Chk1-Dependent S-M Checkpoint Delay in Vertebrate Cells Is Linked to Maintenance of Viable Replication Structures

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Received 27 July 2004/Returned for modification 3 September 2004/Accepted 15 October 2004

We investigated mitotic delay during replication arrest (the S-M checkpoint) in DT40 B-lymphoma cells deficient in the Chk1 or Chk2 kinase. We show here that cells lacking Chk1, but not those lacking Chk2, enter mitosis with incompletely replicated DNA when DNA synthesis is blocked, but only after an initial delay. This initial delay persists when S-M checkpoint failure is induced in Chk2−/− cells with the Chk1 inhibitor UCN-01, indicating that it does not depend on Chk1 or Chk2 activity. Surprisingly, dephosphorylation of tyrosine 15 of Cdc2 did not accompany Cdc2 activation during premature entry to mitosis in Chk1−/− cells, although mitotic phosphorylation of cyclin B did occur. Previous studies have shown that Chk1 is required to stabilize stalled replication forks during replication arrest, and strikingly, premature mitosis occurs only in Chk1-deficient cells which have lost the capacity to synthesize DNA as a result of progressive replication fork inactivation. These results suggest that Chk1 maintains the S-M checkpoint indirectly by preserving the viability of replication structures and that it is the continued presence of such structures, rather than the activation of Chk1 per se, which delays mitosis until DNA replication is complete.

Eukaryotic cells respond to DNA damage or blocks to DNA replication by triggering a variety of checkpoint responses which delay cell cycle progression, promote repair, and protect genome integrity (31). Checkpoints are controlled by signal transduction mechanisms which lead to the activation of two checkpoint effector kinases, Chk1 and Chk2 (the *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* fission and budding yeast) Chk2 homologues are Cds1 and Rad53, respectively, which elicit appropriate checkpoint responses by phosphorylating and modulating the activities of a variety of downstream substrates (31, 40).

Replication arrest triggers at least three mechanistically distinct checkpoints in both yeasts and mammalian cells (31). Two of these are required to protect the inhibited DNA replication machinery and to ensure that replication can resume when conditions permit. In budding and fission yeast, Rad53 and Cds1 are activated when DNA synthesis is inhibited and are required to stabilize slowed or stalled replication structures (12, 26) and to suppress initiation at latent replication origins (20, 43). In the absence of these checkpoint responses, stalled replication forks become inactivated through a poorly defined degenerative process (often referred to as replication fork collapse), while latent replication origins initiate replication even though elongation cannot occur (futile origin firing). Such defects are highly deleterious, and yeast checkpoint mutants which lack these functions exhibit DNA replication abnormalities and diminished cell survival upon release from replication arrest (12, 26).

A third replication checkpoint delays the onset of mitosis while DNA replication is incomplete (the S-M checkpoint). In fission yeast, Cds1 is also considered to be the primary effector of mitotic delay when DNA synthesis is inhibited (25, 30), although paradoxically *cds1* null mutants retain the capacity to delay mitosis in response to replication arrest. This residual delay is dependent on Chk1, since *cds1 chk1* double mutants lack an effective S-M checkpoint (4, 25, 50). Although Chk1 is activated only by DNA damage in wild-type fission yeast (2, 47), in *cds1* null mutant cells Chk1 activation is also observed during replication arrest, either because replication fork collapse gives rise to aberrant DNA structures which are recognized as DNA damage (25) or because in wild-type cells Cds1 suppresses a repair process that leads to Chk1 activation (7).

Regardless of whether Cds1 or Chk1 is responsible, mitotic delay in response to DNA damage and during replication arrest in fission yeast is imposed through the regulation of inhibitory phosphorylation of tyrosine 15 (Y15) of Cdc2 (13, 28). Specifically, the rapid dephosphorylation of Y15-phosphorylated Cdc2, which normally activates Cdc2 catalytic activity and initiates mitosis, is blocked when Cds1 or Chk1 is activated (39, 42). Both Cds1 and Chk1 can phosphorylate and inhibit the Cdc25 phosphatase during replication arrest (50) or DNA damage (15), preventing the dephosphorylation of Cdc2 Y15, and they are required to sustain high levels of Mi1 (10, 41), a Cdc2 Y15 kinase (28). Chk1 also enhances the activity of a second Cdc2 Y15 kinase, Wee1, through direct phosphorylation (32). Although the mechanistic details are not yet fully established, genetic and biochemical evidence suggests that mitotic delay in fission yeast is normally achieved through the coordinated checkpoint modulation of both positive and negative regulators of Cdc2 Y15 phosphorylation (33, 37).

The mechanisms and effectors involved in replication checkpoint responses in higher eukaryotes are less well characterized. In contrast to the case in fission yeast, Chk1 is strongly activated by DNA synthesis inhibitors such as hydroxyurea and aphidicolin as well as by DNA damage in vertebrate cells in culture (14, 49). Furthermore, both biochemical and genetic lines of evidence indicate that the checkpoint functions which preserve replication fork viability and suppress latent origin...
firing during DNA synthesis inhibition in vertebrate cells are controlled primarily or exclusively by Chk1 rather than the Rad53/Cds1 counterpart Chk2 (14, 49).

These considerations suggest that Chk1 is also involved in S-M checkpoint control in vertebrate cells, but conflicting conclusions regarding the role of Chk1 have been reached with different experimental systems. Chk1 is activated by DNA synthesis inhibition in Xenopus laevis egg extracts, and immunodepletion experiments have revealed that Chk1 activity is required for prolonged mitotic delay (22). Also, Chk1−/− mouse blastocysts were observed to enter mitosis in the presence of DNA replication inhibitors (44). Although these results suggest that Chk1 is required for the S-M checkpoint, at least in embryonic cells, checkpoint failure was not observed after moderate periods of replication arrest in Chk1−/− DT40 B-lymphoma somatic cells (49) or in mouse embryonic fibroblasts (MEFs) deficient in ATR, even though the activation of Chk1 in these cells was largely abolished (9).

To account for these apparent discrepancies, we considered the hypothesis that Chk1 and Chk2 might play partially redundant or complementary roles in S-M checkpoint control in vertebrate cells, much as the case proposed for Cds1 and Chk1 in fission yeast. To evaluate this idea, we investigated the effect of replication arrest on mitotic entry in isogenic mutant DT40 cell lines deficient in either Chk1 or Chk2. Unlike embryonic cells from mice and certain other metazoan species, DT40 cells survive in the absence of Chk1 function (49) and thus provide a uniquely tractable experimental system for such studies. We show here that cells deficient in Chk1, but not those deficient in Chk2, activate Cdc2 kinase activity and enter mitosis with incompletely replicated DNA when DNA synthesis is blocked, but only after an initial delay. Surprisingly, this initial delay is not dependent on compensation by Chk2, nor is the eventual activation of Cdc2 and checkpoint failure due to the removal of the inhibitory Y15 phosphorylation of Cdc2. Instead, our results suggest that the role of Chk1 in the S-M checkpoint is to preserve viable replication structures and that it is the continued presence of such structures in arrested cells which precludes Cdc2 activation and thus the onset of mitosis.

MATERIALS AND METHODS

Generation of Chk2-deficient DT40 cells by gene targeting. A detailed description of the construction of the Chk2−/− DT40 cell line will be presented elsewhere (M. D. Rainey and D. A. F. Gillespie, unpublished data). Briefly, an avian Chk2 cDNA was isolated from an avian B-cell cDNA library and then sequenced, and oligonucleotides were designed to amplify segments of the genomic chk2 gene which were then used to construct targeting vectors in which a neomycin or puromycin antibiotic selection cassette replaced essential segments of the Chk2 coding region. The vectors were designed to delete the entire Chk2 FHA domain and a majority of the protein kinase domain so that any potential DNA synthesis inhibition in different experimental systems. Chk2 is activated by DNA synthesis inhibition in Xenopus laevis egg extracts, and immunodepletion experiments have revealed that Chk1 activity is required for prolonged mitotic delay (22). Also, Chk1−/− mouse blastocysts were observed to enter mitosis in the presence of DNA replication inhibitors (44). Although these results suggest that Chk1 is required for the S-M checkpoint, at least in embryonic cells, checkpoint failure was not observed after moderate periods of replication arrest in Chk1−/− DT40 B-lymphoma somatic cells (49) or in mouse embryonic fibroblasts (MEFs) deficient in ATR, even though the activation of Chk1 in these cells was largely abolished (9).

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RESULTS

DT40 cells sustain a prolonged S-M checkpoint delay when DNA synthesis is inhibited. To investigate the requirement for Chk1 and Chk2 in S-M checkpoint control in vertebrate somatic cells, we devised a protocol by which we could rigorously document the effect of DNA synthesis inhibition on the onset of mitosis in DT40 knockout cell lines that are deficient in each checkpoint kinase. The construction of the Chk1-deficient DT40 cell line has been described previously (49), while the generation of its Chk2-deficient counterpart will be described in detail elsewhere (a brief outline is provided in Materials and Methods). As shown in Fig. 1A, each knockout cell line is devoid of a functional Chk1 or Chk2 protein, respectively.

Since by definition the S-M checkpoint delays mitosis in cells which have not yet completed DNA replication, we used centrifugal elutriation to remove the G2- and M-phase cells from asynchronous cultures of wild-type (WT), Chk1−/−, and Chk2−/− DT40 cells (Fig. 1B). This allowed us to purify populations of G1- and S-phase cells, which were then returned to...
Mitotic entry was associated with the activation of Cdc2 catalytic activity, as measured by immunoprecipitation kinase assays with histone H1 as a substrate (see Fig. 4A). Importantly, all of the pS10H3-positive WT cells had a 4N DNA content, indicating that mitosis occurred only after the completion of DNA replication (Fig. 2A).

In contrast, WT cells that were incubated with aphidicolin showed no evidence of cell cycle progression, as judged by an increasing DNA content (Fig. 2A and B), nor was there any significant entry to mitosis or increase in Cdc2 catalytic activity for up to 12 h (see Fig. 4A). Aphidicolin treatment also resulted in a strong and persistent activation of Chk1, as judged by both an electrophoretic mobility shift and phosphorylation on serine 345 (S345) detected by immunoblotting (27, 51), neither of which was observed in untreated cultures (Fig. 2C). Essentially identical results were observed for Chk2-/- cells, which exhibited a similar delay in the appearance of pS10H3-positive cells, the suppression of Cdc2 kinase activity, and the activation of Chk1 when exposed to aphidicolin as that shown by the WT (see Fig. 5B; also data not shown). Taken together, these results demonstrate that WT and Chk2-/- DT40 cells are capable of sustaining a prolonged S-M checkpoint delay when DNA synthesis is blocked and that this delay is associated with a robust activation of Chk1 and an inhibition of Cdc2 kinase activity.

Chk1 is required to maintain but not to initiate mitotic delay during replication arrest. When elutriated Chk1-/- G1/S cells were cultured under control conditions, they also progressed through S and G2, activated Cdc2 catalytic activity, and accumulated in mitosis (Fig. 3A and B). Furthermore, as for WT cells, all of the pS10H3-positive cells that accumulated in the Chk1-/- cultures under these conditions had a 4N DNA content, which is indicative of normal, properly scheduled mitoses (Fig. 3A).

A strikingly different outcome was observed when Chk1-/- cells were cultured in the presence of aphidicolin. Although aphidicolin initially suppressed both the accumulation of pS10H3-positive cells and the increase in Cdc2 kinase activity which occurred in control cultures for up to 9 h, clearly indicating the imposition of an initial checkpoint delay, both then increased abruptly at 12 h (Fig. 3A and B and Fig. 4B). Importantly, a large proportion of the pS10H3-positive Chk1-/- cells which accumulated at 12 h exhibited a 2N DNA content (Fig. 3A), indicating that entry to mitosis had occurred in the absence of DNA replication.

To confirm that pS10H3 staining in this context was a reliable marker of bona fide mitosis, we treated Chk1-/- cells with aphidicolin for 12 h without nocodazole and examined the organization of microtubules in the pS10H3-positive cells by using antibodies against α-tubulin. This revealed that the large majority of the pS10H3-positive Chk1-/- cells possessed bipolar spindles, although unlike natural mitoses these were typically offset from the condensed chromosomes, which often appeared fragmented (Fig. 3D and data not shown). Such structures were not observed in aphidicolin-treated WT cultures (Fig. 3D and data not shown). We also observed that the pS10H3-positive Chk1-/- cells lacked a nuclear envelope, as judged by staining with antibodies specific for lamin B2 (data not shown).

Thus, although Chk1-deficient cells can initially delay mitosis when DNA synthesis is blocked, if the period of arrest exceeds 9 h a premature mitosis characterized by Cdc2 kinase data not shown).
activation, chromosome condensation, nuclear envelope breakdown, and mitotic spindle formation occurs in the absence of genome duplication. To gain more insight into the kinetics of this phenomenon, we performed a more detailed analysis using more time points over a longer time period. As shown in Fig. 3C, this revealed that the mitotic delay induced by aphidicolin treatment persisted at all time points tested up to 14 h and that Chk1-deficient cells entered mitosis in the presence and absence of aphidicolin at broadly comparable rates.

S-M checkpoint failure in Chk1-deficient cells is not associated with loss of Cdc2 tyrosine 15 phosphorylation. In fission yeast, Cds1 or Chk1 delays mitosis during replication arrest by preventing the removal of inhibitory Y15 phosphorylation from Cdc2 (42, 50), but whether this mechanism is solely responsible for S-M checkpoint delay in vertebrate cells is less clear (8). To investigate the relationship between inhibitory phosphorylation of Cdc2 and Chk1-dependent maintenance of the S-M checkpoint, we treated elutriated populations of G1/S WT and Chk1-deficient cells with aphidicolin and nocodazole for 0 to 12 h and determined the levels of total and Y15-phosphorylated Chk1 by immunoblotting analysis.

Consistent with current models of Cdc2 regulation (34), Cdc2 Y15 phosphorylation increased transiently during normal cell cycle progression in both WT and Chk1-deficient cultures but declined again after 12 h, when a majority of cells had arrested in mitosis and Cdc2 kinase activity was maximal (Fig. 4A and B, left panels). We also observed a progressive increase in the level of cyclin B2 (the predominant avian B-type cyclin...
and the appearance of an electrophoretically distinct isoform (asterisks in Fig. 4A and B) which was attributable to phosphorylation since it was reversed when extracts were treated with lambda phosphatase (data not shown). This phosphorylated isoform of cyclin B2 has been shown to accumulate during mitosis and may be associated with nuclear translocation (16). In contrast, when WT cells were treated with aphidicolin, Cdc2 Y15 phosphorylation increased and was maintained at max-

FIG. 3. Chk1 is required to maintain the S-M checkpoint. (A) pSer10H3 fluorescence-DNA content flow cytometry analysis of elutriated Chk1−/− G1/S cells cultured with 1 μg of nocodazole/ml with or without (control [con]) 20 μM aphidicolin for the indicated times. Open boxes indicate total pSer10H3-positive cells (pH3). The open arrow indicates pSer10H3-positive cells with a 4N DNA content, and the solid arrow indicates pSer10H3-positive cells with a 2N DNA content. (B) Quantitation of the data shown in panel A. (C) More detailed kinetic analysis of the rates at which Chk1−/− cells accumulated in premature or natural mitosis in the presence or absence of aphidicolin, performed as described for panel A. (D) Mitotic spindles in pSer10H3-positive Chk1−/− cells. Elutriated G2/S WT or Chk1−/− cells were treated with aphidicolin for 12 h without nocodazole, fixed, and stained with antibodies against pSer10H3 (red) and α-tubulin (green).
imal levels (Fig. 4A, right panel), indicating that, as expected, mitotic delay and the suppression of Cdc2 kinase activity were associated with the persistence of this inhibitory modification. Aphidicolin treatment also prevented the appearance of the mitotic isoform of cyclin B2 (Fig. 4A, right panel). Surprisingly, the inhibitory phosphorylation of Cdc2 was also maintained at high levels in Chk1/−/− cells during aphidicolin treatment (Fig. 4B, right panel). In particular, it was evident that the abrupt increase in Cdc2 kinase activity seen after 12 h of replication arrest was not associated with any corresponding decrease in total Y15 phosphorylation (Fig. 4B, right panel). Essentially identical results were obtained with an antibody which specifically recognizes Cdc2 phosphorylated at both Y 15 and threonine 14 (which also inhibits Cdc2 catalytic activity) (data not shown). In marked contrast to the case for WT cells, however, aphidicolin treatment did not prevent the appearance of the phosphorylated mitotic isoform of cyclin B2 in Chk1/−/− cells.

To monitor the modification state of Cdc2 and cyclin B2, specifically in cells which had entered mitosis prematurely compared with the remaining nonmitotic cells, we treated Chk1/−/− cells with aphidicolin and nocodazole for 12 h, separated the pS10H3-positive and -negative populations by fluorescence-activated cell sorting (see Fig. 6C for an example), and analyzed cell extracts prepared from the resulting fractions by Western blotting. The positions of phosphorylated (asterisk) and nonphosphorylated Cdc2 protein are indicated.

FIG. 4. Cdc2-associated histone H1 kinase activity, Cdc2 Y15 phosphorylation, and cyclin B2 expression in elutriated WT and Chk1/−/− cells during replication arrest. (A) Cdc2-associated H1 kinase activity and Western blot analysis of Tyr15-phosphorylated Cdc2, total Cdc2, and cyclin B2 protein levels in elutriated G1/S WT cultures with 1 μg of nocodazole/ml and with or without (control) 20 μM aphidicolin for the indicated times. The prominent band in the anti-Cdc2 Western blot analysis of the immunoprecipitation-kinase assay corresponds to the light chain of the immunoprecipitating antibody (also shown in panel B). The samples were split in two: one half was analyzed for H1 kinase activity and the second half was used for Western blot analysis. The pY15 Cdc2, total Cdc2, and cyclin B2 results shown in the lower parts of panels A and B were derived from sequential reprobing of the same Western blots. (B) Cdc2-associated H1 kinase activity and Western blot analysis of Tyr15-phosphorylated Cdc2, total Cdc2, and cyclin B2 protein levels in elutriated G1/S Chk1/−/− cultures treated as described for panel A. (C) Levels of Tyr15-phosphorylated Cdc2, total Cdc2, cyclin B2, and actin proteins in total (unsorted) and sorted pSer10H3-positive (mitotic) and -negative (nonmitotic) populations of Chk1/−/− cells treated with 1 μg of nocodazole/ml and 20 μM aphidicolin for 12 h to induce premature mitosis (left) or with 1 μg of nocodazole/ml alone for 12 h to enrich for natural mitotic cells (right). (D) Cyclin B2 was immunoprecipitated from cultures of WT and Chk1/−/− cells treated with 1 μg of nocodazole/ml and 20 μM aphidicolin for 12 h, and the coprecipitated Cdc2 protein was visualized by Western blotting. The positions of phosphorylated (asterisk) and nonphosphorylated Cdc2 protein are indicated.
tates (Fig. 4D) revealed that cyclin B2 was associated with both phosphorylated (asterisk) and nonphosphorylated forms of Cdc2 in aphidicolin-treated WT and Chk1−/− cells, as judged by their electrophoretic mobilities. It was evident, however, that nonphosphorylated Cdc2 predominated in the Chk1−/− immunoprecipitate, whereas both forms were equally abundant in the WT. Thus, although the activation of Cdc2 in Chk1−/− cells during premature entry to mitosis from S phase was not associated with any detectable loss of inhibitory Y15 phosphorylation from the total pool of Cdc2, as occurs during normal mitosis, it was accompanied by the mitotic phosphorylation of cyclin B2, and cyclin B2 was associated with significant amounts of nonphosphorylated and therefore presumably active Cdc2.

Mitotic delay in the absence of Chk1 and Chk2 activity. In wild-type fission yeast, Cds1 rather than Chk1 is activated by DNA synthesis inhibition and is thought to be responsible for delaying mitosis until replication is complete (30). Fission yeast Cds1 mutants nevertheless remain capable of mitotic delay during replication arrest. This residual delay has been ascribed to the activation of Chk1, which occurs in response to replication arrest specifically in Cds1-deficient strains (4, 25, 50). A remarkably similar, if inverted, phenomenon has been described for DT40 cells, in which DNA synthesis inhibition activates the Cds1 homologue Chk2 only in cells lacking Chk1 (49). These considerations raised the possibility that the initial mitotic delay observed for Chk1-deficient cells that were subjected to replication arrest might be due to the compensatory activation of Chk2 (48). To evaluate this idea, we used UCN-01 (17) to inhibit Chk1 function in Chk2-deficient DT40 cells to mimic a double deficiency of Chk1 and Chk2. Elutriated cultures of WT, Chk1−/−, and Chk2−/− DT40 cells were treated with aphidicolin and UCN-01, and the effect on entry into mitosis was determined as described for panel A.

Importantly, UCN-01 induced premature mitosis in aphidicolin-treated WT cells at a similar frequency and after a delay comparable to that observed spontaneously in Chk1−/− cells (Fig. 5A, compare filled circles and shaded triangles). We believe that this premature delay was specifically due to the inhibition of Chk1, since UCN-01 had no effect on the incidence or timing of premature mitosis in the Chk1−/− cells themselves (data not shown). In comparison, Chk2-deficient cells that were treated with UCN-01 and aphidicolin also entered mitosis with incompletely replicated DNA (Fig. 5B, filled circles), although crucially, checkpoint failure was again only observed after an initial delay. Thus, the initial mitotic delay which persists when Chk1 is inhibited does not require and therefore cannot be solely attributable to compensation by Chk2.

Chk1-deficient cells enter mitosis with incompletely replicated DNA only after the loss of viable replication structures. Previous studies have established that Chk1-dependent stabilization of stalled replication forks and suppression of latent origin firing are essential for the resumption of DNA replication after release from a prolonged inhibition of DNA synthesis (14, 49). In the absence of Chk1, replication forks are unstable and become progressively inactivated over a period of hours (14, 49). We therefore explored the possibility that the duration of the initial mitotic delay in Chk1−/− cells during replication arrest might be related to the DNA replication process per se. To this end, we first compared the rates at which cells devoid of detectable replication capacity accumulated during replication arrest in elutriated G1/S populations of WT and Chk1−/− cells. The cells were incubated with aphidicolin and nocodazole for various times, after which they were washed free of drugs and labeled with BrdU for 1 h. Each sample was then split in two; one half was used to determine the proportion of cells that remained capable of resuming DNA synthesis upon release from arrest (Fig. 6A). In contrast, the proportion of cells which incorporated BrdU began to decrease after 5 h, with a particularly marked decline occurring between 9 and 12 h, when mitotic entry was occurring (Fig. 6B).

As shown in Fig. 6A, this revealed that the number of cells incorporating BrdU increased in the first 5 h of culture after elutriation in both WT and Chk1−/− cultures as G1 cells in the starting population entered and then arrested in early S phase. In WT cultures, this percentage then remained constant for 12 h of aphidicolin treatment, indicating that the large majority of cells in the population remained capable of resuming DNA synthesis upon release from arrest (Fig. 6A). In contrast, the proportion of Chk1−/− cells which incorporated BrdU began to decrease after 5 h, with a particularly marked decline occurring between 9 and 12 h, when mitotic entry was occurring (Fig. 6A and B). These results demonstrated a temporal correlation between the appearance of cells devoid of viable replication structures and cells undergoing premature entry into mitosis in the Chk1−/− population, but we could not determine from this analysis alone whether these processes occurred concurrently.
in individual cells. To answer this question, we treated elutriated Chk1−/− cells with aphidicolin and nocodazole for 12 h, after which the drugs were removed and the cells were immediately labeled for 1 h with Clu. The cells were then stained with anti-pS10H3 antibodies to identify mitotic cells, and the pS10H3-negative and -positive populations were isolated by flow cytometry. The data shown are means and standard errors for three independent experiments. (C) Elutriated G1/S Chk1−/− cells were treated with 20 μM aphidicolin and 1 μg of nocodazole/ml for 12 h, washed free of drugs, and pulse-labeled with Clu for 1 h. The pS10H3-positive and -negative populations (top) were then separated by cell sorting and stained for Clu incorporation, and the percentage of cells capable of DNA replication in each population was determined by fluorescence microscopy. Two hundred nuclei were scored in each case. (D) Same data as for panels A and B, except that the cultures were treated with 20 μM aphidicolin and 1 μg of nocodazole/ml for 12 h with or without the continuous presence of 100 μM Roscovitine, washed, and pulse-labeled with BrdU for 1 h, and the percentages of BrdU-labeled and pS10H3-positive cells were then determined.
majority of pS\textsuperscript{10}H3-negative Chk1\textsuperscript{−/−} cells incorporated CldU upon release from aphidicolin arrest, none of those which had become positive for pS\textsuperscript{10}H3 as a consequence of S-M checkpoint failure became labeled (Fig. 6C, lower panel). We do not believe that this was because premature entry to mitosis extinguished DNA replication for several reasons. Firstly, cells devoid of replication capacity were detected before the onset of mitosis, as judged by the appearance of pS\textsuperscript{10}H3-positive cells (Fig. 6, compare panels A and B). Secondly, when similar experiments were performed in the presence of the Cdk inhibitor roscovitine, premature mitosis could be largely or completely blocked in aphidicolin-treated Chk1\textsuperscript{−/−} cells (Fig. 6D, lower panel) without preventing the accumulation of cells devoid of replication capacity (Fig. 6D, upper panel). Finally, the work of others has shown that condensed mitotic chromatin is capable of both the initiation and elongation phases of DNA replication (36).

We also investigated the relationship between premature entry to mitosis and nuclear PCNA accumulation, which concentrates at sites of active DNA replication (5, 6). Elutriated WT and Chk1\textsuperscript{−/−} cells were treated with aphidicolin and nocodazole for 0 to 12 h, after which the cells were washed free of drugs, incubated in fresh medium for 1 h, fixed, and then stained with antibodies specific for pSer\textsuperscript{10}H3 and PCNA. The percentages of cells exhibiting detectable staining for pSer\textsuperscript{10}H3 and PCNA were determined by scoring 200 cells at each time point by fluorescence microscopy. (B) Representative fields of WT and Chk1\textsuperscript{−/−} cells after 12 h of replication arrest. Essentially all of the WT cells exhibited strong nuclear staining for PCNA but were uniformly negative for pSer\textsuperscript{10}H3, whereas Chk1\textsuperscript{−/−} cells exhibited mutually exclusive staining for PCNA (green) and pSer\textsuperscript{10}H3 (red). Individual examples are indicated by white arrows.

FIG. 7. Loss of nuclear PCNA staining precedes premature mitosis in Chk1\textsuperscript{−/−} cells. (A) Elutriated G\textsubscript{1}/S WT and Chk1\textsuperscript{−/−} cells were incubated with 20 μM aphidicolin and 1 μg of nocodazole/ml for the indicated times, washed free of drugs, incubated in fresh medium for 1 h, fixed, and stained with antibodies specific for pSer\textsuperscript{10}H3 and PCNA. The percentages of cells exhibiting detectable staining for pSer\textsuperscript{10}H3 and PCNA were determined by scoring 200 cells at each time point by fluorescence microscopy. (B) Representative fields of WT and Chk1\textsuperscript{−/−} cells after 12 h of replication arrest. Essentially all of the WT cells exhibited strong nuclear staining for PCNA but were uniformly negative for pSer\textsuperscript{10}H3, whereas Chk1\textsuperscript{−/−} cells exhibited mutually exclusive staining for PCNA (green) and pSer\textsuperscript{10}H3 (red). Individual examples are indicated by white arrows.
stained for PCNA and pS10H3. As shown in Fig. 7A, cells with strong nuclear PCNA staining were readily detectable in freshly elutriated G1/S populations of both WT and Chk1−/− cells. In WT cultures, the percentage of cells showing nuclear PCNA staining increased at 5 h and then persisted at this level for at least 12 h of aphidicolin treatment (Fig. 7A). In contrast, in Chk1−/− cultures, cells with weak or undetectable PCNA staining accumulated at later times during replication arrest and constituted approximately 40% of the population after 12 h of aphidicolin treatment, when pS10H3-positive cells were also present (Fig. 7A). Strikingly, strong nuclear PCNA staining was mutually exclusive with pS10H3 staining (Fig. 7B) in individual cells at this time. Since Chk1−/− cells lacking detectable PCNA staining appeared before pS10H3-positive cells (Fig. 7A), we inferred that the loss of PCNA from such sites likely preceded the onset of mitosis and correlated with the loss of replication capacity, as defined by BrdU incorporation. Taken together, these results provide further evidence that premature entry into mitosis from S phase occurs in Chk1−/− cells and constituted approximately 40% of the population after 12 h of aphidicolin treatment, when pS10H3-positive cells were also present (Fig. 7A).

DISCUSSION

To understand the roles of Chk1 and Chk2 in S-M checkpoint control in vertebrate cells, we investigated the effect of DNA synthesis inhibition on mitotic entry in mutant DT40 cell lines deficient in each checkpoint kinase. The results of these studies led to three principal novel conclusions. Firstly, cells deficient in Chk1, but not Chk2, ultimately enter mitosis with incompletely replicated DNA when DNA synthesis is blocked, but only after an initial delay. Secondly, premature entry to mitosis in Chk1−/− cells only when all viable replication structures have been lost and the cells lack the capacity to synthesize DNA, even though genome duplication is incomplete.

Our model also implies the existence of fundamental differences in the mechanisms of S-M checkpoint control between fission yeast and vertebrate cells. In fission yeast, mitosis can be delayed in response to replication arrest through the activation of either Cds1 or Chk1 (4, 25, 50). Although these kinases are not strictly redundant, since Chk1 is activated by replication inhibitors only in the absence of Cds1 (7, 25), both have the potential to impose a mitotic delay under specific circumstances, and yeast mutants that are doubly deficient in both Cds1 and Chk1 lack an effective S-M checkpoint. Initially, we considered that this might also be true for Chk1 and Chk2 and that a compensatory activation of Chk2 (49) might account for the initial mitotic delay in Chk1-deficient cells. Surprisingly, however, a pharmacological inhibition of Chk1 with UCN-01 (17) in Chk2−/− cells (under conditions which induced checkpoint failure in WT DT40 cells) did not eliminate the mitotic delay in response to aphidicolin treatment. Thus, the activation of Chk2 is neither necessary nor sufficient for the initial mitotic delay in the absence of Chk1, which is consistent with other evidence that vertebrate cells can delay mitosis independent of the ATR/Chk1 and ATM/Chk2 pathways (8, 9).

A second major difference concerns the biochemical mechanism of mitotic delay. For fission yeast, a wealth of evidence indicates that maintenance of the inhibitory Y15 phosphorylation of Cdc2 forms the basis of S-M checkpoint delay (13, 28, 42, 50). In contrast, the abrupt activation of Cdc2 kinase ac-
tivity which accompanies checkpoint failure in Chk1−/− cells is not associated with any general loss of Cdc2 Y15 phosphorylation. Since it is unlikely that Y15-phosphorylated Cdc2 acquires catalytic activity, we speculate that S-phase cells contain a subpopulation of Cdc2 molecules which are not subject to this inhibitory modification but whose activity is normally restrained through some other mechanism. Although unexpected, this result is also consistent with studies of ATR-deficient MEFs which failed to identify a correlation between mitotic delay and Cdc2 Y15 phosphorylation during replication arrest (8, 9). Interestingly, other biochemical studies have pointed to the existence of Y15 phosphorylation-independent mechanisms of Cdc2 inhibition which can delay mitotic entry in both vertebrate cells (18, 19) and Xenopus extracts (21). Although the nature of these mechanisms has not yet been fully elucidated, Cdc2 and its associated cyclin B regulatory subunit are known to be subject to regulation at several additional levels, including subcellular localization (29). In particular, regulatory phosphorylation within the cytoplasmic retention sequence of B-type cyclins is thought to play an important role in initiating mitosis by promoting nuclear translocation (45). Furthermore, the forced nuclear localization of cyclin B can override mitotic delay under conditions of DNA damage (46). The correlation between cyclin B2 phosphorylation and premature mitosis in Chk1−/− cells suggests that this modification is a functionally significant target of the S-M checkpoint mechanism, but further work will be required to test this idea explicitly.

Other studies have clearly implicated Cdc25 phosphatase in S-M checkpoint control in HeLa cells (35) and Xenopus extracts (23), suggesting that the regulation of Cdc2 inhibitory phosphorylation must also contribute to mitotic delay in response to replication arrest in vertebrate cells under specific circumstances. It therefore seems possible that Cdc2 Y15 phosphorylation, which accumulates during cell cycle progression, reinforces other checkpoint mechanisms which also restrain the activity of Cdc2 during replication arrest. Furthermore, the relative importance of these mechanisms may vary according to cell type and, possibly, between species (24).

Finally, it is important to stress that the role of Chk1 in our model is indirect, i.e., it is the presence of active, or at least potentially active (viable), replication structures that generates the signal which initially delays mitosis in S-phase cells rather than the action of Chk1 itself. Only when all of these structures have degenerated and become nonfunctional, a situation which ultimately eventuates in Chk1-deficient cells through a combination of replication fork collapse and futile origin firing (14, 49), is the mitotic delay signal lost. An analogous role for Cds1 in mediating an intrinsic link between DNA replication and mitosis by sustaining a stable S-phase state has been proposed (10, 25), although Cdc2 Y15 phosphorylation was envisaged as the ultimate target of the checkpoint regardless of the origin of the signal.

Interestingly, a replication-linked mitotic delay mechanism can be more readily reconciled with earlier, pioneering studies that demonstrated the existence of an intrinsic checkpoint which precludes mitosis during DNA synthesis in vertebrate cells (38) than with models which invoke Chk1 as a direct effector of mitotic delay. In heterokaryons formed by the fusion of S- and G2-phase cells, mitosis was found to be delayed in the G2-phase nucleus until DNA synthesis was completed in the companion S-phase nucleus (38). These experiments, however, did not invoke replication arrest (38), and it seems unlikely that this intrinsic delay could have been imposed by Chk1, since Chk1 is activated only very weakly, if at all, during an unperturbed S phase (Fig. 2C) (14). These observations can be readily explained, however, in terms of an intrinsic mitotic delay signal emanating either from the replication machinery itself (Fig. 8) or from some as yet unidentified checkpoint mechanism which monitors the functional status of this machinery. The future elucidation of the origin and nature of this intrinsic mitotic delay mechanism in vertebrate cells will be of great interest.

ACKNOWLEDGMENTS

We thank Dario Alessi and Carl Smythe for the kind gift of UCN-01, Erich Nigg for anti-cyclin B2 antisera, Tom Gilbey for cell sorting, Tony Carr for comments on the manuscript, Liz Black and Tom McGuire for help with confocal microscopy and image processing, and Michelle Garrett for moral support.

This work was supported by the Association for International Cancer Research (G.Z.) and Cancer Research UK (D.A.F.G. and M.D.R.).

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