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Caffeine can override the S-M checkpoint in fission yeast

Shao-Win Wang[1], Chris Norbury[2,*], Adrian L Harris[2] and Takashi Toda[1]

Imperial Cancer Research Fund, [1]Cell Regulation Laboratory, PO Box 123, Lincoln's Inn Fields, London WC2 A3P, UK and [2]Molecular Oncology Laboratory, University of Oxford, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, UK
*Author for correspondence (E-mail: c.norbury@icrf.icnet.uk)

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SUMMARY

The replication checkpoint (or 'S-M checkpoint') control prevents progression into mitosis when DNA replication is incomplete. Caffeine has been known for some time to have the capacity to override the S-M checkpoint in animal cells. We show here that caffeine also disrupts the S-M checkpoint in the fission yeast *Schizosaccharomyces pombe*. By contrast, no comparable effects of caffeine on the *S. pombe* DNA damage checkpoint were seen. *S. pombe* cells arrested in early S phase and then exposed to caffeine lost viability rapidly as they attempted to enter mitosis, which was accompanied by tyrosine dephosphorylation of Cdc2. Despite this, the caffeine-induced loss of viability was not blocked in a temperature-sensitive *cdc2* mutant incubated at the restrictive temperature, although catastrophic mitosis was prevented under these conditions. This suggests that, in addition to S-M checkpoint control, a caffeine-sensitive function may be important for maintenance of cell viability during S phase arrest. The lethality of a combination of caffeine with the DNA replication inhibitor hydroxyurea was suppressed by overexpression of Cds1 or Chk1, protein kinases previously implicated in S-M checkpoint control and recovery from S phase arrest. In addition, the same combination of drugs was specifically tolerated in cells overexpressing either of two novel *S. pombe* genes isolated in a cDNA library screen. These findings should allow further molecular investigation of the regulation of S phase arrest, and may provide a useful system with which to identify novel drugs that specifically abrogate the checkpoint control.

Key words: Caffeine, Cell cycle, Replication checkpoint, Cdc2, Hydroxyurea, *S. pombe*

INTRODUCTION

Genome integrity is maintained by a complex network of checkpoint mechanisms that co-ordinate DNA replication with repair and ensure the correct ordering of cell cycle events (Hartwell and Weinert, 1989). These checkpoints can be disrupted by a variety of drugs or genetic lesions. In mammalian cells, loss of checkpoint control results in DNA rearrangements, amplification and chromosome loss, events that are causally associated with cancer (Hartwell and Kastan, 1994; Lehmann and Carr, 1995). In fission and budding yeasts, relief-of-dependence mutations have been identified that allow cell cycle progression under conditions that would normally cause cell cycle arrest (reviewed by Murray, 1992). Genetic analysis of these mutants has provided important information about the mechanisms of checkpoint control, and from these studies a picture of how checkpoints may work at the molecular level is beginning to emerge.

Two of the most extensively characterised pathways are the S-M checkpoint, which prevents cells from entering mitosis with incompletely replicated chromosomes, and the DNA damage checkpoint, which prevents entry into mitosis (or anaphase) when DNA integrity is compromised (Stewart and Enoch, 1996). Genetic evidence has indicated that the DNA damage and S-M checkpoint pathways are distinct in the fission yeast *Schizosaccharomyces pombe* (Enoch and Nurse, 1990), although several *rad*/*hus* mutants that are defective in DNA damage checkpoints also show sensitivity to the DNA replication inhibitor hydroxyurea (Al-Khodairy and Carr, 1992; Enoch et al., 1992). This suggests that there is a degree of overlap between the S-M and DNA damage checkpoints in terms of the gene products involved. The checkpoint *rad*/*hus* gene products appear to act as signal transducers that mediate the activation of the Cds1 and Chk1 protein kinases; these in turn serve to inhibit mitotic entry by allowing inhibitory phosphorylation of the mitosis-promoting cyclin-dependent kinase Cdc2, at least in part through Cds1/Chk1-mediated phosphorylation of the Cdc2-specific phosphatase Cdc25 (Francesconi et al., 1997; Boddy et al., 1998; Lindsay et al., 1998; Zeng et al., 1998; Kumagai et al., 1998).

A variety of chemical agents that are capable of overriding checkpoint control have also been identified. These include the phosphatase inhibitors okadaic acid (Yamashita et al., 1990), fostriecin (Roberge et al., 1994; Guo et al., 1995) and calyculin A (Nakamura and Antoku, 1994), protein kinase antagonists, such as staurosporine (Tam and Schlegel, 1992) and aminopurines (Andreassen and Margolis, 1992), and methylxanthines such as caffeine and pentoxifylline. In animal cells, caffeine induces premature chromosome condensation (PCC) when DNA replication is blocked with hydroxyurea (Schlegel and Pardee, 1986) and time-lapse video microscopy of hamster BHK fibroblasts revealed that caffeine can induce

multiple entries into mitosis when DNA synthesis is blocked (Schlegel and Pardee, 1987). Addition of caffeine to sea urchin embryos arrested in S phase with aphidicolin resulted in nuclear envelope breakdown, followed by chromatin condensation (Patel et al., 1997). The abrogation of cell cycle arrest by caffeine is associated with the selective sensitisation of p53-deficient primary and tumour cells to anticancer agents and radiation (Russell et al., 1995; Powell et al., 1995; Fan et al., 1995; Yao et al., 1996). Although the mechanism by which caffeine disrupts the checkpoint control is unknown, there is some evidence to suggest that it works by preventing phosphorylation-mediated inactivation of Cdc2 (Yao et al., 1996; Patel et al., 1997; Poon et al., 1997; Winters et al., 1998). In a very recent study of S-M checkpoint control in *Xenopus* egg extracts, activation of Chk1 in response to inhibition of replication was found to be caffeine-sensitive, suggesting that the relevant caffeine target lies somewhere in a pathway linking the stalled replication complexes and Chk1 (Kumagai et al., 1998).

We have investigated the molecular mechanism of checkpoint disruption by caffeine, using *S. pombe* as a model system. In this unicellular eukaryote, caffeine has been reported to decrease mutation rates induced by DNA-damage agents (Loprieno and Schupbach, 1971) and also to enhance the effect of exogenously added cAMP, possibly by inhibiting a cAMP phosphodiesterase (Beach et al., 1985). The diverse effects caused by caffeine suggest multiple cellular targets exist for this drug (Kumada et al., 1996). In this study, we show that caffeine can also induce mitosis in *S. pombe*, in the presence of unreplicated DNA, by stimulating the tyrosine dephosphorylation of Cdc2. Like checkpoint mutants, cells arrested in S phase and treated with caffeine undergo septation in the absence of chromosome segregation and rapidly lose their viability. By using different *cdc* mutants, we demonstrate that caffeine induces mitosis specifically in S phase. We also show that in addition to the checkpoint pathway inhibiting mitosis, a caffeine-sensitive pathway is also required to enable cells to survive S-phase arrest. The potential mechanisms of action of novel genes involved in these pathways and isolated by a cDNA library screen are also discussed.

MATERIALS AND METHODS

Strains, media and genetic techniques

S. pombe strains used in this study are listed in Table 1. Except where indicated to the contrary, all reagents were obtained from Sigma. Rich medium (YPD; 1% yeast extract, 2% polypeptone, 2% dextrose) and modified minimal medium (EMM2; Moreno et al., 1991) were used for liquid cultures. In some experiments caffeine was added to YPD from a 100 mM stock solution prepared in distilled water. Standard procedures for *S. pombe* genetics were followed according to Moreno et al. (1991). Cell concentration was determined with a Sysmex F-800 cell counter (TOA Medical Electronics, Japan). The lithium acetate method (Ito et al., 1983) was used for yeast transformations.

Table 1. Yeast strains used in this study

Strain	Genotype	Derivation
HM123	*h⁻ leu1-32*	Laboratory stock
cdc2	*h⁻ cdc2-33*	P. Nurse (ICRF, London, UK)
cdc10	*h⁻ cdc10-129 leu1-32*	P. Nurse
cdc17	*h⁻ cdc17-M75*	P. Nurse
cdc22	*h⁻ cdc22-M45*	P. Nurse
cdc25	*h⁺ cdc25-22*	P. Nurse
hus1	*h⁻ hus1-14 leu1-32*	Enoch et al. (1992)

Fig. 1. Caffeine overrides the S-M checkpoint in fission yeast. HU (10 mM) was added to an asynchronous culture of *S. pombe* strain HM123 growing at 30°C in YPD medium. After 3 hours of further incubation caffeine (10 mM) was added to one half of the culture. (A) Samples were taken at the time of HU addition and at hourly intervals thereafter and percentages of septated cells were scored. (B) Samples taken at the time of caffeine addition and at hourly intervals thereafter were assessed microscopically for the appearance of the cut phenotype. (C) Cell viability was measured at the same time points by plating appropriate dilutions of cells onto YPD agar plates without drugs and scoring colony formation after 3 days incubation at 30°C. Viability is expressed as a percentage of the number of colonies obtained in the sample taken from the culture exposed to HU alone for 3 hours. (D) Examples of cells harvested after 6 hours HU treatment, with or without caffeine added at 3 hours. Cells were stained with calcofluor (upper panels) to reveal septa, or with DAPI (lower panels) to reveal nuclei. Septated cells exhibiting the cut phenotype are indicated by arrowheads. Bar, 10 μm. (E) Flow cytometric analysis of the DNA content of propidium iodide-stained, ethanol-fixed samples from the experiment shown in A-D. Cells were harvested at the time of HU addition (0) and at hourly intervals thereafter. Data from 10,000 cells per time point are plotted, and the positions of G_1 (1C) and G_2/M (2C) cell populations are indicated. Note that the asynchronous starting population consists mainly of G_2 cells, as expected of a wild type *S. pombe* exponential population (the G_2 phase occupies most of the cell cycle and cells replicate their DNA close to the time of septation).

Flow cytometry

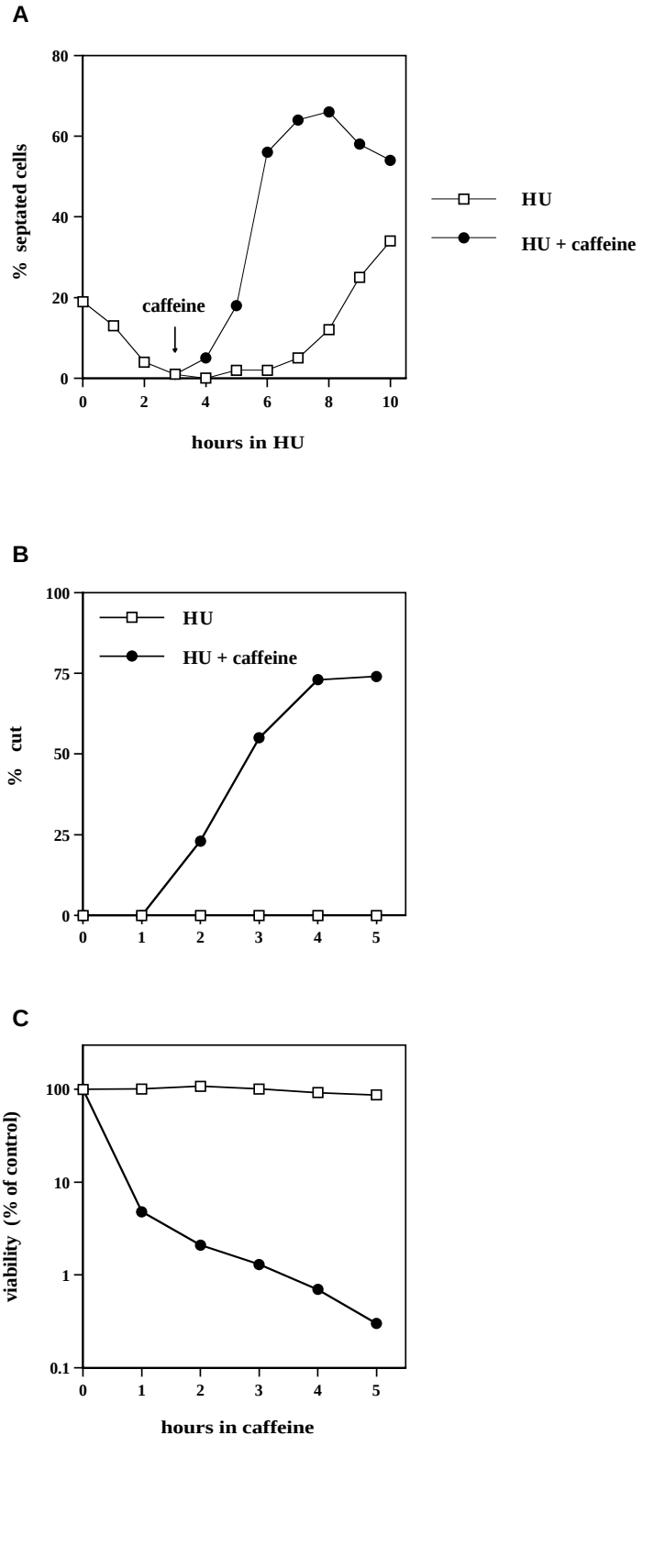
Cells were fixed at a density of 10^7/ml in 70% ethanol at 4°C overnight. Fixed cells were washed once with 50 mM sodium citrate buffer (pH 7.0), resuspended in the same buffer containing 100 μg/ml DNAse-free RNAse and incubated at 37°C overnight. After staining with propidium iodide (4 μg/ml final concentration), cells were analysed by flow cytometry (FACScan, Becton Dickinson) using a 488 nm laser. Red fluorescence (DNA) data were collected for 10,000 cells per time point and were analysed using CellQuest software.

Immunochemistry

Cell extracts were prepared by trichloroacetic acid precipitation following glass bead disruption (Watanabe et al., 1997). Total cellular protein preparations were separated on a 10% SDS-PAGE gel and blotted to nitrocellulose membranes (Hybond ECL, Amersham) as described elsewhere (Ausubel et al., 1995). Tyrosine 15 phosphorylation of Cdc2 was detected with a phosphoepitope-specific rabbit polyclonal antibody (New England Biolabs). Total Cdc2 was visualised with the mouse monoclonal antibody Y100 (generated by Dr J. Gannon and kindly provided by Dr H. Yamano). Horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad) and enhanced chemiluminescence (ECL, Amersham) were used to detect bound antibody.

cDNA library screen

S. pombe strain HM123 was transformed with a *S. pombe* cDNA library (B. Edgar and C. Norbury, unpublished) constructed in the vector pREP3X, a *LEU2*-containing multicopy plasmid (Forsburg, 1993). 10^5 leu^+ transformants obtained were tested for their resistance to caffeine with hydroxyurea (HU) by replica plating to YPD medium containing 5 mM caffeine and 10 mM HU. Cells capable of forming colonies were isolated and tested their cross-resistance to other drugs by replica plating to YPD medium containing the spindle poison MBC (10 μg/ml) or 1 μg/ml staurosporine (generously provided by Dr H.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HU
HU + caffeine
time (hours)
cell count
1C 2C
caffeine
DNA content

Nakano, Kyowa Hakko Co., Japan). Three transformants showing no such cross resistance were isolated. Plasmids recovered from these transformants were sequenced by the dideoxy method (Sanger et al., 1977) using an automated sequencer (ABI 377, Perkin Elmer).

RESULTS

Caffeine overrides the S-M checkpoint in fission yeast

To determine whether caffeine was capable of triggering mitosis in the presence of unreplicated DNA, we used a fission yeast culture arrested in early S phase by the ribonucleotide reductase inhibitor hydroxyurea (HU). The septation index (a marker for progression through mitosis) dropped from approximately 20% to <1% by 3 hours after addition of HU (Fig. 1A). The number of septated cells started to increase after 7 hours exposure to the inhibitor, showing that the cells had adapted to the HU-induced block and had re-entered the cell cycle. These data are consistent with previous studies showing that HU blocks cell cycle progression only temporarily in *S. pombe*, as in other organisms (Sazer and Nurse, 1994). Caffeine was added to one half of the culture 3 hours after HU; septation, abnormal mitosis and viability were measured in both half-cultures at hourly intervals. The control cells remained in interphase and became elongated between 3 and 7 hours after HU addition, continuing to grow without dividing (Fig. 1A,B,D). By contrast the caffeine-treated cells underwent septation in the absence of chromosome segregation. This resulted in cells where the septum had either bisected the single nucleus or had divided the cell such that one daughter was anucleate (Fig. 1D), which resembles the phenotype previously described in *cut* mutants (Hirano et al., 1986) and in S-M checkpoint mutants arrested in S phase (Enoch and Nurse, 1990). After exposure to caffeine for 4 hours, 75% of the HU-treated cells displayed the cut phenotype. In contrast, at the same time point, none of the cells exposed to HU alone had this phenotype (Fig. 1B).

Approximately 90% of the HU-arrested cells were committed to loss of viability by 1 hour after the addition of caffeine (Fig. 1C), at which time septated cells had only just started to accumulate, indicating that cells became committed to losing their viability before they entered mitosis. A similar phenotype has been described previously in *S. pombe* for certain of the *rad*/*hus* checkpoint mutants (Al-Khodairy and Carr, 1992; Kostrub et al., 1997). This result suggests that, in addition to the defect in the coupling of mitosis to the completion of DNA replication, a function that is required to maintain viability early in S-phase is also lacking in these cells (Enoch et al., 1992).

To determine if the caffeine-treated cells had entered mitosis prior to the completion of S-phase, we monitored the DNA content of cells using flow cytometry. DNA synthesis was completely blocked by 3 hours after the addition of HU, at which time cells displayed a 1C DNA content (Fig. 1E). As cells adapted to the HU block, DNA synthesis was observed after 5 hours, but this was not completed until about 7 hours after HU addition. Cells treated with caffeine resumed and completed DNA replication with similar kinetics, indicating that caffeine has no obvious effect on DNA synthesis in this system. Cells displaying the cut phenotype were first observed 2 hours after addition of caffeine (5 hours in HU) and cut cells accumulated at 3 hours, well before DNA replication was completed (Fig. 1B,E). Thus, *S. pombe* cells treated with caffeine entered mitosis prematurely when DNA synthesis was blocked by HU. In addition, cells with DNA contents less than 1C appeared 3 hours after addition of caffeine, further suggesting that defective mitoses and cell divisions were taking place during this period.

Caffeine acts at S and S/G_2 but not at the pre-start or G_2/M period

The phenotype described above strongly suggests that caffeine causes a defect in the prevention of premature mitosis when S phase is delayed or arrested. To examine this further, temperature-sensitive cell cycle mutants were used to arrest cells at different points of the cell cycle, to test the ability of caffeine to override checkpoint control from these arrest points. Cells were arrested in pre-start G_1 by *cdc10-129*, in S phase by *cdc22-M45* (a temperature-sensitive ribonucleotide reductase mutant), in late S/G_2 phase by *cdc17-M75* (a DNA ligase I mutant) or in late G_2 phase by *cdc25-22*. After 3 hours at the restrictive temperature, caffeine was added to half of each culture and viability was measured in both half-cultures at hourly intervals. Since each *cdc* mutant has different viability kinetics after the temperature shift, the cell viability after addition of caffeine was expressed relative to that of the respective *cdc* mutant in the absence of caffeine. Results of the effects of caffeine in these *cdc* backgrounds are summarised in Table 2. Addition of caffeine had no effect on the viability of the pre-start arrested *cdc10-129* cells (Fig. 2); cells with a cdc phenotype were observed both in caffeine-treated and in untreated cultures. In contrast, the *cdc22-M45* cells rapidly entered mitosis, displayed the cut phenotype and lost viability after the addition of caffeine. Thus caffeine treatment of cells

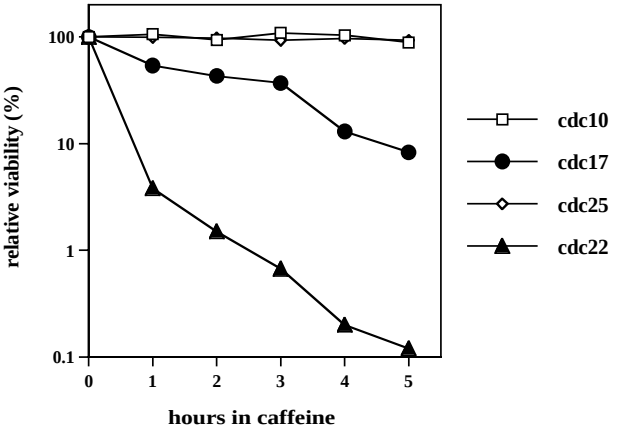


Fig. 2. Caffeine-induced loss of cell viability is specific to S phase arrest. Fresh overnight liquid cultures of the temperature-sensitive *cdc* strains indicated were grown at 26°C in YPD medium and were diluted to 10^6 cells/ml before being shifted to the restrictive temperature of 35.5°C for further incubation. After 3 hours at 35.5°C caffeine was added to 10 mM, and the cell viability (relative to that of cells of the same strain harvested at the time of caffeine addition) was determined at hourly intervals by plating equivalent dilutions of each culture onto YPD agar plates and scoring colony formation after 5 days incubation at 26°C.

Table 2. Effect of caffeine on different *cdc* mutants*

Strain	Arrest point	Phenotype after addition of caffeine	Viability after caffeine
cdc10	Pre-start G_1	Elongated cells with single nuclei	Viable
cdc22	Early S	Uneven cell division (*cut*)	Inviable
cdc17	Late S	Uneven cell division (*cut*)	Inviable
cdc25	G_2	Elongated cells with single nuclei	Viable

*See also Fig. 2.

in which ribonucleotide reductase was inhibited by mutation gave results indistinguishable from those obtained with HU and caffeine (Fig. 1C). The checkpoint-inhibitory effect of caffeine cannot therefore be due to some unforeseen interaction of caffeine with HU. The *cdc17-M75* cells also displayed the cut phenotype and lost viability following addition of caffeine, though the decrease in viability was less rapid than that seen in *cdc22-M45* (Fig. 2); this difference might reflect a more prominent role in early S phase than in late S phase for a function required during recovery from S-phase arrest (Enoch et al., 1992). Unlike cells arrested in S-phase, *cdc25-22* cells were viable after up to 5 hours at the restrictive temperature in the presence of caffeine. No aberrant mitoses were observed in these cultures. These results suggest that caffeine has no effect on cells arrested in G_2. We conclude that caffeine acts to inhibit a mechanism that is normally capable of preventing unscheduled mitosis during S and the S/G_2 transition, but not during G_2 or pre-start G_1.

Caffeine causes tyrosine dephosphorylation of Cdc2 in HU-treated cells

Activation of the S-M checkpoint in *S. pombe* ultimately results in inhibition of Cdc2 by tyrosine phosphorylation at residue 15. Inhibitory phosphorylation of Cdc2 is crucial for integrity of the replication checkpoint (Enoch et al., 1991; Boddy et al., 1998; Rhind and Russell, 1998). To investigate how caffeine disrupts the S-M checkpoint, we monitored the phosphorylation status of Cdc2 upon treatment of HU and caffeine. Western blots of whole cell extracts were probed with

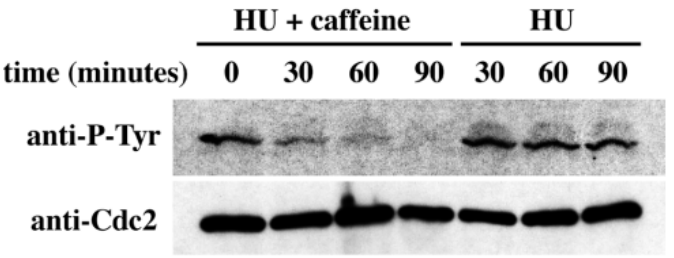


Fig. 3. Caffeine causes tyrosine dephosphorylation of Cdc2 in HU treated cells. HU (10 mM) was added to an asynchronous culture of strain HM123 growing at 30°C in YPD medium. After 3 hours of further incubation caffeine (10 mM) was added to one half of the culture. At the indicated times, cell extracts were prepared by TCA precipitation followed by glass bead disruption. Total cellular proteins were separated by SDS/PAGE and were transferred to nitrocellulose. Cdc2 Tyr15 phosphorylation (upper panel) was detected with a phospho-epitope-specific rabbit polyclonal antibody, while total Cdc2 (lower panel) was detected with a mouse monoclonal antibody (Y100).

polyclonal antibodies specific for Tyr15-phosphorylated Cdc2 and, as an internal control, with monoclonal antibodies against Cdc2. As shown in Fig. 3, Cdc2 remained in its tyrosine-phosphorylated form as cells arrested in S-phase 3 hours after addition of HU. Addition of caffeine caused tyrosine dephosphorylation of Cdc2 in these cells. The level of Cdc2 phosphorylated at Tyr15 decreased shortly after addition of caffeine, such that it was undetectable by 90 minutes after caffeine addition. These results suggest that the caffeine-treated cells underwent authentic activation of Cdc2, and by this criterion passed through mitosis.

As noted above, the relative timings of loss of viability and entry into mitosis suggest that unscheduled M phase progression is not the primary cause of cell death in the caffeine-treated cultures. To address this point, we investigated loss of viability in a *cdc2* temperature-sensitive strain upon treatment of HU and caffeine. Cells grown at the permissive

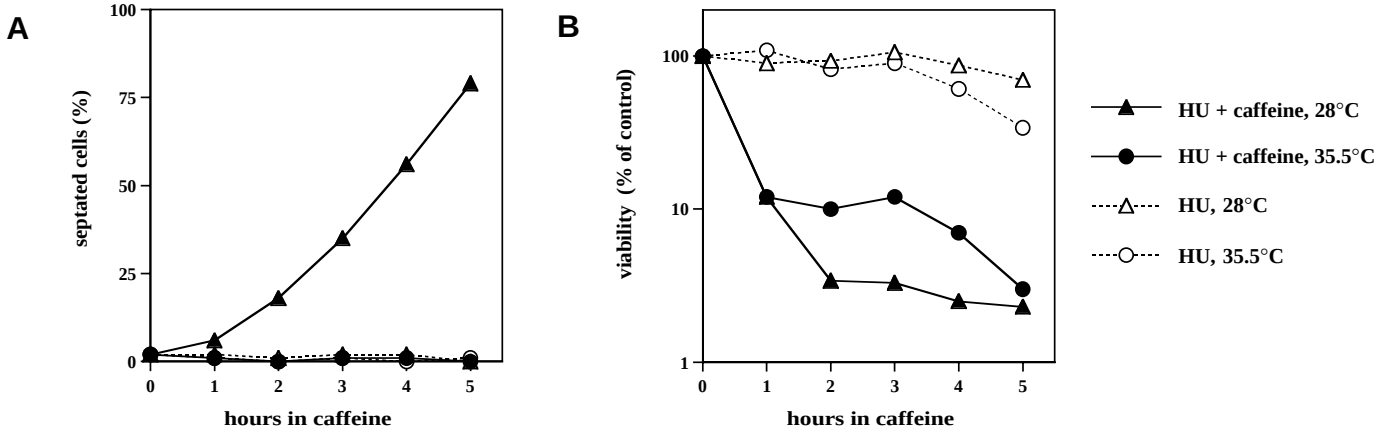


Fig. 4. Inactivation of Cdc2 can only partially suppress the loss of viability on exposure of HU-arrested cells to caffeine. HU (10 mM) was added to a fresh overnight liquid culture of a temperature-sensitive *cdc2* strain growing at 28°C. The culture was then divided between four flasks. After a further 2.5 hours of growth two of the cultures were shifted to 35.5°C, and after a further 30 minutes caffeine (10 mM) was added to one of the flasks at each of the two temperatures. Incubation was continued at either 28° or 35.5°C, and cells harvested at hourly intervals were used to assess septation (A) and viability (B) as described in the legends to Figs 1 and 2.

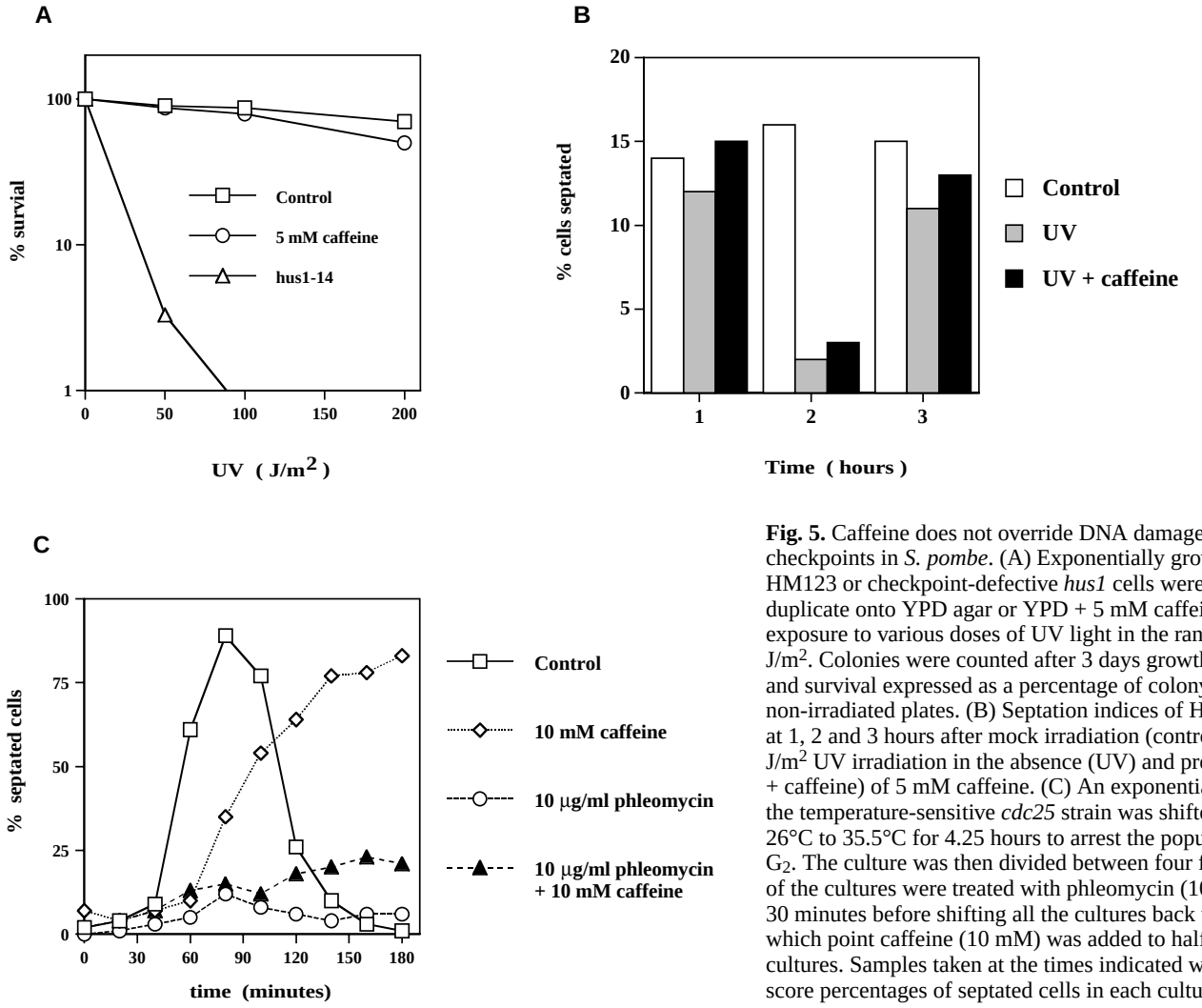


Fig. 5. Caffeine does not override DNA damage checkpoints in *S. pombe*. (A) Exponentially growing HM123 or checkpoint-defective *hus1* cells were plated in duplicate onto YPD agar or YPD + 5 mM caffeine before exposure to various doses of UV light in the range 0-200 J/m^2. Colonies were counted after 3 days growth at 30°C, and survival expressed as a percentage of colony number on non-irradiated plates. (B) Septation indices of HM123 cells at 1, 2 and 3 hours after mock irradiation (control) or 200 J/m^2 UV irradiation in the absence (UV) and presence (UV + caffeine) of 5 mM caffeine. (C) An exponential culture of the temperature-sensitive *cdc25* strain was shifted from 26°C to 35.5°C for 4.25 hours to arrest the population in G_2. The culture was then divided between four flasks. Two of the cultures were treated with phleomycin (10 μg/ml) for 30 minutes before shifting all the cultures back to 26°C, at which point caffeine (10 mM) was added to half of the cultures. Samples taken at the times indicated were used to score percentages of septated cells in each culture.

temperature were treated with HU for 3 hours to arrest cells in S phase. To inactivate Cdc2, half of the culture was then shifted to the restrictive temperature 30 minutes before addition of caffeine. Cells grown at the permissive temperature entered mitosis and lost their viability following addition of caffeine (Fig. 4A,B). In contrast, no septated cells were observed in cultures grown at the restrictive temperature in the presence of caffeine. Although the proportion of viable cells decreased slightly less rapidly in the culture grown at the restrictive temperature, after 1 hour 90% of the cells had become committed to losing their viability despite the inactivation of Cdc2 (Fig. 4B). These results strongly suggest that mitosis is not the sole reason for cell death, as inactivation of Cdc2 could only partially rescue the cells from caffeine-induced death. We propose that an additional function is required to enable cells to survive S phase arrest, and that this function, like the S-M checkpoint, is also inhibited by caffeine.

Caffeine has no effect on G_2 DNA damage checkpoint

To determine whether caffeine was capable of overriding the G_2 DNA damage checkpoint, we investigated the effect of caffeine on cell viability following ultraviolet light (UV) irradiation. Cells plated on caffeine-containing medium were exposed to various doses of UV. Caffeine very slightly enhanced the lethality of UV irradiation (Fig. 5A), but the additional loss of viability induced by caffeine was much less than that seen after UV treatment of the checkpoint mutant *hus1-14*. This result suggests that the G_2 DNA damage checkpoint is still functional after caffeine treatment. To confirm this point, we also scored septation of cells up to 3 hours after UV irradiation at 200 J/m^2 in the presence and absence of caffeine (Fig. 5B). Like irradiated cells incubated in the absence of caffeine, cells treated with caffeine responded to UV-induced DNA damage and transiently delayed mitosis (as judged by septation index at 2 hours) as compared to non-irradiated cells. In contrast, *hus1-14* cells underwent mitosis despite UV-induced DNA damage and displayed the cut phenotype (data not shown). Thus caffeine has no significant effect on UV-induced cell cycle arrest under these conditions.

In order to investigate whether the same might hold for double-strand DNA breaks, we tested the effect of caffeine on phleomycin-treated cells in a synchronous culture. Phleomycin, a bleomycin-like radiomimetic drug, binds to DNA and produces DNA strand breaks (Moore, 1988), and *S. pombe* cells have previously been shown to arrest in G_2 in

response to phleomycin-induced DNA damage (Belenguer et al., 1995). A *cdc25-22* temperature-sensitive strain was arrested at the restrictive temperature for 4.25 hours, allowing the cells to accumulate in late G_2. Phleomycin was then added to the synchronised culture for 30 minutes before transfer to the permissive temperature (Fig. 5C). Upon temperature shift down, all the untreated cells underwent a synchronous mitosis, as monitored by detection of the percentage of septated cells. A delayed mitosis was observed in cells treated with caffeine alone, which may be due to the previously described inhibition of cytokinesis by caffeine (Kumada et al., 1996). In contrast, cells treated with phleomycin did not enter mitosis, but remained arrested in G_2 in response to the drug-induced DNA damage. Addition of caffeine had no effect on phleomycin-induced cell cycle arrest as the percentage of septated cells remained low in these cells. We therefore conclude that caffeine has no effect on G_2 DNA damage checkpoint, whether the DNA damage is UV- or phleomycin-induced.

Overexpression of *cds1*$^+$ or *chk1*$^+$ rescues caffeine-induced cell death

The results described above strongly suggests that caffeine can disrupt the S-M checkpoint. To investigate further the relationship between caffeine and checkpoint override, we asked whether or not overexpression of *cds1*$^+$ could rescue caffeine-induced cell death in HU-treated cells. The *cds1*$^+$ gene was originally identified as a multicopy suppressor of the temperature-sensitive *swi7-H4* DNA polymerase-α mutant (Murakami and Okayama, 1995). Cds1 is a protein kinase that shows significant similarity to budding yeast Spk1/Rad53/Mec2/Sad1 (Stern et al., 1991; Zheng et al., 1993; Allen et al., 1994; Weinert et al., 1994) and has been proposed to be a component of the S-M checkpoint pathway (Murakami and Okayama, 1995; Lindsay et al., 1998; Boddy et al., 1998; Zeng et al., 1998). It has been shown previously that *cds1*$^+$ overproduction can suppress the HU sensitivity of checkpoint *rad* mutants (*rad1*, *3* and *9*) that are defective in the S-M checkpoint (Murakami and Okayama, 1995). If the checkpoint-inhibitory effect of caffeine works through disruption of the checkpoint *rad* pathway, it might also be expected that overexpression of *cds1*$^+$ would rescue caffeine-induced cell death; this indeed was the case. As shown in Fig. 6A, wild-type cells containing pREP*cds1*$^+$ (a generous gift of Dr H. Murakami, ICRF) became resistant specifically to the combination of caffeine and HU. No resistance to caffeine as a single agent up to concentrations of 15 mM was conferred by this plasmid (data not shown). This result suggests that, although caffeine affects a wide variety of cellular processes, the effect we describe here is checkpoint-related. It also indicates that caffeine may interfere with a process upstream of Cds1 by disrupting checkpoint *rad* gene products mediating the checkpoint signal transduction pathway. Note that, although it has been reported that expression of a GST-Cds1 fusion causes cell cycle arrest in G_2 (Boddy et al., 1998), we have not seen such an effect with overexpression of the unmodified cds1 cDNA.

It has recently become apparent that there is a degree of functional overlap between Cds1 and a second protein kinase, Chk1, in the replication checkpoint pathway in *S. pombe* (Francesconi et al., 1997; Boddy et al., 1998; Zeng et al., 1998). Furthermore, Chk1 appears to act in a caffeine-sensitive

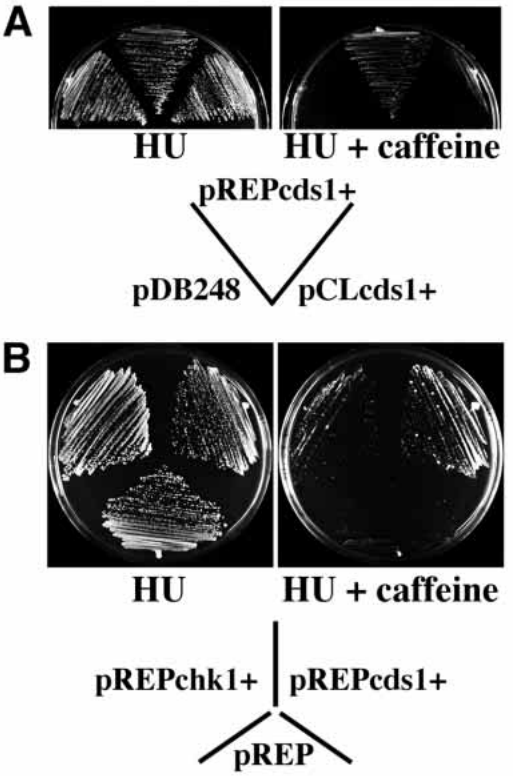


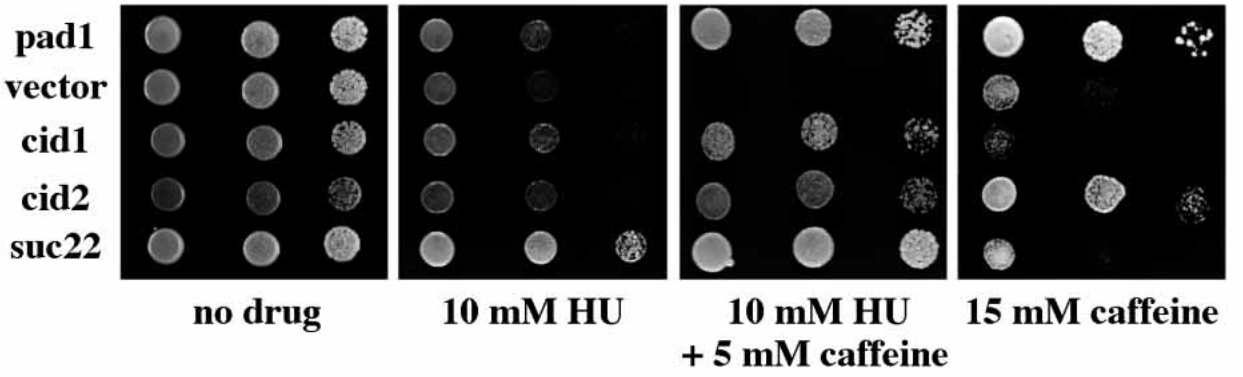
Fig. 6. Overexpression of *cds1*$^+$ or *chk1*$^+$ suppresses the combined toxicity of caffeine and HU. (A) *S. pombe* HM123 transformed with the multi-copy plasmids pDB248 (control vector), pREP*cds1*$^+$ (high level *cds1*$^+$ expression) or pcL*cds1*$^+$ (low level *cds1*$^+$ expression) as indicated were streaked onto minimal agar plates containing 10 mM HU alone (left hand side) or 5 mM caffeine and 10 mM HU (right hand side). Plates were photographed after 4 days growth at 28°C. (B) An analogous experiment comparing the abilities of pREP (control, empty vector), pREP*cds1*$^+$ and pREP*chk1*$^+$ to allow growth on HU or HU + caffeine.

pathway of S-M checkpoint control in *Xenopus* eggs (Kumagai et al., 1998). We therefore investigated the possibility that overexpression of Chk1 might also suppress the combined toxicity of caffeine and hydroxyurea. Very high level expression of Chk1 in *S. pombe* can induce cell cycle arrest (O'Connell et al., 1997), but *nmt1* promoter-driven expression of a *chk1*$^+$ cDNA including a C-G tail in its 5′ untranslated sequence in the plasmid pREP*chk1*$^+$ (kindly provided by H. Murakami) does not inhibit colony formation significantly. *S. pombe* cells transformed with the pREP*chk1*$^+$ were able to grow on plates containing caffeine and HU (but lacking thiamine) almost as well as pREP*cds1*$^+$ transformants (Fig. 6B). As with Cds1, overexpression of Chk1 did not confer resistance to caffeine alone at concentrations up to 15 mM (data not shown). Thus the combined toxicity of HU and caffeine can be suppressed by overexpression of either of the protein kinases thought to act in the S-M checkpoint pathway downstream from the checkpoint *rad* proteins.

Novel cDNAs suppressing the combined toxicity of HU and caffeine

On the basis of the above results we set out to investigate the possibility that additional genes might be involved in this

A



B

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AGTTGTAGCTACCAAAAGGTCCCTAATTCGCACAAGGAATTTACGAAGTTTTGCTATGAAGTGTATAATGAGATTAAAATTAGTGACAAA 180
 S  C  S  Y  Q  K  V  P  N  S  H  K  E  F  T  K  F  C  Y  E  V  Y  N  E  I  K  I  S  D  K

GAGTTTAAAGAAAAGAGAGCGGCATTAGATACACTTCGGCTATGCCTTAAACGAATATCCCCTGATGCTGAATTGGTAGCCTTTGGAAGT 270
 E  F  K  E  K  R  A  A  L  D  T  L  R  L  C  L  K  R  I  S  P  D  A  E  L  V  A  F  G  S

TTGGAATCTGGTTTAGCACTTAAAAATTCGGATATGGATTTGTGCGTGCTTATGGATTCGCGCGTCCAAAGTGATACAATTGCGCTCCAA 360
 L  E  S  G  L  A  L  K  N  S  D  M  D  L  C  V  L  M  D  S  R  V  Q  S  D  T  I  A  L  Q

TTCTATGAAGAGCTTATAGCTGAAGGATTTGAAGGAAAATTTTTACAAAGGGCAAGAATTCCCATTATCAAATTAACATCTGATACGAAA 450
 F  Y  E  E  L  I  A  E  G  F  E  G  K  F  L  Q  R  A  R  I  P  I  I  K  L  T  S  D  T  K

AATGGATTTGGGGCTTCGTTTCAATGTGATATTGGATTTAACAATCGTCTAGCTATTCATAATACGCTTTTACTTTCTTCATATACAAAA 540
 N  G  F  G  A  S  F  Q  C  D  I  G  F  N  N  R  L  A  I  H  N  T  L  L  L  S  S  Y  T  K

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```

C

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 F  E  G  W  L  S  R  D  Q  F  L  K  E  C  Q  T  I  V  R  S  Q  L  D  Q  D  R  N  T  S  K

TCACCCTTAAAATCACAGCAGCAATTGCCTTCATCATCAACGACTCAGGTTTCCGAACGTTTGGATCCTTACGCTAAAGAGGTGCAAGTG 360
 S  P  L  K  S  Q  Q  Q  L  P  S  S  S  T  T  Q  V  S  E  R  L  D  P  Y  A  K  E  V  Q  V

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ACAAATGCTTGTCCTGGAATGTTTAAGGATTGGAGAGACACTTATAAAGACTAA 504
 T  N  A  C  P  G  M  F  K  D  W  R  D  T  Y  K  D  .
```

Fig. 7. Identification of novel cDNAs that confer resistance to HU and / or caffeine. (A) Fresh overnight cultures of *S. pombe* HM123 transformed with the multi-copy plasmids pST23, directing expression of the multidrug resistance determinant Pad1 (Shimanuki et al., 1995), pREP3X (control vector), or pREP3X cDNA library plasmids encoding Cid1, Cid2 or Suc22 were spotted in tenfold serial dilutions onto YPD plates containing no drug, HU and/or caffeine, as indicated. Plates were photographed after 4 days growth at 28°C. (B,C) Sequences of the *cid1* (B) and *cid2* (C) cDNAs and their predicted protein products. GenBank accession numbers for the two sequences are AF105076 and AF105077, respectively.

caffeine-sensitive pathway. A screen was performed to identify cDNAs that could suppress (HU+caffeine)-induced cell death. *S. pombe leu1-32* cells transformed with a cDNA library were tested for their sensitivity to the combination of caffeine and HU. As overexpression of a variety of multi-drug resistance (MDR) genes may suppress caffeine-induced cell death in a non-specific manner (Kumada et al., 1996), the clones isolated were further tested for their cross-resistance to unrelated drugs such as the spindle poison methyl benzimidazole-2-yl carbamate (MBC) and staurosporine. In this way, three non-MDR genes were identified; the gene encoding the small subunit of ribonucleotide reductase (*suc22*$^+$) and two novel *cid* (caffeine induced cell death) genes. As shown in Fig. 7A, *leu1-32* cells containing pREP*cid* plasmids were resistant to (HU+caffeine) treatment. Cells overexpressing the Pad1 protein were also caffeine resistant, but showed cross-resistance to MBC and staurosporine (Shimanuki et al., 1995 and data not shown). In addition, differential sensitivities to the single drugs were observed. Cells containing pREP*cid2* were found to be resistant to caffeine as a single agent. As amplification of target molecules could allow cells to withstand higher concentrations of the corresponding drugs, these results suggest that Cid2 might be a target of caffeine (by analogy with the case of *suc22*, which can induce resistance to HU). In contrast, *cid1*$^+$ overexpression induced resistance specifically to the combination of caffeine and HU, suggesting that the phenotype observed is not due to the interaction of Cid1 with either drug. In addition, overexpression of *cid1*$^+$ also suppressed the hydroxyurea sensitivity of a number of checkpoint *rad* mutants (data not shown), indicating that Cid1 may be involved in the checkpoint pathway.

The primary sequences of the *cid1* and *cid2* cDNAs (Fig. 7B,C) give no conclusive indication of the likely functions of the respective protein products. Database comparisons indicate that Cid2 is not significantly related to any other protein of known function, although potential Cid2 homologues are encoded by several mammalian expressed sequence tags (ESTs). Cid1 is a member of a divergent protein family that includes Trf4 and Trf5 in *Saccharomyces cerevisiae* (Castaño et al., 1996a) and multiple other members in *S. pombe* and other species (S.-W. W., unpublished data).

DISCUSSION

It has been known for some time that caffeine has the capacity to induce mitotic events in S phase-arrested cells from a variety of multicellular organisms. This effect of caffeine could, in principle, shed light on the molecular mechanisms by which mitosis is normally held in check until DNA replication is complete. Until now, investigations of this phenomenon have been performed in systems that are not amenable to rapid genetic analysis. We show here that caffeine can override the replication (S-M) checkpoint in *S. pombe*. This effect of caffeine was seen whether DNA replication was inhibited chemically with HU (Fig. 1) or genetically with temperature-sensitive ribonucleotide reductase or DNA ligase mutants (Fig. 2). This finding makes possible a genetic analysis of this effect of caffeine in a system where the S-M checkpoint mechanism is increasingly well-defined at the molecular level.

Caffeine-induced override of the *S. pombe* S-M checkpoint was correlated with activation of Cdc2, as judged by removal of inhibitory Tyr15 phosphorylation (Fig. 3) and subsequent septation (Fig. 1A), a cell cycle event normally dependent on progression through mitosis. It might be imagined that promotion of mitosis from S-phase arrest would be sufficient to induce cell death. Indeed, loss of viability in a checkpoint-defective *cdc2-3w* strain exposed to HU was shown to coincide with progression through mitosis (Enoch and Nurse, 1990). In contrast, other checkpoint mutants, such as those defective in *hus1* or other checkpoint *rad* genes, lose viability much more rapidly than *cdc2-3w* when arrested in S phase. In these mutants the major cause of HU-induced death appears not to be progression through unscheduled mitosis, but the loss of a separable function required for maintenance of viability during S phase arrest (Enoch et al., 1992). In our experiments, most of the commitment to death in cells exposed to HU and caffeine occurred before progression through mitosis (Fig. 1) and could not be blocked by inhibition of Cdc2 activity (Fig. 4). The effect of caffeine on S phase-arrested fission yeast cells is, therefore, reminiscent of the effect of mutations of checkpoint *rad*/*hus* genes. Thus, caffeine may (either directly or indirectly) interfere with the function of the Rad/Hus proteins.

What might be the caffeine-sensitive function required for maintenance of viability during S phase arrest? One possibility is that the integrity of stalled DNA replication complexes must be actively maintained if subsequent resumption of synthesis is to take place. Alternative explanations could implicate a form of DNA repair specifically required for recovery from S phase arrest (Enoch et al., 1992). Caffeine has long been known to inhibit repair processes in *S. pombe* and other experimental systems (Loprieno and Schupbach, 1971; Gentner and Werner, 1975; Roberts et al., 1974). Although some enhancement of UV-induced killing was seen on caffeine treatment in our experiments (Fig. 5), this was much less dramatic than the effect of caffeine on HU-arrested cells. It follows that general inhibition of repair is insufficient to explain the dramatic loss of viability seen here on treatment of S phase-arrested cells with caffeine. Instead, we consider it more likely that the relevant caffeine target is functionally related to the S-M checkpoint machinery itself. This view is supported by the observation that the combined toxicity of caffeine and HU can be suppressed by overexpression of the replication checkpoint determinants Cds1 and Chk1 (Fig. 6). Such a conclusion would also be in line with the recently described effect of caffeine on signalling through Chk1 after replication inhibition in *Xenopus* (Kumagai et al., 1998).

Caffeine does not appear to override the DNA damage-induced checkpoint arrest induced in *S. pombe* either by UV light or by phleomycin (Fig. 5). This is in contrast to the ability of caffeine and other methylxanthines to suppress DNA damage-induced cell cycle arrest in metazoans (Walters et al., 1974; Zampetti-Bosseler et al., 1985; Musk et al., 1988), but the reason for this difference is not yet clear. Our data show that caffeine is able to disrupt the S-M checkpoint pathway in *S. pombe*, so the lack of effect on the DNA damage checkpoint could be taken as evidence that distinct caffeine targets are involved in override of the S-M and damage checkpoint pathways. In this case the damage checkpoint caffeine target might be insufficiently well conserved in *S. pombe* for an effect to be seen. Alternatively, a single caffeine target might be involved in both types of checkpoint arrest, with species-

specific, caffeine-insensitive mechanisms acting in parallel and determining the precise nature of the checkpoint defects seen on caffeine treatment. Clarification of this point will be made more straightforward if the appropriate caffeine target(s) can be identified, perhaps by genetic strategies such as the one described here (Fig. 7).

By using a functional screen we have identified two novel *S. pombe* cDNAs on the basis of their ability to allow cell growth in the presence of HU and caffeine. While Cid2, the protein encoded by one of these cDNAs, is a candidate for the S-M checkpoint-related target of caffeine itself, the Trf4/Trf5-related Cid1 protein may act elsewhere in the checkpoint pathway. *TRF4* and *TRF5* were identified through the synthetic lethality of *trf4* and *trf5* mutations with mutations in the otherwise inessential DNA topoisomerase I (*TOP1*) gene (Castaño et al., 1996a). Trf4 and Trf5 are required for chromatin segregation and condensation (Castaño et al., 1996a,b), but it is not clear at this stage to what extent Cid1 performs functions analogous function(s) in *S. pombe*. Studies of the biological functions of Cid1 and Cid2 are now in progress, and are expected to shed further light on the molecular mechanisms of checkpoint control.

Much interest has been generated in the biomedical community by the capacity of methylxanthines to override cell cycle checkpoint controls and thus synergise with conventional anticancer agents. In particular, cells lacking the tumour suppressor p53 were found to be selectively sensitive to the checkpoint-inhibitory activity of caffeine or the related methylxanthine pentoxifylline (Fan et al., 1995; Powell et al., 1995; Russell et al., 1995; Yao et al., 1996). While caffeine itself is unlikely ever to be of use in this respect, due to the high (millimolar) concentrations required to elicit checkpoint-inhibitory activity, other related compounds may be substantially more specific and effective at lower doses. Identification of the relevant methylxanthine target could be made easier by the application of fission yeast genetics to the problem, and may ultimately allow the refinement of more specific checkpoint-overriding drugs.

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