Topoisomerase II and histone deacetylase inhibitors delay the G2/M transition by triggering the p38 MAPK checkpoint pathway

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When early prophase PtK1 or Indian muntjac cells are exposed to topoisomerase II (topo II) inhibitors that induce little if any DNA damage, they are delayed from entering mitosis. We show that this delay is overridden by inhibiting the p38, but not the ATM, kinase. Treating early prophase cells with hyperosmotic medium or a histone deacetylase inhibitor similarly delays entry into mitosis, and this delay can also be prevented by inhibiting p38. Together, these results reveal that agents or stresses that induce global changes in chromatin topology during G2 delay entry into mitosis, independent of the ATM-mediated DNA damage checkpoint, by activating the p38 MAPK checkpoint. The presence of this pathway obviates the necessity of postulating the existence of multiple “chromatin modification” checkpoints during G2. Lastly, cells that enter mitosis in the presence of topo II inhibitors form metaphase spindles that are delayed in entering anaphase via the spindle assembly, and not the p38, checkpoint.

Introduction

The term “antephase” was coined in the mid 20th century to denote the period in late G2, “just before . . . prophase becomes visible,” where cells delay in response to chemical and physical insults (Bullough and Johnson, 1951). However, in organisms containing large chromosomes, like rat kangaroos (PtK1), newts, and Indian muntjacs, prophase can last for >1 h. Importantly, in these cells the commitment to mitosis does not occur until the nucleoli begin to fade, ~15 min before nuclear envelope breakdown (NEB; for review see Pines and Rieder, 2001). Before NEB, chromosome condensation can be arrested, and even reversed, by various treatments (for review see Mikhailov and Rieder, 2002). Thus, in cells like PtK1 the end of G2, or antephase, can be expanded to encompass the early stages of chromosome condensation.

The arrest or reversal of chromosome condensation during prophase provides a unique visible cue that entry into mitosis has been delayed, and we have been using this feature to study how the G2/M transition is regulated. This assay has a numeric readout, the duration of prophase, and also a qualitative readout, the degree of chromatin condensation (a measure of CDK activity). In our initial studies we found that disassembling microtubules induces a 3–4-h delay in completing prophase (Rieder and Cole, 2000), a behavior that is likely mediated by a checkpoint involving the Chfr protein (Scolnick and Halazonetis, 2000; Chaturvedi et al., 2002; Matsuoka and Pines, 2004). Recently, we used this assay to explore how inhibiting topoisomerase II (topo II) and other enzymes involved in chromatin structure affect the G2/M transition. The results of these studies, which are described here, reveal that drugs which modify chromatin topology during late G2 delay entry into mitosis, independent of the ATM kinase, by activating the p38 MAPK checkpoint pathway.

Results

All topo II inhibitors delay the G2/M transition. To explore the mechanism behind this delay we treated antephase PtK1 and Indian muntjac cells with various topo II inhibitors, and then followed their behavior by time-lapse video light microscopy. We used a topo II poison (adriamycin) known to...
produce double-strand breaks (DSBs), as well as catalytic inhibitors (ICRF-193, merbarone) that are not supposed to damage DNA. We also used aclarubicin which, by intercalating into DNA, inhibits decatenation (Perrin et al., 1998) by preventing topo II from binding to chromatin (Kellner et al., 2002). Not unexpectedly all of these agents delayed progression through prophase (Table I). When exposed to 4 μM ICRF-193 or 1 μM aclarubicin the chromosomes in early to mid prophase cells decondensed, and then slowly recon-densed, or they continued to slowly condense over a pro-longed prophase period (Table I; Fig. 1, A and B). When these cells finally entered mitosis their chromosomes exhibited the typical nondecatenated phenotype (Gorbsky, 1994), i.e., they were less compacted than normal and the chromatids failed to separate during the ensuing anaphase (Fig. 1, A and B). Adriamycin “froze” cells in a prophase-like state for 10 h (Fig. 1 C), whereas merbarone arrested the cells in anaphase after the chromosomes had decondensed (not depicted).

ICRF-193 and merbarone, but not aclarubicin, induce the formation of γ–H2AX complexes during anaphase

To explore if catalytic inhibitors of topo II delay G2 in the absence of DSBs, we used the phosphorylation of histone H2AX on Ser139 (i.e., the formation of γ–H2AX complexes) as a sensitive visible assay for DSB formation (Rogakou et al., 1998; Caspari and Carr, 2002). We found that a 1-h treatment with 4 μM ICRF-193 or 40 μM merbarone produced multiple γ–H2AX foci in anaphase cells (Fig. 2 A), although many fewer than adriamycin treatment. In contrast, treatment with aclarubicin did not produce γ–H2AX foci above that of the background (Fig. 2 A), even at concentrations that strip topo IIα from chromosomes in 40 min (Fig. 2 B). During interphase and mitosis chromatin bound topo IIα is in a rapid dynamic exchange with unbound topo II (Christensen et al., 2002; Tavormina et al., 2002). Thus, the induction of multiple γ–H2AX foci by ICRF-193 and merbarone imply (Caspari and Carr, 2002) that these drugs induce significant DSBs in vivo.

ATM is not involved in the anaphase delay induced by catalytic inhibitors of topo II

During anaphase adriamycin, ICRF-193 and merbarone activate the ATM kinase as evident from the fact that they in-
duce γ–H2AX foci (Fig. 2 A), which requires ATM activity (Fernandez-Capetillo et al., 2002). Does aclarubicin, which does not induce γ–H2AX foci above that of background, similarly activate ATM? To answer this question we treated CFPAC-1 cultures for 1 h with adriamycin or aclarubicin before preparing whole cell extracts. We then stained Western blots of these extracts for total and activated (serine 1981-P; Bakkenist and Kastan, 2003) ATM. As predicted from our γ–H2AX studies, adriamycin activates the ATM kinase but aclarubicin does not, even at high concentrations (Fig. 3 A).

Figure 2. Aclarubicin does not induce DSBs and depletes chromosomes of topo II. (A) ICRF-193, merbarone, and adriamycin induce DSBs (γ–H2AX complexes) in prophase cells, whereas aclarubicin does not. CFPAC-1 cells were treated with DMSO (Control), 4 μM ICRF-193, 40 μM merbarone, 10 μM adriamycin, or 1 μM aclarubicin in conditioned media for 1 h. They were then fixed and stained as described previously for γ–H2AX complexes (Mikhailov et al., 2002). (Left) Hoechst 33342 and γ–H2AX antibody. (Right) γ–H2AX antibody channel. Note that all topo II inhibitors except aclarubicin induce formation of γ–H2AX complexes above that of the background. Bar, 10 μm. (B) PtK1 cells were fixed 10, 20, 30, and 40 min after incubation in 1 μM aclarubicin, and stained for chromatin (left, Hoechst 33342) and the IMF localization of topo II (right). Note that aclarubicin depletes the chromosomes of topo IIa in 30–40 min. Bars, 10 μm.

Figure 3. ATM is activated by adriamycin but not aclarubicin, and both topo II inhibitors activate p38. (A) CFPAC cells were grown for 4 h in the presence of DMSO (control) or SB203580. They were then exposed to different concentrations of aclarubicin or adriamycin. Whole cell lysates were separated on 6% acrylamide gels and blotted for activated (pS1981) and general ATM. Note that ATM is activated by adriamycin, but not aclarubicin, even at high concentrations. (B) Hela cells enriched in S, G2 and M-phase were collected after release from double thymidine block. Cells were exposed to different concentrations of aclarubicin (ACLA) and adriamycin (ADR) for 30 min during the G2/M transition. Whole cell lysates were then separated on a 12% acrylamide gel and blotted for activated (T*GY*) and general p38. Note that p38 is activated during G2/M by adriamycin and aclarubicin in a dose-dependent manner. White lines indicate that intervening lanes have been spliced out.
Next, we asked if aclarubicin causes cells lacking the ATM kinase to delay in late G2. For this experiment, we filmed fields of human GM16666A (−/− ATM) cell cultures in the presence or absence of 1 μM aclarubicin. From these records, we determined the percentage of cells in mitosis every hour, over a 7-h period, as well as the number that entered mitosis (underwent NEB) each hour. These data (Fig. 4) clearly reveal that aclarubicin rapidly delays the G2/M transition in cells lacking ATM.

Because aclarubicin delays the G2/M transition in the absence of ATM activation, the delay induced by this catalytic topo II inhibitor is not due to the DNA damage checkpoint. To determine if this is also true for other topo II inhibitors, we pretreated PtK1 cultures for 4 h with 5–10 mM caffeine or wortmannin, potent inhibitors of the ATM kinase (Zhou et al., 2000), before adding topo II inhibitors. We found that caffeine or wortmannin did not prevent the antephase delay induced by adriamycin, merbarone, or aclarubicin (Table I). It did, however, shorten the ICRF-193 induced delay, which is consistent with reports that ICRF-193 delays cells in G2, at least in part via the ATM/ATR kinase (Deming et al., 2001). The inability of caffeine to override the adriamycin-induced antephase delay may be due to the extensive DNA damage caused by this poison (Fig. 2A), which may trigger other pathways in addition to ATM. Regardless, because caffeine does not override the antephase delay induced by merbarone or aclarubicin, which compared with adriamycin produce significantly few DSBs (Fig. 2A), the delay must be due to pathways not involving the ATM kinase.

### Triggering p38 MAPK during antephase delays entry into mitosis

In addition to the ATM/DNA damage checkpoint, a caffeine-insensitive pathway appears to exist that delays cells in G2 in response to UV, IR, or γ-irradiation (Goldstone et al., 2001; Jha et al., 2002; Xu et al., 2002). In the case of γ and UV irradiation this arrest is mediated by the p38 MAPK (Bulavin et al., 2001; Dmitrieva et al., 2002; for review see Bulavin et al., 2002). This prompted us to ask if activating p38 during antephase, with concentrations of anisomycin (5–7 ng/ml) that do not affect protein synthesis (Bunyard et al., 2003), delays entry into mitosis. We found that anisomycin rapidly induced early to mid prophase PtK1 cells to decondense their chromosomes and return to G2, for >3 h (Table I). Osmotic stress, which is also a potent activator of p38 (Han et al., 1994; Dmitrieva et al., 2002), similarly induced early to mid prophase cells to decondense their chromosomes and delay in antephase (unpublished data).

By binding to the ATP site on p38, the small molecule SB203580 potently and selectively inhibits the downstream activity of p38 without preventing its activating phosphorylation (Gum et al., 1998; Lisnock et al., 1998). Not unexpectedly, if p38 activity was prevented in PtK1 with SB203580, before treating antephase cells with anisomycin (Table I) or hypertonic medium (not depicted), they entered prometaphase with near normal kinetics. Thus, activating p38 during antephase delays entry into mitosis, and this delay can be eliminated by inhibiting p38 with SB203580.
mycin treatment (Table I; Fig. 5 A). During aclarubicin treatment the cells entered mitosis with little or no chromosome-bound topo II (Fig. 2 B). Pre-incubating PtK1 cells with SB202474, an inactive analogue of SB203580, did not prevent the anaphase delay (unpublished data). We then repeated these experiments with another potent p38 inhibitor, 2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one (de Laszlo et al., 1998), and obtained the same results (unpublished data). Finally, the Jun-N-terminal (JNK) MAPK shares a high degree of structural and functional homology with p38. To determine if JNK is involved in the G2 delay induced by topo II inhibitors we inhibited this MAPK during prophase with 30 \mu M SP600125 (Bennett et al., 2001) and found that it did not prevent the anaphase delay (unpublished data).

We then repeated the p38 inhibitor experiments on Indian muntjac (Table II; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200405167/DC1), human CFPAC-1, and hTERT-RPE-1 cells (Fig. 5, B and C) and obtained similar results. The duration of visible prophase in CFPAC-1 and RPE-1 is \sim 15 min, and by the time chromosome condensation is evident the cells are committed to mitosis. To determine how these cells respond to inhibiting topo II in late G2, in the presence or absence of active p38, we used video light microscopy to follow populations for 6–8 h after drug addition. When treated only with SB203580 the cells entered and completed prophase with normal kinetics for at least 7 h (Fig. 5, B and C). As with PtK1 and Indian

### Table II. Duration of prophase in Indian muntjac cells treated with topo II inhibitors

| Treatment           | None           | SB203580 (50 \mu M/1 h) |
|---------------------|----------------|------------------------|
| None                | 55 ± 13 (16)   | 49 ± 16 (5)            |
| Aclarubicin (0.75–1 \mu M) | 300 ± 141 (3) | 57 ± 4 (6)             |
| ICRF-193 (4 \mu M) | 384 ± 187 (3)  | 101 ± 35 (2)           |

Duration of prophase (min) in Indian muntjac cells treated with aclarubicin or ICRF-193 in the absence or presence of SB203580. Note that inhibiting p38 significantly reduces the duration of prophase in cells treated with topo II inhibitors. The data in A and B represent the average ± SD; the number of cells followed is shown in parentheses.
muntjac cells, both ICRF-193 and aclarubicin rapidly inhibited entry into mitosis in CFPAC-1 (Fig. 5 B) and RPE-1 (Fig. 5 C) cultures. However, the inhibition could be largely overridden during the first several hours by first treating the cultures with SB203580. After 4–7 h in aclarubicin, CFPAC-1 and hTERT-RPE1 cells fail to enter mitosis even when p38 is inhibited. This is likely due to toxic effects arising, e.g., from the inability of late S or early G2 cells in aclarubicin-treated cultures to transcribe genes required for cell cycle progression.

Inhibiting histone deacetylase also delays the G2/M transition via p38

One interpretation of our results is that topo II inhibitors and osmotic shock impede the G2/M transition because they induce abnormal chromatin topology which activates the p38 pathway. To explore whether other agents that globally disrupt chromatin structure delay cells in antephase via p38 we treated PtK1 cells with apicidin, a potent histone deacetylase inhibitor (Witt et al., 2003). Because histone deacetylase is recruited to DNA by other proteins, inhibiting its activity during antephase with 0.5 μM apicidin should not, and does not (Fig. 6 C), induce γ-H2AX foci (DSBs) above that of background. However, others have shown that inhibiting histone deacetylase alters chromatin structure (Grunstein, 1997) and arrests cell cycle progression via an undefined checkpoint (Qiu et al., 2000). Not unexpectedly we found that inhibiting histone deacetylase during early prophase delayed entry into mitosis by either inducing the chromosomes to decondense (n = 2; Fig. 6 A), or by prolonging prophase (159 ± 87 min; n = 3). Importantly, this delay was eliminated when cells were pretreated with SB203580 (52 ± 11 min; n = 3; Fig. 6 B), but not caffeine (not depicted). For controls we treated early prophase cells with lumicolcemid or cytochalasin D, which do not affect chromatin structure, and found that they entered mitosis with normal kinetics (not depicted; for review see Rieder and Cole, 2000).

Localized DSBs delay entry into mitosis via the ATM and not p38 checkpoint pathway

So far our data support the idea that global disruptions in chromatin topology delay cell cycle progression via the p38

![Figure 6. Inhibiting histone deacetylase delays progression through antephase via a p38-dependent mechanism.](image)

![Figure 7. Inhibiting p38 does not override the ATM/DNA damage checkpoint.](image)
pathway independent of DSBs. This model predicts that inducing DSBs in just a few highly localized regions of the nucleus will not arrest anaphase cells via the p38 pathway. To test this we “stitched” nuclei in anaphase PtK₁ cells with 50 pulses of 546-nm laser light. This produces highly localized regions of γ-H2AX foci (unpublished data; for review see Rogakou et al., 1999) and delays anaphase cells from entering mitosis (Rieder and Cole, 1998). When we repeated these experiments after inhibiting p38 with SB203580, the cells continued to decondense their chromosomes and were blocked in anaphase (Table I; Fig. 7 A). However, if we pretreated cultures with 5–10 mM caffeine before stitching early prophase nuclei, the cells progressed into mitosis with normal kinetics even though they contained numerous DSBs (Table I; Fig. 7 B). This experiment reveals that SB203580 does not inhibit the ATM kinase. It also demonstrates that the localized disruption of chromatin does not activate p38, or that if it is activated under this condition it does not contribute to the cell cycle delay.

**P38 activity is not required for progression through mitosis or for the spindle assembly checkpoint**

Cells that enter mitosis in the presence of ICRF-193 form metaphase spindles that are delayed in entering anaphase (Mikhailov et al., 2002). Here, we report that aclarubicin-treated cells, driven into mitosis by inhibiting p38, also form spindles that are delayed in metaphase (Fig. 5 A). This was true for all cell types tested, including PtK₁ (Table III), Indian muntjac (Fig. S1 B), CFPAC (185–200 min, n = 4 vs. 60 ± 7 min, n = 9), HeLa (160 ± 56 min, n = 56 vs. 46 ± 6 min, n = 2), and U2OS (238 ± 90 min, n = 5 vs. 43 ± 14 min, n = 5).

As reported by others (Deacon et al., 2003), we found that p38 is not activated as untreated HeLa cells transit from G2 into mitosis (Fig. 3 B). Therefore, it is not surprising that inhibiting p38 with SB203580 had no effect on the duration of mitosis in untreated PtK₁ cells (Table III). From our live cell studies it was also clear that the delay in mitosis caused by aclarubicin, or disrupting microtubule assembly with nocodazole, is not overridden by inhibiting p38 in PtK₁ (Table III) or Indian muntjac (Fig. S1 C). Under both conditions it is, however, rapidly eliminated when the cells are injected with a dominant negative Mad2 spindle assembly checkpoint component (see Mikhailov et al., 2002; unpublished data). Thus, p38 activation is not required for normal mitotic progression or for the spindle assembly checkpoint in PtK₁ or Indian muntjac cells.

**Discussion**

Topo II is the only enzyme that can cut and rejoin double-strand DNA, and it is used to relieve torsional stress caused e.g., during DNA replication, transcription and repair. The enzyme is particularly active during the G2 and M phases of the cell cycle where it is involved in many aspects of DNA metabolism and chromatin topology. Two features have made topo II a primary target for some of the most widely prescribed antibiotics and anticancer drugs currently in clinical use: it serves an indispensable function and it lacks biological redundancy (Froelich-Ammon and Osheroff, 1995; Kellner et al., 2002). Because deleting topo II is usually lethal, its function is studied primarily by disrupting its activity with “poisons” that stabilize the enzyme on cleaved DNA, or with “catalytic” inhibitors that bind the enzyme before it cuts DNA, or after it has been rejoined.

The synthesis of topo IIα starts in G1 and peaks in G2, whereas topo IIβ expression is continuous throughout the cell cycle (Kellner et al., 2002). As a result, poisons like ICRF-193 and merbarone, that preferentially bind to and inhibit topo IIα-catalyzed decatenation (Perrin et al., 1998), delay cells selectively in G2 (Deming et al., 2002). In contrast, by intercalating directly into chromatin aclarubicin prevents decatenation by both topo IIα and β (Perrin et al., 1998). As a result, this drug delays progression through all stages of the cell cycle including G2 (Teillaud et al., 1998).

The mechanism(s) by which topo II inhibitors delay cell cycle progression are only vaguely understood. Work with the catalytic inhibitor ICRF-193 suggested that this delay is mediated by a caffeine-sensitive pathway that monitors chromatin decatenation (Downes et al., 1994; Clifford et al., 2003). Subsequent work suggested that this “decatenation checkpoint” delays entry into mitosis, independent of the p53 pathway (Kaufmann et al., 2002), by using the ATR kinase and BRCA1 to inhibit the polo-like kinase (Deming et al., 2001, 2002; Kaufmann et al., 2002). The idea that a G2 “decatenation” checkpoint exists, distinct from the DNA damage checkpoint, is based largely on indirect observations and biochemical data that ICRF-193 does not induce DSBs. However, recent work (Huang et al., 2001; Wang and Eastmond, 2002), as well as our own data (Fig. 2), reveals that many of these drugs, including ICRF-193 and merbarone, do damage DNA in vivo. Furthermore, it is now clear that the ATR kinase implicated in the so called decatenation checkpoint has considerable overlap with the ATM kinase which arrests cells in response to DSBs (Durocher and Jackson, 2001). Finally, because sister chromatids do not become fully decatenated until the metaphase/anaphase transition, it is difficult to envision how a checkpoint monitoring the state of DNA catenation can delay the G2/M transition when cells normally enter mitosis with catenated chromatids.

**Table III. Duration of mitosis in PtK₁ cells treated with nocodazole, anisomycin, or aclarubicin**

| Treatment       | None          | SB203580    |
|-----------------|---------------|-------------|
| None            | 50 ± 2 (7)    | 57 ± 10 (8) |
| Nocodazole      | 136 ± 20 (13) | 337 ± 107 (5) |
| Anisomycin (5–7.5 ng/ml) | 46 ± 18 (6) | 40 ± 12 (5) |
| Aclarubicin (1.5–3 μM) | 128 ± 38 (10) | 158 ± 54 (4) |

The duration of mitosis (NEB to anaphase onset) in PtK₁ cells treated at 37°C with nocodazole, anisomycin, or aclarubicin, with or without prior treatment with the p38 inhibitor SB203580. The data represents the average ± SD; number of experiments is shown in parentheses.

*Nuclear envelope breakdown to anaphase onset.*

**Topo II and histone deacetylase inhibitors delay the G2/M transition by activating the p38 pathway**

Our data reveal that, when applied to anaphase (late G2) cells, topo II inhibitors delay entry into mitosis via the p38 pathway.
MAPK, and not the ATM, pathway. Because this delay is triggered in minutes, by a route that functions well into prophase, it does not require activation of transcription factors (like p53) or new protein synthesis. The p38 MAPK pathway fulfills the criteria for a checkpoint control, at least during late G2: at this time it is normally not active and, when activated, it delays cell cycle progression via a route that shows a relief of dependence. Importantly, under many conditions this delay is transient and it is ultimately bypassed by an adaptation process, even when the problem cannot be fixed. This control provides a mechanism during the G2/M transition, as it appears to also do during the G1/S transition (Kyriakis and Avruch, 2001), for quickly delaying cell cycle progression in response to diverse stresses. In the absence of such a rapid response system, such stresses may well lead to chromosome segregation problems during mitosis independent of those generated by DNA damage. The p38 checkpoint pathway thus gives the cell time to recover, just before important transitional events, in cases where the insult is transient. If need be, it also allows other checkpoints that require transcription time to work.

What does the “topo II checkpoint” monitor if not chromatin decatenation? Topo II inhibitors either bind to chromatin (aclacinomycin) or lock the enzyme on chromatin in an inactive form (adriamycin, ICRF-193, merbarone). One possibility is that as these drugs bind, they induce a global change in chromatin topology that delays the G2/M transition by activating the p38 pathway. This idea is supported by our data, and those of others, that osmotic shock and histone deacetylase inhibitors, which similarly induce global changes in chromatin topology, also delay the G2/M transition via the p38 pathway. It is also consistent with our finding that selectively damaging chromatin in just a few regions of the antephase nucleus delays entry into mitosis via the ATM and not p38 kinase pathway.

How could global changes in chromatin topology during antephase activate p38? One possibility is that regions of chromatin bind an unidentified factor that is released in response to abnormal chromatin topology. Once released this factor may interact with c-Abl and/or DNA-protein kinase (Kharbanda et al., 1997) to initiate a kinase cascade (Brancho et al., 2003) that activates p38. Active p38 can influence cell behavior by activating transcription factors or other kinases. Because the antephase response we describe is rapid, and occurs as chromosomes are condensing, it is not due to transcription factors like p53. Rather, the activation of p38 by abnormal chromatin topology likely initiates another kinase cascade, perhaps involving MNK1 (Fukunaga and Hunter, 1997), that produces the cell cycle delay. P38 can also directly interact with Cdc25B (Bulavin et al., 2001). The antephase checkpoint may work by ultimately blocking activation of cyclin A/CDK2 via Cdc25, which in response can occur independent of ATM/ATR (Goldstone et al., 2001; Mitra and Enders, 2004).

We find that the delay in entering mitosis induced in late G2 cells by topo II inhibitors is caffeine insensitive and does not involve the ATM kinase. Bakkenist and Kastan (2003) report that based primarily on immunofluorescence (IMF) data, osmotic stress, and histone deacetylase inhibitors induce a diffuse phosphorylation of ATM in the absence of DSBs. This suggested that ATM is activated globally by changes in chromatin structure, and then later accumulates at DSBs when present. Our results reveal that these same treatments delay the G2/M transition. However, we find that this delay is not overridden by inhibiting ATM with caffeine or wortmannin (or in −/− ATM cells), yet it is eliminated by preventing p38 kinase activity. We also find that topo II inhibitors which induce DSBs activate both ATM (as evidenced by γ–H2AX foci formation) and p38, but that inhibitors that do not induce DSBs (aclacinomycin) do not activate ATM. Regardless, with the exception of adriamycin, which induces massive numbers of DSBs, all of these inhibitors delay entry into mitosis via the p38 and not ATM pathway. These results imply that, by itself, the global activation of ATM by changes in chromatin topology does not produce a late G2 delay independent of the p38 pathway.

**P38 activity is not required for entry into mitosis or the spindle assembly checkpoint**

The spindle assembly checkpoint delays anaphase when kinetochores are not stably associated with the spindle. Work on 3T3 and HeLa cell populations suggests that p38 is activated in response to spindle poisons (Deacon et al., 2003), and that this activity is required for the spindle assembly checkpoint (Takenaka et al., 1998). However, in situ studies conclude that p38 is normally active during mitosis, and that this activity is required to overcome this checkpoint (Campos et al., 2002). Cell sorting studies even suggest that inhibiting p38 does not influence the mitotic arrest or slippage of HeLa cells treated with nocodazole (Tsukui et al., 2001).

As reported by others (Deacon et al., 2003) we found that p38 is not activated as untreated HeLa cells transit from G2 into mitosis (Fig. 3 B). We also found that inhibiting p38 does not influence the rate at which CFPAC-1 or hTERT-RPE1 cells enter mitosis (Fig. 5, B and C), or the duration of the mitotic delay induced in live PtK2, or Indian muntjac cells by nocodazole or topo II inhibitors. This latter delay is, however, rapidly abrogated when cells are injected with a dominant negative construct of Mad2 (Mikhailov et al., 2002). From these observations we conclude that p38 activity is neither required for entry into mitosis, for normal mitotic progression, or for the spindle assembly checkpoint in PtK2, or Indian muntjac cells.

Many of the chemical or physical insults that delay the G2/M transition also delay the metaphase/anaphase transition. With few exceptions, most of these globally perturb chromatin topology. Good examples here include chromatin damage caused by radiation (Mikhailov et al., 2002), and inhibitors of topo II (Illidge et al., 2000; Mikhailov et al., 2002) or histone deacetylase (Cimini et al., 2003). We propose that topo II and histone deacetylase inhibitors delay entry into and exit from mitosis because they bind to and induce structural changes in chromatin. During antephase these changes are detected by the p38 pathway. During mitosis they impede satisfaction of the spindle assembly checkpoint by deleteriously affecting kinetochore structure and thus their stable attachment to the spindle.

Finally, we found that cells arrested in G2 by drugs that prevent normal topo II function can be driven into a highly aberrant mitosis by simply overriding activation of the P38
MAPK. Many of these drugs are currently used as a primary or adjunct chemotherapy in cancer treatment (Froelich-Ammon and Osheroff, 1995; Kellner et al., 2002). One interesting avenue may therefore be to explore the clinical effects of combining topo II and p38 inhibitors.

Materials and methods

Cell culture

PtK1, HeLa, U2OS, CFPAC-1, and hTERT-RPE1 were cultured on coverslips as detailed previously (Mikhailov et al., 2002). Indian muntjac cells were grown in DME supplemented with 10% FBS. GM1666A cells were grown on coverslips in DME supplemented with 10% FBS and 100 μg/ml hygromycin-μc; 12 h before the experiment cells were placed in hygromycin-free media. Coverslip cultures of PtK1 and Indian muntjac cells were assembled into Rose chambers (Khodjakov and Rieder, 2004) at least 5 h before the start of each experiment, whereas those containing CFPAC, hTERT-RPE, and GM1666A cells at least 12 h before each experiment. Once assembled the Rose chambers were then incubated at 37°C.

Sequential images were then assembled into movie stacks which were using Image Pro Plus (Media Cybernetics) or Scion Image (Scion Corp.). Coverslip cultures of PtK1 and Indian muntjac cells were assembled into Rose chambers (Khodjakov and Rieder, 2004) at least 5 h before the start of each experiment, whereas those containing CFPAC, hTERT-RPE, and GM1666A cells at least 12 h before each experiment. Once assembled the Rose chambers were then incubated at 37°C.

Immunological techniques

Rabbit antibody to human topo II was purchased from Topogen. Cells were fixed and stained for IFM, including γ-H2AX, as detailed previously (Mikhailov et al., 2002).

Laser irradiation and live cell microscopy

Laser irradiation of early prophase nuclei was conducted with pulses of 532 nm light (Rieder and Cole, 1998). Nuclei were irradiated with 50 μJ pulses of 5–10 mW 300 nm light (Rieder and Cole, 1998). Nuclei were irradiated with 50 μJ pulses of 5–10 mW 300 nm light (Rieder and Cole, 1998).

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