A,B). However no such reduction was observed in the *cdc25-22* strain, supporting the idea that the mechanism of G2 arrest in *cdc37ts* mutants is quite different from that imposed by reduced phosphatase activity on Cdc2-tyr15. Rather, the reduced Cdc2 activity in *cdc37* mutants at 36°C appears to result from a breakdown of the Cdc2-Cdc13 complex, or an inability to maintain the complex.

Cdc2 and Cdc37 interact in vivo

The results in the previous sections indicate that Cdc2 is a client protein of Cdc37. To investigate this possibility further, the biochemical interaction between Cdc2 and Cdc37 was investigated by immunoprecipitation experiments. Immunoprecipitates of Cdc37 from *cdc37*$^{+}$, *cdc37-681* and *cdc37-184* cells grown at 28 and 36°C contained Cdc2, identifying a biochemical interaction between the Cdk and the molecular chaperone protein (Fig. 6C). The level of Cdc2 bound to Cdc37 was reduced in *cdc37-681* and *cdc37-184* protein extracts from cells cultured at both 28 and 36°C compared with immunoprecipitation experiments with the *cdc37*$^{+}$ strain ED1022 (Fig. 6C). In the reverse immunoprecipitation experiment using a Cdc2-HA-tagged strain ED1576, we were unable to detect a biochemical interaction between Cdc2 and Cdc37. It is possible that the HA

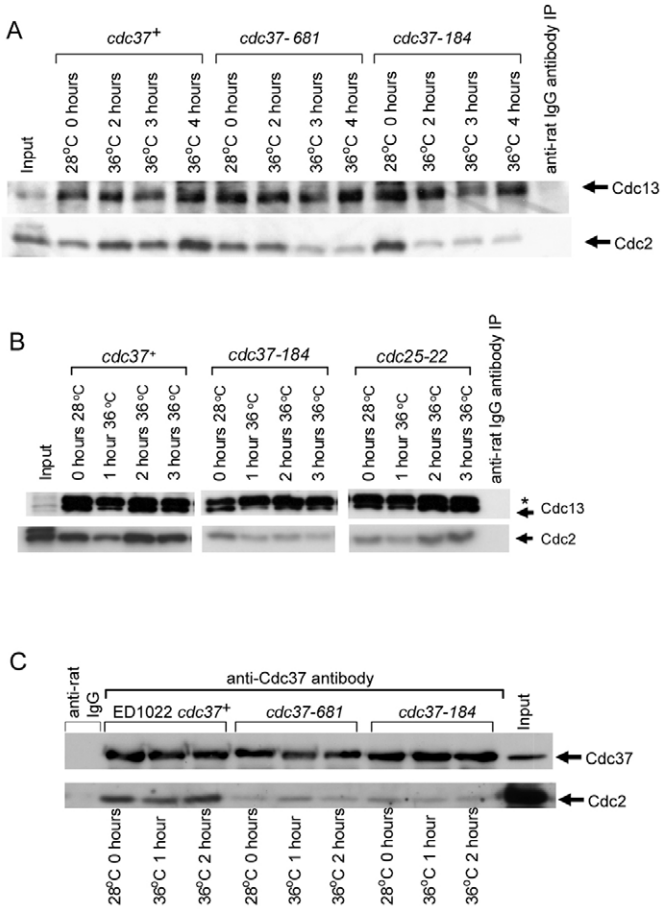

Fig. 6. (A) The interaction between Cdc2 and Cdc13 in *cdc37-184*, *cdc37-681* and *cdc37*$^{+}$ strains was analysed. Strains were cultured at 28 and 36°C over a 4 hour time course and samples of cells were taken as shown. Cdc13 was immunoprecipitated from protein extracts with the anti-Cdc13 6F 10/11 antibody. Western blot analysis was carried out with anti-Cdc13 6F 10/11 and anti-PSTAIR antibodies. (B) The interaction between Cdc2 and Cdc13 in *cdc37-184*, *cdc25-22* and *cdc37*$^{+}$ strains was analysed. Strains were cultured at 28 and 36°C over a 3 hour time course and samples of cells were taken hourly. Cdc13 was immunoprecipitated from protein extracts with the anti-Cdc13 6F 10/11 antibody. Western blot analysis was carried out with anti-Cdc13 6F 10/11 and anti-PSTAIR antibodies. (C) Immunoprecipitation experiments to detect a biochemical interaction between Cdc2 and Cdc37 in native *S. pombe* protein extracts from *cdc37+*, *cdc37-681* and *cdc37-184* cells cultured at both 28 and 36°C. Immunoprecipitates with the anti-*S. pombe* Cdc37 and anti-rat IgG (control) antibodies were run on SDS-PAGE and analysed by western blot to determine whether Cdc2 precipitates with Cdc37 from native *S. pombe* protein extracts.

tag subtly affects stability of the complex, making it harder to detect by immunoprecipitation.

Discussion

In this work three temperature-sensitive mutants of *cdc37* were generated in *S. pombe* using either random or directed mutagenesis, and were analysed in parallel with a fourth mutant, *cdc37-681*. It is interesting to note that all the mutations identified in these mutants are located in the same region of the protein. By alignment with the human Cdc37 protein these mutations are found around the Hsp90-binding domain and the homodimerisation domain in the large six-helix bundle of the middle domain (Roe et al., 1999). It is possible that temperature-sensitive mutants of *cdc37* in *S. pombe* only results from mutations in this region, though it would be necessary to examine a larger sample of mutants to test this possibility. Mutations in this region may produce temperature-sensitive proteins by interfering with proper folding and conformation of the six-helix bundle of Cdc37, which may affect essential activities such as interacting with client proteins. The data presented here indicate that reduced Cdc37 function is the cause of lethality at the restrictive temperature, as protein levels of this molecular chaperone were unchanged in *cdc37*^{ts} cells incubated at 36°C.

At 36°C all four *cdc37*^{ts} mutants were unable to produce colonies from single cells. The effect of loss of Cdc37 function in *cdc37*^{ts} mutants was rapid, stopping cell division at 36°C within 2 hours. The arrested cells displayed an elongated morphology, characteristic of *cdc* phenotype. Previous studies on *cdc37-681* reported that this mutant did not show cell elongation at the restrictive temperature (Tatebe and Shiozaki, 2003). The reason for the discrepancy is not clear, although growth and cell elongation may be impaired as in two other cdc37ts mutants examined, and perhaps subtle differences in growth conditions also play a role. The observation here that all *cdc37*^{ts} mutants become elongated at 36°C suggests that a major component involved in cell-cycle progression is inhibited by impairment of Cdc37 function.

All four *cdc37*^{ts} mutants were found to arrest the cell cycle in G2 with a single nucleus. The majority of *S. cerevisiae* temperature-sensitive mutants of *cdc37* arrest the cell cycle at Start (Dey et al., 1996; Farrell and Morgan, 2000; Gerber et al., 1995; Reed, 1980a; Reed, 1980b; Valay et al., 1995). This may reflect differences in cell-cycle control between the two yeasts: the major control point in *S. cerevisiae* is at Start within G1, whereas the fission yeast cell cycle is primarily regulated during G2. Interestingly, a proportion of *cdc37-184* cells was seen to contain an unreplicated DNA content after 2 hours at the restrictive temperature, but all arrested with a replicated DNA content after 4 hours. These cells may undergo a delay in G1 owing to defects caused by loss of Cdc37 function, and then finally arrest the cell cycle in G2, similar to the other three *cdc37*^{ts} mutants.

The arrest phenotype of *cdc37*^{ts} mutants is reminiscent of that of temperature-sensitive *cdc2* mutants, namely, elongated cells that arrest mainly at the G2-M boundary with an undivided interphase nucleus (MacNeill et al., 1991). It may be pertinent that like some *cdc2*^{ts} mutants, *cdc37-184* shows a G1 defect in addition to G2 arrest. We report in this work that Cdc2 activity is dramatically reduced at 36°C in *cdc37*^{ts} mutants. A modest increase in the level of Cdc2 expression fully rescues the temperature-sensitive growth defect of *cdc37-13* and *cdc37-J*, and partially rescues *cdc37-184* and *cdc37-681*. These data indicate that loss of Cdc2 activity is the principal cause for the cell-cycle arrest. One explanation for the suppression is that Cdc37 function is reduced in *cdc37*^{ts} mutants below a critical threshold level, but not abolished, so that elevating the cellular level of Cdc2 increases the chances of Cdc37 carrying out its required chaperone activity on Cdc2. Alternatively, Cdc2 activity may only partially depend on Cdc37 function, so that artificially increasing the Cdc2 level allows enough of the protein to form active complexes and drive cell-cycle progress. Differences in the ability of Cdc2 to rescue the *cdc37*^{ts} mutants could arise from the different types of mutations that affect Cdc37 at varying levels of severity.

Our investigations have shown that, in contrast to the situation in *S. cerevisiae*, reduced Cdc2 activity in *cdc37*^{ts} mutants is not the result of lower Cdc2 protein levels nor because Cdc2 aggregates into insoluble complexes. Furthermore, there is no indication of an increase in the level of phosphorylation of Tyr15 of Cdc2, which might have accounted for loss of Cdc2 activity, as occurs in arrested *cdc25* mutants (Nurse, 1997). The data we present here show that Cdc2 activity in *cdc37*^{ts} mutants is reduced because of its inability to maintain a stable complex with the cyclin Cdc13. Cdc2 may be a client of Cdc37 that relies on this molecular chaperone to promote its activation by aiding in the assembly of complexes with Cdc13. Further evidence supporting this idea comes from the identification of both genetic and biochemical interactions between Cdc2 and Cdc37. Mutants containing temperature-sensitive mutant alleles for both Cdc2 and Cdc37 are synthetically lethal and Cdc2 co-immunoprecipitates with Cdc37. The level of Cdc2 that precipitated with Cdc37 was reduced in *cdc37*^{ts} mutants at both 28 and 36°C compared with a *cdc37*$^{+}$ strain. In these *cdc37*^{ts} mutants at the permissive temperature, the level of Cdc37 binding of Cdc2 may be reduced but sufficient to maintain cell viability and promote cell-cycle progression. When these mutants are shifted to the restrictive temperature, Cdc37 loses function, and although bound to Cdc2 cannot carry out its required chaperone function, which presumably promotes the interaction between Cdc2 and Cdc13.

The complex between Cdc2 and Cdc13 may be extremely dynamic and require functional Cdc37 to maintain the interaction. Cdc37 has been seen to promote the assembly of Cdk-cyclin complexes in other systems, such as Cdk4 and its cyclin partners (Lamphere et al., 1997; Stepanova et al., 1996). Similar observations have been reported for the *S. cerevisiae* temperature-sensitive mutant *cdc37-1*, where Cdc28, the *S. cerevisiae* homologue of Cdc2, failed to bind cyclin partners, G1 cyclin Cln2 and the mitotic cyclin Clb2, at the restrictive temperature (Farrell and Morgan, 2000; Gerber et al., 1995). The Cdk-activating kinase (CAK) Cak1 that phosphorylates Thr169 was present at reduced protein levels and displayed decreased activity (Farrell and Morgan, 2000). This may be a direct or indirect consequence of reduced Cdc37 function in *S. cerevisiae* temperature-sensitive *cdc37*^{ts} mutants. It is likely to contribute to the reduction in Cdc28 activity by reducing the stability and hence levels of Cdc28-cyclin complexes, as the equivalent threonine needs to be phosphorylated for the formation of stable complexes in some cases (Gould et al., 1991). In *S. pombe*, reduction in the level of complex with

Cdc13 is the only effect on Cdc2 we have detected, as the level of Cdc2 protein and its solubility appear to be unaffected in *cdc37*^{ts} mutants. This should prove to be an interesting avenue for investigation using the *S. pombe cdc37* mutants generated in this work.

Materials and Methods

Cloning, expression vectors and generation of *cdc37*^{ts} mutants.

S. pombe cdc37 (Westwood et al., 2004) was cloned into pREP vectors (*pREP1, pREP81*) for expression in *S. pombe*. *pIRT2-cdc2* was a kind gift from Stuart MacNeill (Institute for Molecular Biology and Physiology, University of Copenhagen, Denmark). The DNA sequences of all constructs were verified by DNA sequencing.

Temperature-sensitive mutants of *cdc37* were generated by random mutagenic PCR amplification of the random mutagenesis (RM) template. The RM template was constructed by amplification by PCR of the *S. pombe cdc37*$^{+}$ gene plus 300 bp of 5′ genomic flanking sequence from the cosmid c9b6 (Sanger Centre). New restriction sites, *Pac*I at the 5′ and *Bgl*II at the 3′ end, were introduced by the oligonucleotide primers for cloning into the *pFA6a-KanMX6* vector (Bahler et al., 1998) upstream of the G418 cassette. The 300 bp of *cdc37* 3′ chromosomal flanking sequence was also PCR amplified, introducing *Eco*RI restriction sites at both ends, and was cloned downstream of the G418 cassette. Random mutagenic PCR amplification of the RM template was carried out using 0.1-0.5 mM $MnCl_2$ in PCR reactions. PCR fragments were purified by phenol-chloroform extraction and ethanol precipitation and transformed into the *S. pombe* strain ED1090 for homologous recombination into the genome as described (Bahler et al., 1998). Stable transformants were selected on medium containing G418, and were replicated and incubated at 28 and 36°C to identify temperature-sensitive mutants.

Temperature-sensitive mutants of *cdc37* were also generated by directed mutagenesis using the RM template. The equivalent mutation to the *S. cerevisiae* temperature-sensitive mutant *cdc37-184* (Valay et al., 1995) was introduced into *S. pombe cdc37* gene in the vector pREP81 by overlap PCR mutagenesis, changing Ala275 to Asp. A DNA fragment containing the mutation was then inserted into the RM template using unique restriction sites *Blp*I and *Swa*I. This construct was amplified by PCR, transformed into the *S. pombe* strain ED1090 and stable transformants with temperature-sensitive phenotypes were identified. To determine the sequence of each mutant *cdc37* gene, genomic DNA was prepared essentially as described (Alfa et al., 1993) and was used as template in PCR reactions. Three PCR reactions were carried out to amplify individual regions of the *cdc37* gene, and the products were combined and purified. Fragments were sequenced in the forward and reverse direction at least twice for precision. This protocol was carried out over the entire *cdc37* gene and flanking sequence in duplicate to accurately determine the genomic sequence of *cdc37* temperature-sensitive mutants.

Yeast strains

S. pombe cdc37 temperature-sensitive mutant strains used were ED1565 (*cdc37-184 ura4-D18 leu1-32 h*$^{-}$), ED1566 (*cdc37-13 ura4-D18 leu1-32 h*$^{-}$), ED1567 (*cdc37-J ura4-D18 leu1-32 h*$^{-}$) and ED1538 (*cdc37-681 ura4-D18 leu1-32 h*$^{-}$) (a kind gift from Kazuhiro Shiozaki (Tatebe and Shiozaki, 2003)). Other *S. pombe* strains used were ED1090 (*ura4-D18 leu1-32 h*$^{-}$), ED1576 (*cdc2:3HA (KanMX) ura4-D18 leu1-32 h*$^{-}$), a kind gift from Paul Russell, and ED0824 (*cdc10-129 leu1-32 h*$^{-}$) for flow cytometry analysis (Nurse et al., 1976). The *cdc2* temperature-sensitive mutants used in this work were ED1446 (*cdc2-33 ura4-D18 leu1-32*) and ED1123 (*cdc2-L7 ura4-D18 h*$^{+}$). The temperature-sensitive mutant ED0865 (*cdc25-22 ura4-D18 leu1-32 h*$^{+}$) was also used.

Determination of *S. pombe* cell number and DNA content

Cell number in liquid fission yeast cultures was determined in a Coulter counter as described (Alfa et al., 1993). Flow cytometry was carried out as essentially described (Alfa et al., 1993), but the cells were subjected to an initial incubation step at room temperature for 1 hour in 1 ml of 0.1 M HCl plus 2 mg/ml pepsin (Erik Boye, Institute for Cancer Biology, Montebello, Oslo, Norway, personal communication) and processed in a FACScan (Becton Dickson).

Antibodies

The anti-*S. pombe* Cdc37 antibody used in this work was generated and affinity-purified in our lab (Turnbull et al., 2005). Depletion of the *S. pombe* Cdc37 serum in immunofluorescence experiments was carried out by incubation with recombinant GST-Cdc37 bound to glutathione beads and depleted antiserum was used in western blots against GST-Cdc37 and total *S. pombe* protein extracts to show loss of anti-*S. pombe* Cdc37 antibodies from serum (Turnbull et al., 2005). Cdc2 antibodies used were anti-PSTAIR, a kind gift from Jeremy Hyams (Institute of Molecular BioSciences, Massey University, New Zealand), also available from Sigma (#7962) and anti-phospho-Cdc2 (Tyr15) antibody (NEB #9111). Anti-Cdc2 Y63.2 and anti-Cdc13 6F 10/11 antibodies were kind gifts from Paul Nurse's laboratory (Cancer Research UK, London Research Institute, UK). The anti-HA 12CA5 monoclonal antibody (Roche) was used in immunoprecipitation experiments. The antibody used in immunofluorescence with the anti-*S. pombe* Cdc37 antibody was Alexa Fluor 680-tagged anti-rabbit IgG antibody (Molecular Probes) and Alexa Fluor 488 anti-mouse IgG antibody in parallel with the TAT1 antibody which was a kind gift from Keith Gull (Sir William Dunn School of Pathology, University of Oxford, UK). Anti-rabbit IgG HRP-linked antibody (Amersham), anti-mouse IgG HRP-linked antibody (Amersham) and anti-rat IgG HRP-linked antibody (Amersham) were used as appropriate.

Cytological staining

Fission yeast cells from liquid YE cultures were processed for staining with 1 μl Calcofluor (1 mg/ml), a fluorescent brightener that efficiently binds the yeast cell wall and septum (Streiblova et al., 1984) (Sigma-Aldrich F3543) or 1 μl DAPI (4′-6-diamidino-2-phenylindole) (50 μg/ml) (Sigma D9542) as described (Alfa et al., 1993). Immunofluorescence of fission yeast cells with the anti-*S. pombe* Cdc37 antibody was carried out according to published methods (Snaith and Sawin, 2003) and with TAT1 antibodies as described (Sawin and Nurse, 1998). Cells were visualized under a 63× oil objective Axioskop 2 lens on a fluorescence microscope (Zeiss) and photographs were taken with a digital camera (Princeton Instruments) using IPLab scientific imaging software (Scanalytics).

Protein extracts and western blots

Protein extracts (native or denatured) were made from fission yeast by harvesting 50-200 ml cells at an OD_{600} of 0.4 at 5000 ***g*** and washing once in STOP buffer (Lyapina et al., 2001). Samples were resuspended in 100 μl buffer C (10 mM NaCl, 0.35% Triton-X, 50 mM Tris-HCl pH 7.5, 20 mM molybdate, 10% glycerol and 1× Complete protease inhibitors) or HB15 [1× Complete protease inhibitors, 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 25 mM MOPS (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4 (pH 8), 1% Triton X-100] and placed in a tube containing 1 ml sterile acid-washed glass beads (Sigma G8772). Samples were vortexed four times for 20 seconds, with 1 minute intervals on ice. A 500 μl volume of buffer was added to each tube and the contents mixed thoroughly. The supernatant was transferred to a fresh tube and centrifuged at 20,000 ***g*** to remove insoluble material. For native extracts, the protein concentration of the supernatant was determined by Bradford Protein Assay (Bio-Rad), and equal amounts of protein were loaded into each gel lane. For denatured extracts this was not possible because the SDS interferes with the protein assay. Therefore equal loading was ensured by extracting total protein from an equivalent biomass of cells (OD_{600} × volume) for each sample. Western blotting was carried out using PBS buffer and non-fat dried milk, except with the anti-phospho-Cdc2 (Tyr15) antibody when TBS buffer with BSA was used.

Immunoprecipitation

Immunoprecipitation from *S. pombe* protein extracts of Cdc37 and Cdc2-HA were carried out with buffer C. Immunoprecipitation of Cdc13 and Cdc2 used HB15 buffer. Protein A Sepharose™ CL-4B beads (Amersham) were incubated with anti-*S. pombe* Cdc37 antibody, anti-Cdc13 6F 10/11 antibody, anti-HA antibody or anti-rat IgG (Amersham) for 30 minutes at 4°C on a rotating wheel, then washed three times. Native *S. pombe* protein extracts were added and incubated at 4°C for 2 hours on a rotating wheel. Immunoprecipitates were washed four times with appropriate buffer and resuspended in 2× SDS loading buffer. Samples were run on SDS-PAGE and western blots were carried out with appropriate antibodies.

Cdc2 kinase assays

Cdc2 was precipitated from native *S. pombe* protein extracts using $p13^{Suc1}$ beads (Amersham) and its kinase activity was assayed using histone H1 (Upstate Biotechnologies) as described (Stern and Nurse, 1997).

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