

# Caffeine mimics adenine and 2'-deoxyadenosine, both of which inhibit the guanine-nucleotide exchange activity of RCC1 and the kinase activity of ATR

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## Abstract

**Background:** Both caffeine and the inactivation of RCC1, the guanine-nucleotide exchange factor (GEF) of Ran, induce premature chromatin condensation (PCC) in hamster BHK21 cells arrested in the S-phase, suggesting that RCC1 is a target for caffeine.

**Results:** Caffeine inhibited the Ran-GEF activity of RCC1 by preventing the binary complex formation of Ran-RCC1. Inhibition of the Ran-GEF activity of RCC1 by caffeine and its derivatives was correlated with their ability to induce PCC. Since caffeine is a derivative of xanthine, the bases and nucleosides were screened for their ability to inhibit RCC1. Adenine, adenosine, and all of the 2'-deoxynucleosides

inhibited the Ran-GEF activity of RCC1; however, only adenine and 2'-deoxyadenosine (2'-dA) induced PCC. A factor(s) other than RCC1, should therefore be involved in PCC-induction. We found that both adenine and 2'-dA, but none of the other 2'-deoxynucleosides, inhibited the kinase activity of ATR, similar to that of caffeine. The ATR pathway was also abrogated by the inactivation of RCC1 in tsBN2 cells.

**Conclusion:** The effect of caffeine on cell-cycle control mimics the biological effect of adenine and 2'-dA, both of which inhibit ATR. dATP, a final metabolite of adenine and 2'-dA, is suggested to inhibit ATR, resulting in PCC.

## Introduction

Progression of the cell cycle is regulated by a checkpoint control which ensures that the cells do not enter mitosis until the completion of DNA replication (Murray & Hunt 1993). In mitosis, MPF, which is composed of p34<sup>cdc2</sup> kinase and cyclin B, is activated so that chromatin is condensed into mitotic chromosomes. If mitotic events occur in the S-phase, the chromatin ceases to be replicated, having become fragmented due to premature chromatin condensation (PCC). Cells showing PCC enter apoptosis due to a loss of genomic integrity (Canman 2001). When tsBN2 cells arrested in the S-phase, which have a defect in the *RCC1* gene (Kai *et al.* 1986), are incubated at the

non-permissive temperature, 39.5 °C, they prematurely enter mitosis, show PCC (Nishimoto *et al.* 1978), and subsequently enter apoptosis (T. Sekiguchi, Kyushu-University, personal communication). We hypothesized that RCC1 is involved in coupling the completion of DNA replication and the initiation of mitosis. Caffeine has been reported to induce PCC in BHK21 cells arrested in the S-phase (Schlegel & Pardee 1986), similar to the tsBN2 mutation. In the case of both tsBN2- and caffeine-induced PCC, cells showing PCC appeared with similar kinetics, and the induction of PCC was blocked by the addition of a protein-synthesis inhibitor (Nishimoto *et al.* 1981; Schlegel *et al.* 1987). Cdc25B, an activator of MPF, was lost in tsBN2 cells arrested in the S-phase by the addition of cycloheximide, a protein synthesis inhibitor, resulting in the blocking of PCC-induction (Nishijima *et al.* 1997). Over-expression of Cdc25B also enhanced caffeine-induced PCC (Nishijima *et al.* 1997). Taken together, these findings suggest that RCC1 and caffeine are both involved in cell-cycle checkpoint control in a similar manner.

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RCC1 is the only known guanine nucleotide-exchange factor (GEF) for Ran, a Ras-like nuclear small GTPase (Bischoff & Ponstingl 1991), which is required for the nucleocytoplasmic transport, microtubule assembly, rRNA processing and other unknown cellular processes (Nishimoto 2000; Clarke & Zhang 2001; Dasso 2002; Hetzer *et al.* 2002). However, it is not yet clear how RCC1 regulates cell-cycle progression. RCC1 is abundantly localized on the chromatin (Ohtsubo *et al.* 1989), and RanGAP1, a Ran GTPase-activating protein, is located within the cytoplasm (Hetzer *et al.* 2002). Therefore, there is a steep gradient of Ran-GTP concentration from the nucleus to the cytoplasm which is important for the nucleocytoplasmic transport of macromolecules through the nuclear pore (Weise 2002). Nuclear import and export are important for coordinating the components of the cell-cycle machinery, so that they function at an appropriate time in the cell cycle (Pines 1999). In this regard, it is noticeable that RCC1 plays a crucial role in steady-state flux across the nuclear pore complex (Smith *et al.* 2002). Thus, RCC1 should play an important role on the chromatin, for coupling the completion of DNA replication with MPF activation.

Caffeine has been reported to inhibit the kinase activity of ATM and ATR (Sarkaria *et al.* 1999; Hall-Jackson *et al.* 1999; Blasina *et al.* 1999; Guo *et al.* 2000; Zhou *et al.* 2000; Feijoo *et al.* 2001), both of which cause cell-cycle arrest and apoptosis in response to chromosomal insults (Abraham 2001). Deletion of the *ATR* gene in mice leads to early embryonic cell death due to the apoptosis caused by extensive chromosomal fragmentation similar to PCC (Brown & Baltimore 2000). The *ATM* gene is not essential for animal growth. Both ATM and ATR proteins are diffused in the nucleus. Following either DNA damage or the inhibition of DNA replication in the S-phase, they rapidly generate a protein complex on the chromatin to activate the downstream kinases of Chk1 or Chk2, resulting in either cell-cycle arrest or S-phase delay (Osborn *et al.* 2002). These findings suggest that caffeine overrides cell-cycle checkpoint control through inhibition of ATR and ATM. Cell cycle checkpoint control is weak in most cancer cells. Therefore, an appropriate dose of caffeine (if it is possible) could kill cancer cells, but not normal cells, by forcing them to enter apoptosis. However, caffeine has a myriad of pharmacological effects (Kihlman 1997), which means that caffeine itself can not be used as an anti-cancer drug. In this context, the bases and the nucleosides were screened for their abilities to inhibit the Ran-GEF activity of RCC1 and the kinase activity of ATM and ATR. We found that adenine and 2'-deoxyadenosine (2'-dA) inhibited the Ran-GEF activity of RCC1 and the

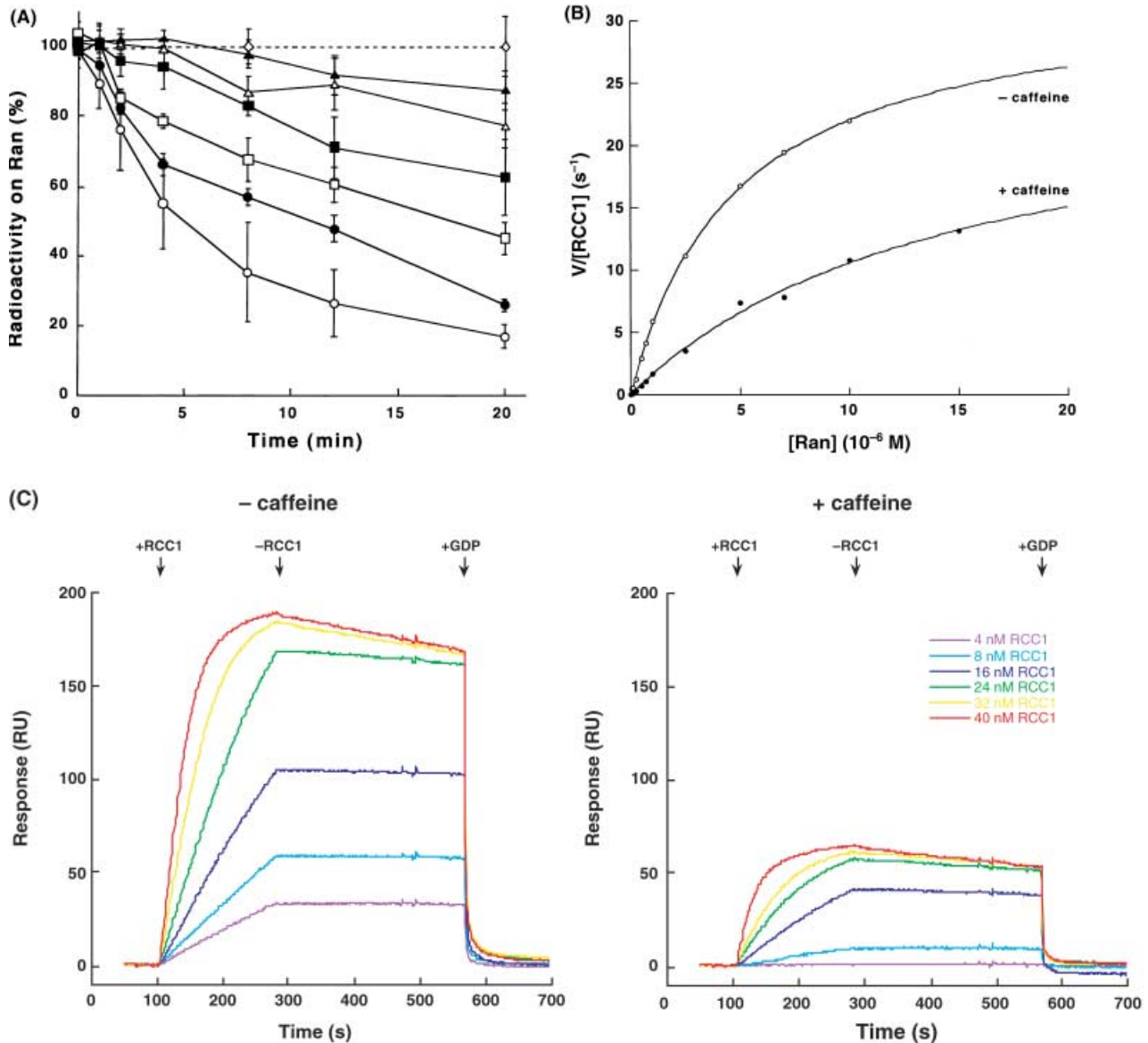
kinase activity of ATR. They induced PCC in BHK21 cells arrested in the S-phase. Other 2'-deoxynucleosides which surprisingly inhibited the Ran-GEF activity of RCC1, neither induced PCC nor inhibited the kinase activity of ATR. We postulate that caffeine mimics adenine and 2'-dA *in vivo*, with regard to cell-cycle control. Using tsBN2 cells, the inactivation of RCC1 was found to abrogate the ATR cell-cycle checkpoint pathway, which suggests that the RCC1-Ran cycle has a relationship with ATR-dependent cell-cycle checkpoint control. Taken together, the inactivation of ATR could be enough to induce PCC.

## Results

### Nucleotide-exchange activity of RCC1 was inhibited by caffeine

Recombinant RCC1 and Ran were purified as described in the Experimental procedures. When Ran- $^3\text{H}$ GDP was incubated with RCC1, the remaining radioactivity on Ran was rapidly reduced depending on the incubation time, as previously reported (Bischoff & Ponstingl 1991). This indicated that  $^3\text{H}$ GDP was released by the action of RCC1 (Fig. 1A). A release of  $^3\text{H}$ GDP from Ran- $^3\text{H}$ GDP was inhibited by the addition of caffeine in a dose-dependent manner. Five mM caffeine was able to induce PCC in BHK21 cells arrested with hydroxyurea (HU) (Fig. 5) as previously noted (Schlegel & Pardee 1986). At a concentration of 4–8 mM caffeine, RCC1-stimulated  $^3\text{H}$ GDP release from Ran- $^3\text{H}$ GDP was consistently inhibited.

The interaction of Ran with RCC1 is carried out by a four-step mechanism which involves the formation of a ternary complex consisting of Ran, RCC1 and GXP, as well as a nucleotide-free binary Ran-RCC1 complex (Klebe *et al.* 1995). To determine which of the steps involved in the Ran-RCC1 interaction were inhibited by caffeine, RCC1-stimulated nucleotide release was analysed by Michaelis-Menten kinetics, either in the presence or the absence of caffeine, using the fluorescent GDP analogue, 2',3'-O-methylanthraniloyl-GDP (mantGDP) bound to Ran, as previously described (Azuma *et al.* 1996). The RCC1-stimulated release of mantGDP from Ran-mantGDP was inhibited by the addition of caffeine (data not shown). Using a constant amount of RCC1 and an increasing concentration of Ran-mantGDP, the reaction rate was calculated either in the presence or the absence of 10 mM caffeine. The observed rates were plotted against the concentration of Ran-mantGDP in order to calculate the  $K_M$  and  $k_{\text{cat}}$  of the reaction (Fig. 1B and Table 1). Both the  $K_M$



**Figure 1** Caffeine inhibits RCC1-stimulated GDP release from Ran-GDP. (A) Highly purified recombinant RCC1 and Ran were prepared as described in Experimental procedures. Ran- $[^3H]$ GDP (final concentration 50 nM) was either mixed with RCC1 (final concentration 120  $\mu$ M) (solid line) or not (dotted line), and then incubated at 37 °C in GEF buffer containing 5 mM GTP and an increasing amount of caffeine (○, 0 mM; ●, 1 mM; □, 2 mM; ■, 4 mM; △, 6 mM; ▲, 10 mM). At the indicated time, the remaining radioactivity on Ran was estimated by liquid scintillation counting. The vertical axis shows the ratio (percentage) of radioactivity remaining on Ran after incubation, compared with the value without incubation. (B) Kinetics of Ran-GEF activity of RCC1 using fluorescence. Recombinant RCC1 proteins (final concentration 1.6 nM) were mixed with an increasing concentration of Ran-mantGDP and incubated at 37 °C, in the presence or absence of 10 mM caffeine. The intensity of fluorescence (emission at 450 nm, excitation at 355 nm) was measured at intervals of every second. The estimated initial velocity of reaction was divided by the concentration of RCC1 in order to determine the dissociation coefficient. The calculated values were plotted against the concentration of Ran and fitted to the Michaelis-Menten equation to give the  $K_M$  and  $k_{cat}$ . The curvilinear fittings were achieved using the KALEIDAGRAPH program on a Macintosh computer. (C) Plasmon surface resonance analysis of the RCC1-Ran interaction. 120  $\mu$ M of GST-Ran and as a control, GST, were captured by the anti-GST antibody immobilized on to a sensor chip. The indicated concentration of RCC1 (magenta, 4 nM; cyan, 8 nM; blue, 16 nM; green, 24 nM; yellow, 32 nM; red, 40 nM) was incubated with either 10 mM caffeine in GEF buffer, or else with GEF buffer alone, for 10 min at 37 °C, and then injected. After 180 s, lanes were washed with GEF buffer, either containing or not containing 10 mM caffeine. At the indicated time, 1  $\mu$ M of GDP was added. The sensorgrams were evaluated, as described (Azuma *et al.* 1999).

**Table 1** Kinetics analysis of RCC-stimulated nucleotide exchange on Ran, either in the presence or absence of caffeine

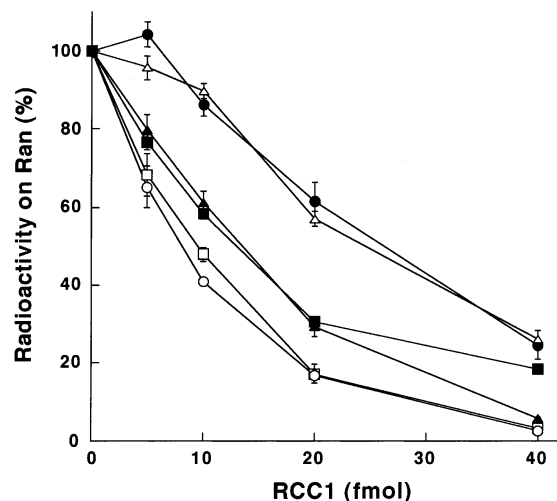
Caffeine	$K_M$ ( $10^{-6}$ M)	$k_{cat}$ (/s)	$k_{cat}/K_M$ ( $10^6$ /M/s)
–	$4.81 \pm 0.17$	$32.8 \pm 0.53$	6.81
+	$14.9 \pm 2.24$	$26.4 \pm 2.42$	1.77

Based on Fig. 1B, the first-order rate constants obtained were plotted against the concentration of Ran to determine  $K_M$  and  $k_{cat}$  using the Michaelis–Menten equation, as previously described (Azuma *et al.* 1996).

and  $k_{cat}$  values were affected by caffeine. Specifically, the  $K_M$  values, which reflect the interaction between RCC1 and Ran, were dramatically changed in contrast to the  $k_{cat}$  values which represent the reaction rate, so that the total enzymatic activity ( $k_{cat}/K_M$  ratio) of RCC1 was reduced to about 26% of the original values (Table 1) by the addition of caffeine. In order to obtain more information on the partial reaction step(s) affected by caffeine, a real-time interaction analysis was carried out using plasmon surface resonance (Biacore), as previously described (Azuma *et al.* 1999). GDP-bound GST-fused Ran was captured through an anti-GST antibody on to the sensor chip. The lanes were flushed with an increasing amount of RCC1 proteins which either had, or had not, been incubated with 10 mM caffeine (Fig. 1C). At the indicated time, 1  $\mu$ M of GDP was added to dissociate the RCC1–Ran complex. The stability and the dissociation of the RCC1–Ran binary complex were not affected by the addition of caffeine. However, caffeine strongly inhibited the formation of the ternary complexes consisting of Ran, RCC1 and GDP. The observed association rate constant ( $k_a$ ) and the dissociation rate constant ( $k_d$ ) of RCC1, which were  $1.78 \times 10^6$ /M/s and  $1.45 \times 10^{-6}$ /s, respectively, changed to  $7.99 \times 10^4$ /M/s and  $7.48 \times 10^{-4}$ /s, respectively, following the addition of caffeine. Accordingly, the dissociation constant ( $K_p$ ) was actually increased 11 000-fold. Thus, it can be concluded that caffeine inhibits the interaction of RCC1 with RanGDP, subsequently inhibiting the formation of RCC1–Ran binary complexes.

#### Effect of the derivatives of caffeine on the Ran-GEF activity of RCC1

If caffeine inhibits the Ran-GEF activity of RCC1, its derivatives might also affect the Ran-GEF activity of RCC1. Caffeine is 1,3,7-trimethylxanthine. Theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine), 3-isobutyl-1-methylxanthine and



**Figure 2** Inhibition of the Ran-GEF activity of RCC1 by caffeine-derivatives. Indicated concentration of recombinant RCC1 was mixed with Ran- $[^3\text{H}]$  GDP (final concentration 50 nM), and then incubated at 37 °C for 20 min in the presence of 2.5 mM caffeine (●) and its derivatives: theophylline (■), theobromine (△), 3-isobutyl-1-methylxanthine (□), and 1,7-dimethylxanthine (▲), and as a control, buffer alone (○). The remaining radioactivity on Ran was estimated by liquid scintillation counting. The vertical axis shows the ratio (percentage) of radioactivity remaining on Ran after incubation, compared with the value incubated in buffer without RCC1. In parallel, the ability of caffeine and its derivatives to induce PCC in BHK21 cells arrested with HU was determined. The PCC ratio induced by caffeine and its derivatives was as follows: caffeine,  $56.0 \pm 0.98\%$ ; theophylline,  $18.4 \pm 2.0\%$ ; theobromine,  $55.3 \pm 1.4\%$ ; 3-isobutyl-1-methylxanthine, 0%; 1,7-dimethylxanthine,  $19.3 \pm 0.83\%$ . More than 600 cells were counted.

1,7-dimethylxanthine are all known derivatives of caffeine. Among these caffeine derivatives, theobromine has been reported to be as active as caffeine in inducing PCC, whereas 3-isobutyl-1-methylxanthine, which is an inhibitor of phosphodiesterase, does not induce PCC, in BHK21 cells arrested with HU (Schlegel & Pardee 1986). In order to address the correlation between caffeine-induced PCC and the inhibitory activity of caffeine on the Ran-GEF activity of RCC1, we compared the ability of caffeine and its derivatives to induce PCC with their ability to inhibit the Ran-GEF activity of RCC1. Firstly we re-examined a previous report from Schlegel & Pardee (1986).

When caffeine was given to BHK21 cells arrested with HU, PCC appeared in 56% of the cells (Fig. 2, legend). Similar to caffeine, theobromine (3,7-dimethylxanthine) induced PCC in 55% of the cells, but 3-isobutyl-1-methylxanthine did not induce PCC, consistent with the previous report (Schlegel & Pardee 1986). The other

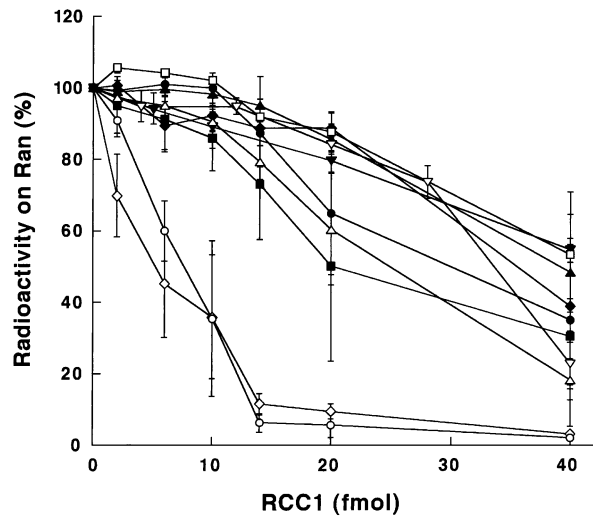
dimethylxanthines, theophylline (1,3-dimethylxanthine) and 1,7-dimethylxanthine induced PCC in 18% and 19% of BHK21 cells arrested with HU, respectively.

Next, we addressed their ability to inhibit the Ran-GEF activity of RCC1. Theobromine (3,7-dimethylxanthine) induced PCC, inhibited RCC1-stimulated nucleotide release. 3-isobutyl-1-methylxanthine did not induce PCC, and did not inhibit RCC1-stimulated nucleotide release (Fig. 2). Furthermore, two dimethylxanthines, 1,7-dimethylxanthine and theophylline (1,3-dimethylxanthine), were 40% as active as caffeine with regard to PCC-induction; they partially inhibited RCC1-stimulated nucleotide release. Thus, the ability of caffeine and its derivatives to induce PCC in BHK21 cells arrested with HU was correlated with their ability to inhibit the Ran-GEF activity of RCC1.

### Adenine and 2'-dA inhibited the Ran-GEF activity of RCC1 and induced PCC

In order to search for an agent(s) which caffeine mimics *in vivo*, the bases and the nucleosides were examined for their ability to inhibit the Ran-GEF activity of RCC1. In addition to adenine and adenosine, all of the examined 2'-deoxynucleosides inhibited the Ran-GEF activity of RCC1. A representative result is shown in Fig. 3. The 2'-ribose, which was used as a control, did not inhibit the Ran-GEF activity of RCC1. The next question was whether all of the agents which inhibit the Ran-GEF activity of RCC1 could induce PCC in HU arrested BHK21 cells or not. As shown in Fig. 4A, only adenine and 2'-deoxyadenosine (2'-dA), but not the other agents, prematurely activated MPF in BHK21 cells arrested with HU.

In order to confirm that RCC1 is involved in PCC induced by 2'-dA, the recombinant wild-type RCC1 and, as a control, mutated RCC1, D182, which has no Ran-GEF activity (Azuma *et al.* 1996, 1999) was prepared. An indicated amount of the wild-type or mutated RCC1 was mixed with gelatin to adjust the final protein concentration to 12 mg/mL, and then with 0.4 mg/mL of rabbit IgG (injection marker). RCC1-mixtures were injected into BHK21 cells arrested in the S-phase with HU. After the injection, cells were incubated in HU-medium containing 5 mM of 2'-dA for 5 h at 37 °C, and stained with Alexa Fluor 568-conjugated goat anti-rabbit IgG antibodies and with the mAb, MPM2 which recognizes a group of related M-phase phosphorylated proteins (Davis *et al.* 1983). A representative result is shown in Fig. 4B. In injected cells that had been stained with Alexa Fluor 568 (IgG), the frequency of cells stained with the mAb, MPM2 (indicated by a yellow



**Figure 3** Ran-GEF activity was inhibited by adenine and 2'-deoxynucleosides. The indicated concentration of recombinant RCC1 was mixed with Ran-[<sup>3</sup>H] GDP (final concentration 50 nM), and then incubated at 37 °C for 20 min in the presence of 5.0 mM caffeine (●), 2'-dA (■), 2'-dG (□), 2'-dT (▲), 2'-dC (△), 2'-dI (◆), 2'-deoxyribose (◇), adenine (▼), adenosine (▽) and as a control, buffer alone (○). The remaining radioactivity on Ran was estimated by liquid scintillation counting. The vertical axis shows the ratio (percentage) of radioactivity remaining on Ran after incubation, compared with the value incubated in buffer without RCC1.

arrow) was determined (Fig. 4C). Previously, we reported that 1.7 mg/mL of RCC1 did not inhibit caffeine-induced PCC in BHK21 cells arrested with HU (Seino *et al.* 1991). Consistent with this, 3 mg/mL of RCC1 only partially inhibited PCC; however, when an increasing dose of wild-type RCC1 was microinjected, the frequency of PCC was clearly reduced by the microinjected RCC1. Compared to the case of injection with the wild-type RCC1, PCC was not strongly inhibited when the mutated RCC1, D182, was microinjected into HU arrested BHK21 cells.

### ATR, but not ATM, is involved in caffeine-induced PCC

Caffeine has been reported to be able to inhibit the protein kinase activity of ATM and ATR, both of which play an important role in cell-cycle checkpoint control (Abraham 2001; Osborn *et al.* 2002). According to these previous reports, it is possible that caffeine induces PCC in HU arrested BHK21 cells through the inactivation of either ATM or ATR. Both ATM and ATR kinases belong to the family of PI3 kinases (Fruman *et al.* 1998), a specific inhibitor of which is wortmannin (Wymann

*et al.* 1996). Thus, if either ATR or ATM kinases are the only targets of caffeine, wortmannin should also induce PCC in BHK cells arrested with HU. To address this question, wortmannin and as a control, caffeine, were added to cultures of BHK21 cells arrested in the S-phase with HU.

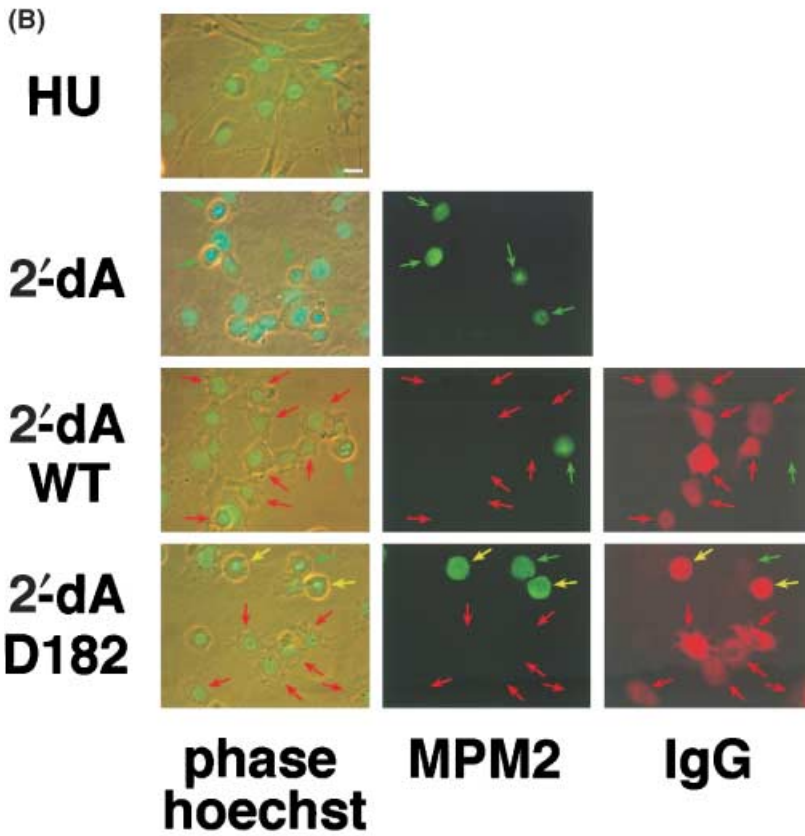
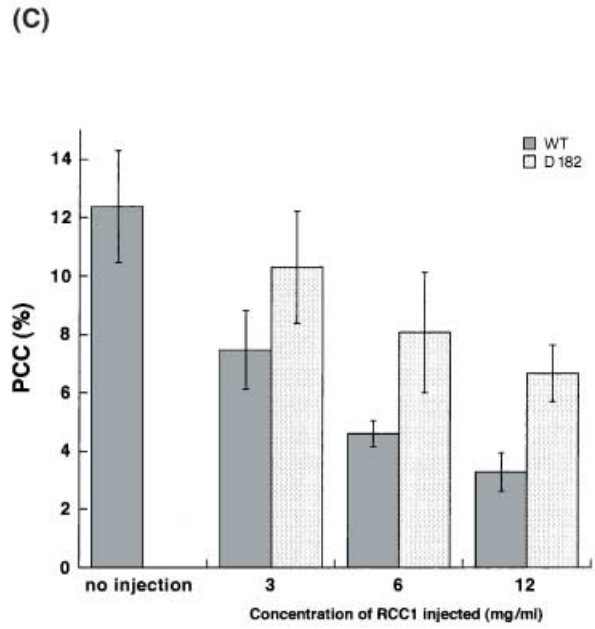
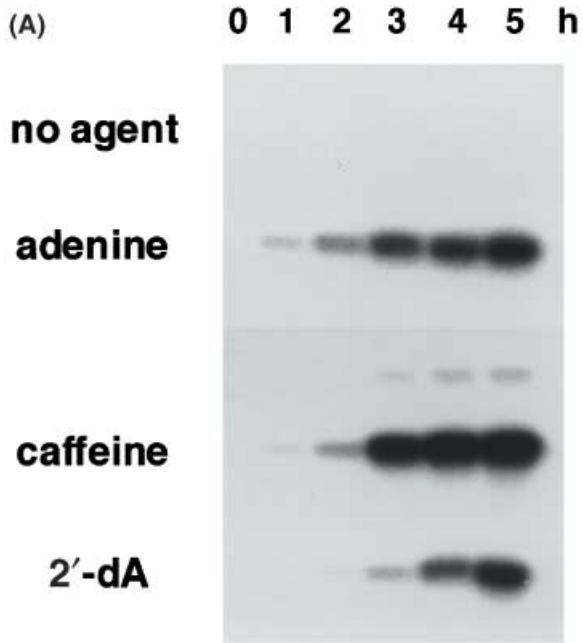
As previously reported (Schlegel & Pardee 1986), PCC was induced in HU arrested BHK cells by the addition of 5 mM caffeine (Fig. 5, column 2, PCC ratio). Consistent with this, the activity of MPF, which was estimated by the phosphorylation of histone H1, was increased by the addition of caffeine (Fig. 5, column 2, H1 kinase activity). In contrast, wortmannin neither induced PCC nor activated histone H1 kinase activity when added to HU arrested BHK21 cells (Fig. 5, columns 3 and 4). Even at a higher dose of 150  $\mu$ M wortmannin (Fig. 5, column 4), which completely inhibits the kinase activity of ATM, but only partially inhibits ATR kinase *in vitro* (Sarkaria *et al.* 1998), histone H1 kinase activity did not increase. Since PI3 kinases are involved in the regulation of cell growth and survival, metabolism, transcription, vesicular trafficking and cytoskeletal organization (Fruman *et al.* 1998), it was thought that wortmannin would be able to inhibit the activation of histone H1 kinase. However, even in the presence of 150  $\mu$ M wortmannin, caffeine activated the MPF and induced PCC in HU arrested BHK21 cells (Fig. 5, column 6, H1 kinase activity and PCC ratio). Based on these results, we excluded the possibility that ATM is involved in caffeine-induced PCC. The inhibition of ATR should therefore be important for PCC induction.

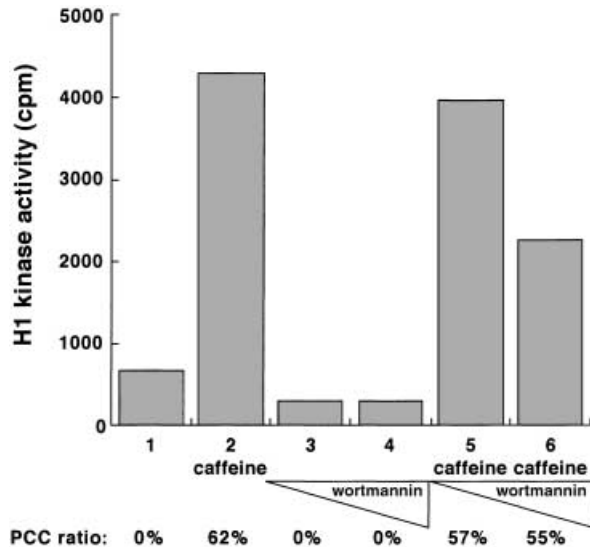
We next addressed the question of whether adenine and 2'-dA could inhibit the protein kinase activity of ATR. To do that, FLAG-tagged wild-type ATR (ATRwt) or kinase-inactive ATR (ATRkd) cDNA

were expressed in 293T cells and then immunoprecipitated using the anti-FLAG antibody. As a control, proteins derived from the extracts of cells expressing either FLAG-tagged ATRwt or ATRkd were also immunoprecipitated using the anti-GST mAb. The kinase activity of immunoprecipitated proteins was assayed using PHAS-1 peptide as a substrate. After incubation at 30 °C for 25 min in the absence of any agents (Fig. 6, no agent), proteins immunoprecipitated from 293T cells expressing FLAG-tagged ATRwt with the anti-FLAG mAb, significantly phosphorylated PHAS-1 peptide (Fig. 6A, right lane of no agent in ATRwt), whereas proteins immunoprecipitated with the control anti-GST mAb did not show any phosphorylation of it (Fig. 6A, left lane of no agent in ATRwt). Proteins which were immunoprecipitated from 293T cells expressing FLAG-tagged ATRkd, either with the anti-FLAG mAb or the control anti-GST mAb, did not phosphorylate PHAS-1 peptide (Fig. 6A, both lanes of no agent in ATRkd). Our kinase assay of immunoprecipitated proteins therefore specifically shows the kinase activity of ATR.

As previously reported, 5 mM caffeine efficiently inhibited the kinase activity of ATR. When the kinase assay was performed in the presence of 5 mM of adenine and 2'-deoxynucleosides, both adenine and 2'-dA, but not the other 2'-deoxynucleosides, inhibited the kinase activity of ATR, similar to caffeine. In order to confirm the present results, the kinase assay was carried out repeatedly. The obtained kinase activities are shown in Fig. 6B as arbitrary units compared to the value of the kinase activity of proteins immunoprecipitated using the control anti-GST mAb from 293T cells expressing ATRwt, which had been assayed in the absence of any agents (no agent). These results indicated that, in addition to caffeine, both adenine and 2'-dA inhibited the kinase activity of ATR.

**Figure 4** Adenine and 2'-dA induced PCC in BHK21 cells arrested in the S-phase. (A) Premature activation of MPF. Cultures of BHK21 cells ( $1.5 \times 10^6/100$  mm dish) were synchronized in the S-phase as described in Experimental procedures at 37.5 °C. After the addition of 5 mM adenine, caffeine or 2'-dA (2'-dA), and as a control, buffer alone (no agent), cells were collected every hour, washed with TD and then with EB buffer. p34<sup>cdc2</sup>/cyclin B1 complexes of S100 extracts prepared from collected cells were immunoprecipitated using the anti-cyclin B1 antibody and assayed for histone H1 kinase activity using [ $\gamma$ -<sup>32</sup>P]. Phosphorylated histone H1 was resolved by SDS-PAGE and visualized by autoradiography. (B) and (C) Microinjection of RCC1. An indicated amount of RCC1 was mixed with gelatin to adjust the final protein concentration to 12 mg/mL, and then with 0.4 mg/mL of rabbit IgG (injection marker). RCC1-mixtures were injected into BHK21 cells arrested with HU, as described (Ohba *et al.* 1996). After the injection, cells were incubated in HU-medium containing 5 mM 2'-dA for 5 h at 37 °C, then fixed with cold methanol (-20 °C). Fixed cells were stained initially with the mAb, MPM2, and then with Alexa Fluor 568-conjugated goat anti-rabbit IgG antibody (red) and then with Alexa Fluor 488-conjugated goat anti-mouse IgG (green). (B) Representative staining results. HU: cells with neither injection nor addition of an agent. 2'-dA: cells were given 2'-dA, but no injection. 2'-dA/WT: cells were injected with 12 mg/mL of wild-type RCC1 and then given 2'-dA. 2'-dA/D182: cells were injected with 12 mg/mL of mutated RCC1, D182, and then given 2'-dA. Cells demonstrated by phase-contrast were stained by Alexa Fluor 568-conjugated goat anti-rabbit IgG antibodies (IgG) and Alexa Fluor 488-conjugated goat anti-mouse IgG (MPM2), as indicated. The yellow arrows indicate the injected cells showing PCC. (C) The vertical axis shows the frequency (percentage) of cells stained with the mAb, MPM2, among the injected cells. As a control, the PCC-frequency of no injected cells is also shown. More than 400 cells were counted.





**Figure 5** Caffeine, but not wortmannin, prematurely activates MPF in BHK21 cells arrested with HU. BHK21 cells synchronized in the S-phase with isoleucine-free medium and then 2.5 mM HU, were incubated at 37 °C for 5 h in fresh-medium containing either caffeine, or wortmannin, or else both, in addition to 2.5 mM HU as indicated. Histone H1 kinase activity and PCC-frequency were determined as described (Nishijima *et al.* 1997). Columns: 1, no drug; 2, 5 mM caffeine; 3, 50 μM wortmannin; 4, 150 μM wortmannin; 5, 5 mM caffeine plus 50 μM wortmannin; 6, 5 mM caffeine plus 150 μM wortmannin. The vertical axis shows the radioactivity (c.p.m.) of the phosphorylated histone H1. Below the columns, the frequency of cells showing PCC is shown as the PCC ratio. More than 600 cells were counted.

### Inactivation of RCC1 abrogated ATR pathway

Taking the above results into consideration, it has become evident that the agents which inhibit both the Ran-GEF activity of RCC1 and the protein kinase activity of ATR can induce PCC in HU arrested BHK21 cells. In this regard, it is curious as to why PCC was induced in tsBN2 cells arrested in the S-phase with HU at the non-permissive temperature (Nishimoto *et al.* 1978; Nishijima *et al.* 1997; Nishitani *et al.* 1991), since this mutant has a single mutation in the *RCC1* gene (Kai *et al.* 1986). In order to address the question of whether or not the inactivation of RCC1 causes the inactivation of the kinase activity of ATR, we examined the phosphorylation of Chk1, which is carried out by ATR and which is recognized as a mobility shift of Chk1 (Feijoo *et al.* 2001).

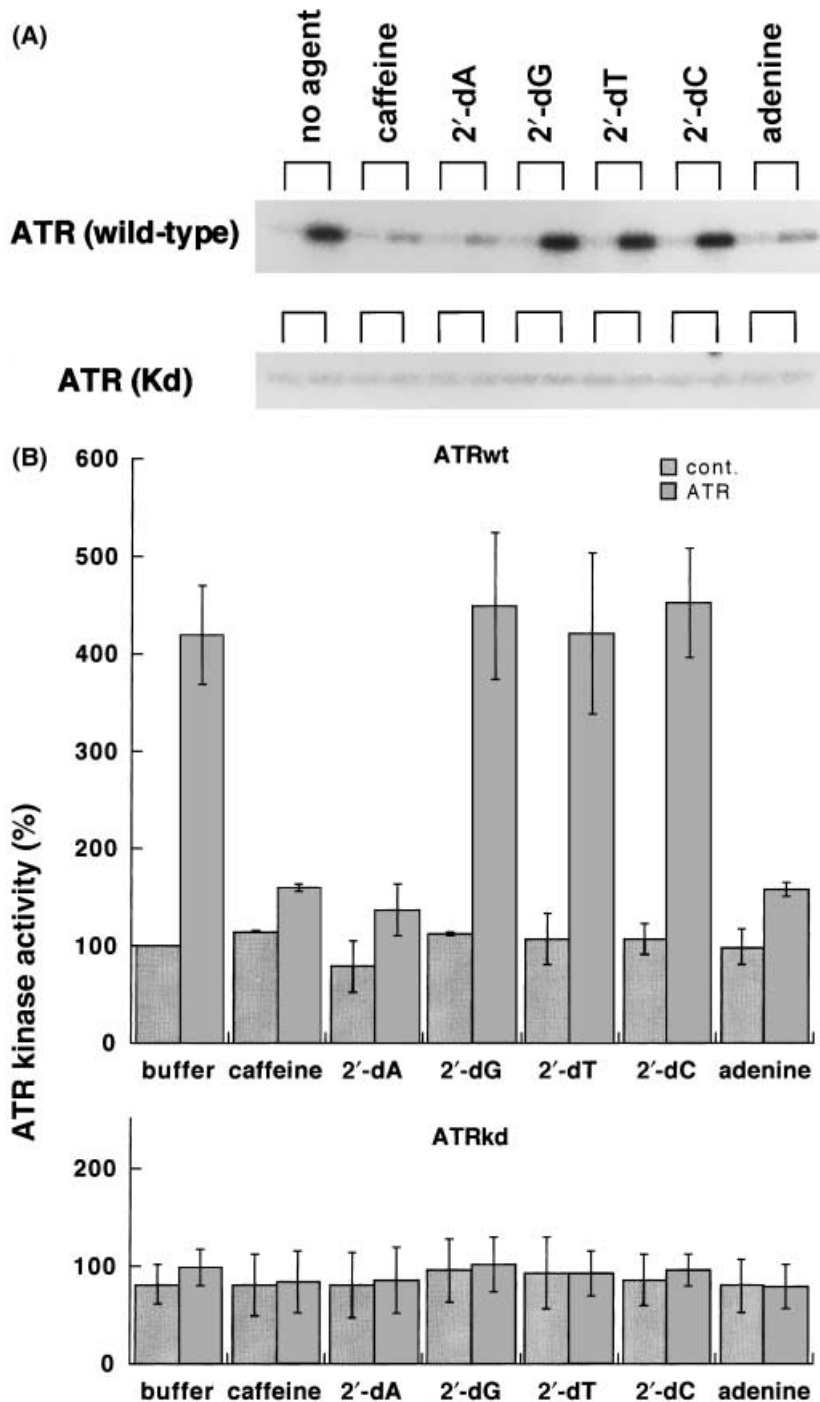
Cultures of tsBN2 and wtBHK21 cells were synchronized in the G1-phase and then incubated in a fresh complete-medium with or without 2.5 mM HU for

16 h. One series of each culture was then incubated as indicated. Every hour, cell-lysates were prepared and analysed with immunoblotting using the anti-Chk1 antibodies (Fig. 7). In the absence of HU, a mobility shift of Chk1 was not observed, but in the presence of HU, the mobility shift of Chk1 was observed in both tsBN2 and wtBHK21 cells (Fig. 7, compare -HU/32 and +HU/32) (a mobility-shifted form is indicated by the arrows in Fig. 7). Such a mobility-shifted form of Chk1 disappeared within 1 h following the addition of caffeine (Fig. 7, +HU +Caf/32), as reported (Feijoo *et al.* 2001). Thus, the mobility-shifted form of Chk1 should actually be the phosphorylated form of Chk1. When a series of cultures of tsBN2 and wtBHK21 cells were incubated at 40 °C in the presence of HU, the mobility-shifted form of Chk1 significantly decreased in tsBN2 cells after 1 h of incubation, when RCC1 was lost due to the tsBN2-mutation (Nishitani *et al.* 1991), but not in wild-type BHK21 cells (Fig. 7, +HU/40). These results indicated that the inactivation of RCC1 abrogated the ATR-pathway.

### Discussion

Since both caffeine and the inactivation of RCC1 cause PCC in hamster BHK21 cells arrested in the S-phase, it has been a long-standing question as to how caffeine is correlated with the function of RCC1. In this present work, we confirmed that caffeine inhibits the Ran-GEF activity of RCC1. Caffeine is 1,3,7-trimethylxanthine, a xanthine derivative. Other xanthine derivatives are theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine), 3-isobutyl-1-methylxanthine and 1,7-dimethylxanthine. With the exception of 3-isobutyl-1-methylxanthine, these other xanthine-derivatives induced PCC in BHK21 cells arrested in the S-phase, either strongly, or else partially (Fig. 2, legend) as previously reported (Schlegel & Pardee 1986). The ability of caffeine and its derivatives to induce PCC was correlated with their ability to inhibit the Ran-GEF activity of RCC1, indicating that RCC1 is a possible target for caffeine. From the viewpoint of cancer chemotherapy, caffeine has been considered to be a potential anti-cancer drug, since it can override cell-cycle checkpoint control, which is weak in most of cancer cells. However, caffeine has a myriad of pharmacological effects (Kihlman 1997). In this regard, we searched for an agent(s) which has the same effect as caffeine on cell-cycle checkpoint control.

It is known that adenine or adenine-containing nucleoside and nucleotide are involved in the biological systems affected by caffeine (Kihlman 1997). As expected, adenine and 2'-dA inhibited the Ran-GEF activity of RCC1 and induced PCC in BHK21 cells arrested in the

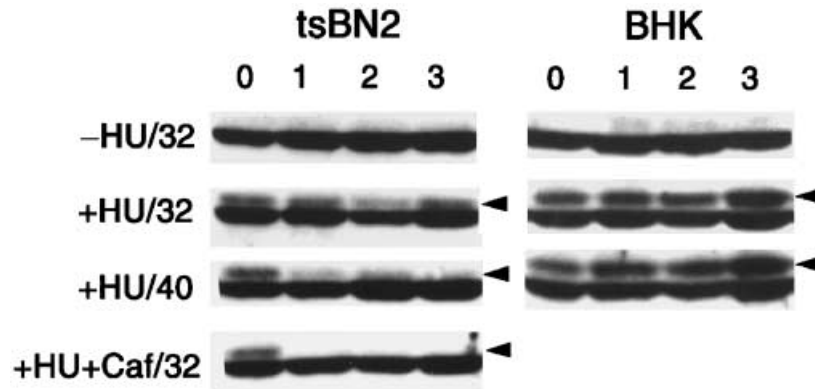


**Figure 6** Inhibition of the kinase activity of ATR. (A) Cultures of 293T cells were transfected with pcDEBA carrying the FLAG-fused ATRwt or ATRkd. Thirty-nine hours later, transfected cells were collected, washed with TD and then lysed in ATR lysis buffer as described in Experimental procedures. Proteins immunoprecipitated with the anti-FLAG M2 mAb (right lane of each panel) or the anti-GST mAb (left lane of each panel) from the S100 extracts derived from transfected cells were assayed for their ability to phosphorylate PHAS-1 using [ $\gamma$ - $^{32}$ P] ATP in the presence or absence of 5 mM of indicated agents. Phosphorylated PHAS-1 was resolved by SDS-PAGE and visualized by autoradiography. (B) The vertical axis shows the ratio (percentage) of radioactivity of PHAS-1 phosphorylated in the presence of indicated agents, compared with the radioactivity of PHAS-1 phosphorylated by proteins immunoprecipitated with the anti-GST mAb, from ATRwt-transfected cells (left column of 'buffer' in the panel of ATRwt).

S-phase. Since PCC induction by 2'-dA was prevented by the microinjection of wild-type, but not mutated, RCC1, it is evident that RCC1 is involved in PCC-induction. However, microinjected RCC1 did not completely inhibit PCC, which was induced by 2'-dA. Furthermore, all of the examined 2'-deoxynucleosides inhibited the Ran-GEF activity of RCC1, but only adenine and 2'-dA acti-

vated MPF in BHK21 cells arrested in the S-phase, resulting in PCC-induction. These results suggest that a factor(s) other than RCC1 is involved in PCC-induction.

Previously, it was reported that caffeine inhibits the kinase activity of both ATM and ATR (Sarkaria *et al.* 1999; Hall-Jackson *et al.* 1999; Blasina *et al.* 1999; Guo *et al.* 2000; Zhou *et al.* 2000; Feijoo *et al.* 2001), which



**Figure 7** Inactivation of RCC1 inhibited the phosphorylation of Chk1. Cultures of tsBN2 and wtBHK21 cells ( $2 \times 10^5$  cells/60 mm dish) were synchronized in the G1-phase and then incubated in fresh medium either containing, or not containing 2.5 mM HU for 16 h. A series of cultures were then incubated in the absence of 2.5 mM HU at 32 °C (-HU/32), in the presence of 2.5 mM HU at 32 °C (+HU/32) or at 40 °C (+HU/40), or in the presence of 2.5 mM HU and 5 mM caffeine at 32 °C (+HU+Caf/32). Every hour, total cell lysates were prepared, subjected to SDS-PAGE, and immunoblotted for Chk1. Arrows indicate phosphorylated Chk1.

play an important role in coupling the completion of DNA replication with the initiation of mitosis (Abraham 2001; Osborn *et al.* 2002). The finding that PCC can be induced by caffeine, even in the presence of 150  $\mu$ M of wortmannin, excluded the possibility that ATM is involved in PCC-induction, since the kinase activity of ATM is reported to be inhibited by 30  $\mu$ M wortmannin (Sarkaria *et al.* 1998). Subsequent to this, adenine and 2'-dA, both of which induced PCC in BHK21 cells arrested in the S-phase, were found to inhibit the kinase activity of ATR, similar to caffeine. Other 2'-deoxynucleosides, which did not induce PCC, did not inhibit the kinase activity of ATR. These results suggest that the inactivation of ATR is essential for PCC-induction.

RCC1 is involved in the nucleocytoplasmic transport of macromolecules which are important for coordinating the components of the cell-cycle (Pines 1999). In this context, microinjected RCC1 probably prevented PCC induction by maintaining the traffic of macromolecules between the nucleus and the cytoplasm. It has been reported that the activation of ATR causes the phosphorylation of Cdc25 through the activation of Chk1 kinase, and then the phosphorylated Cdc25 binds to 14-3-3 proteins, being exported to the cytoplasm (Lopez-Girona *et al.* 1999; Peng *et al.* 1997). Thus, ATR prevents the initiation of mitosis through the nucleocytoplasmic transport, which is consistent with the fact that microinjected RCC1 partially prevented PCC-induction. We showed that the inactivation of RCC1 indeed abrogates the ATR pathway *in vivo*, by using the tsBN2 cell line, a temperature-sensitive mutant of the BHK21 cell line, which has a defect in the *RCC1* gene (Kai *et al.* 1986). The inhibition of Chk1 phosphorylation by the

inactivation of RCC1 was delayed, compared to the addition of caffeine. When shifted down to 33.5 °C within 1 h of incubation at 40 °C, PCC induction was inhibited in tsBN2 cells (Nishimoto *et al.* 1981). The residual activity of RCC1 in tsBN2 cells probably delays the inhibition of Chk1 phosphorylation at 40 °C.

It is not yet known how the inactivation of RCC1 abrogates the ATR pathway. The most intriguing possibility is that the ATR-complexes on the chromatin, which are formed following DNA damage or by the inhibition of DNA-replication (Abraham 2001; Osborn *et al.* 2002), contain RCC1. The inactivation of RCC1 could thus disrupt the protein-complexes comprised of ATR, RCC1 and other unknown proteins, thereby inhibiting the activation of ATR. From the view point of that RCC1 is involved in the Ran-dependent nucleocytoplasmic transport, it is possible that abolishing the nucleocytoplasmic transport, which was caused by the inactivation of RCC1, inhibits the activation of ATR. If Ran is required for recruiting ATR on damaged DNAs or on blocked DNA-replication forks, the inactivation of RCC1 could inhibit the activation of ATR through nucleocytoplasmic transport. It is also possible that a defect in the nucleocytoplasmic transport leads to the abrogation of the checkpoint signal from ATR to Chk1. To answer these questions, we need an agent, which inhibits the kinase activity of ATR alone at a reasonable dose, because a high dose of wortmannin (which almost kills cells) is required in order to inhibit the kinase activity of ATR (Sarkaria *et al.* 1998). Consistent with our results, Sarkaria *et al.* (1998) reported that a low dose of wortmannin (30  $\mu$ M) is unable to affect the histone H1 kinase activity.

The finding that adenine and 2'-dA inhibit ATR kinase is very important. It paves the way for the development of a new anti-cancer drug. Normally, 2'-dA, which is derived from degraded DNA and dietary sources, is rapidly metabolized by adenosine deaminase (ADA) (Wakade *et al.* 1995). Such a metabolism of 2'-dA may cause a lower frequency of PCC which was induced by 2'-dA, compared to the case of caffeine-induced PCC (compare Figs 4 and 5). In the absence of ADA, the concentration of 2'-dA increases in cells, resulting in cell-killing, as if in a genetic disorder responsible for severe combined immunodeficiency syndrome (SCIDS) (Giblett *et al.* 1972). Wakade *et al.* suggested that the cellular effect of 2'-dA is caused by the formation of dATP (Wakade *et al.* 1995). The metabolite responsible for the inhibition of thymocyte development and neuronal apoptosis may therefore not be adenosine or 2'-dA, but a phosphorylated derivative of adenosine or 2'-dA. The correction of ADA-deficient foetal thymic organ cultures by adenosine kinase inhibitors is reported to be correlated with the reduced accumulation of dATP (Wiele *et al.* 2002). Even in cultured BHK21 cells, adenosine is toxic for cell proliferation (Mittal *et al.* 2000). The toxic effect of adenosine is not rescued by the inhibitors of the adenosine receptor, but is modulated by a defect in adenosine kinase (Mittal *et al.* 2000). Taken together, these results suggest that adenine and 2'-dA might induce PCC in HU arrested BHK21 cells by being metabolized to dATP. The cellular concentration of dATP is strictly regulated. Even so, the concentration of dATP may transiently increase following completion of the S-phase. Such a transient increase in cellular dATP concentration may inactivate the kinase activity of ATR, thereby triggering the initiation of mitosis in the normal cell cycle. In this context, we propose that caffeine mimics an *in vivo* effect of adenine and 2'-dA, which is finally metabolized into dATP. We are currently investigating whether dATP inhibits the kinase activity of ATR and whether the concentration of dATP increases upon the completion of DNA replication.

## Experimental procedures

### Cells and cell culture

The hamster wild-type and temperature-sensitive BHK21 cell lines wtBHK21 and tsBN2, and human 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS) in a humidified atmosphere containing 10% CO<sub>2</sub>, at 32 °C for tsBN2 and at 37.5 °C for the other cell lines. For synchronization in the S-phase, cells were cultured in isoleucine-free medium for 28 h, and then cultured in normal medium containing 2.5 mM HU for 16 h as previously described

(Nishijima *et al.* 1997). For tsBN2 cells 40 °C was used as the non-permissive temperature.

### Analysis of guanine nucleotide-exchange reaction on Ran by RCC1

Ran and RCC1 were purified as previously described (Azuma *et al.* 1996; Dasso *et al.* 1994). Highly purified recombinant human Ran was mixed with [<sup>3</sup>H]GDP or fluorescent GDP analogue: 2',3'-bis-O-(*N*-methylantraniloyl) guanosine diphosphate (mantGDP) (Molecular Probes Inc.) in a buffer containing 20 mM Tris (pH 7.4), 25 mM NaCl, 2 mM DTT, 1 mM CHAPS and 10 mM EDTA. After incubation at 37 °C for 20 min, Ran-[<sup>3</sup>H]GDP or Ran-mantGDP was collected through a PD10 column (Amersham Pharmacia Biotech) equilibrated with GEF buffer (20 mM Tris (pH 7.4), 25 mM NaCl, 20 mM MgCl<sub>2</sub> and 1 mM CHAPS).

RCC1 was pre-incubated in GEF buffer either with or without the indicated agent at 37 °C for 10 min, and the reactions were started by the addition of Ran-[<sup>3</sup>H]GDP or Ran-mantGDP. The mixtures were incubated in GEF buffer containing 5 mM GTP. Reactions were stopped by the addition of an ice-cold stop buffer (20 mM Tris (pH 7.5), 25 mM MgCl<sub>2</sub> and 100 mM NaCl). The mixture was then filtered through a nitrocellulose filter (0.45 µm, NC45, Schleicher & Schuell) in order to estimate the [<sup>3</sup>H]GDP remaining on the Ran, and the intensity of fluorescence emission at 450 nm, excited at 355 nm, measured in the time-drive mode of a fluorescence photometer, LS50B (Perkin-Elmer), in order to estimate the amount of mantGDP remaining on Ran.

### Biosensor analysis

Real-time interaction analysis of the binding between RCC1 and Ran, either in the presence or absence of 10 mM caffeine was carried out using a BIAcore biosensor instrument (Biacore), as previously described (Azuma *et al.* 1999).

### Assay of histone H1 kinase activity and PCC-induction

Cells arrested in the S-phase were incubated in fresh medium containing 2.5 mM HU and the indicated dose of agents. After incubation for the indicated time, cells were collected, washed with TD (136.8 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>PO<sub>4</sub>, and 25 mM Tris (pH 7.4)), and then with EB buffer (40 mM HEPES (pH 7.4), 50 mM KCl, 50 mM β-glycerophosphate, 15 mM EGTA and 10 mM MgCl<sub>2</sub>), before being disrupted in 0.5 × EB buffer containing 5 µg/mL cytochalasin B, 5 mM DTT, mixtures of protease inhibitors, and 0.5% NP40, by pipetting and vortexing. Total cell-lysates were centrifuged for 30 min at 100 000 g. The anti-cyclin B1 antibody (Santa Cruz Biotechnology, GNS1) was added to the supernatant, which was then incubated for 1 h on ice, and then the protein G beads were added.

The resulting mixtures were rotated for 1 h. Beads were spun down, washed with buffer containing 60 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>, 250 mM NaCl, 15 mM MgCl<sub>2</sub>, 1% Triton X-100, 1 mM DTT and 40 mM HEPES (pH 7.5), and then with histone H1 kinase buffer containing

20 mM HEPES (pH 7.5), 15 mM EGTA, 1 mM DTT and 20 mM MgCl<sub>2</sub>. The resulting immunoprecipitates were incubated with histone H1 (4 µg/30 µL) in the presence of 3 µCi of [ $\gamma$ -<sup>32</sup>P] ATP (final ATP concentration 5 µM). <sup>32</sup>P-labelled histone H1 was resolved by SDS-PAGE and its radioactivity was calculated by a liquid scintillation counter.

BHK21 cells cultured on a coverglass, which had been arrested in the S-phase, were given fresh medium with or without 0.4 µg/mL nocodazole, plus 40 mM HEPES (pH 7.2) and 2.5 mM HU. They were then microinjected with RCC1 mixtures. After incubation at 37 °C for 5 h in the presence of 2'-dA, the injected cells were fixed with cold methanol (-20 °C), stained initially with the mAb, MPM2 (Davis *et al.* 1983), and then with Alexa Fluor 568-conjugated goat anti-rabbit IgG antibodies (Molecular Probes) and Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes).

### ATR kinase assays

Both wild-type (wt) and kinase-dead (kd) ATR fused to FLAG-tags at the N-terminus were digested-out with the restriction enzyme, *Bam*HI from p<sub>tet</sub>-FLAG-ATRwt and p<sub>tet</sub>-FLAG-ATRkd (Cilby *et al.* 1998), and then inserted into the *Xho*I site of pcDEBΔ. The orientation of each insert relative to the promoter was confirmed by DNA sequencing. 293T cells (3 × 10<sup>6</sup>) were transfected with 18 µg of the resulting FLAG-fused ATRwt or ATRkd plasmid by using *TransIT*<sup>®</sup>-293 (Mirus Corp.). Thirty-nine hours after transfection, the cells were collected, washed with TD and then disrupted in ATR-lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP40, 5 mM EDTA, 10% glycerol, 1 µM microcystin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM β-glycerophosphate, 1 mM NaF, 2 mM DTT and a mixture of protease inhibitors) (Zhao & Piwnicka-Worms 2001). Lysates were centrifuged at 100 000 *g* for 30 min. The resulting supernatant, representing 6.3 mg of total cell protein, was incubated with the anti-FLAG M2 mAb (Sigma F-3165) or the anti-GST mAb (Santa Cruz Biotechnology, SC-138) for 1 h on ice, and then mixed with protein G beads for 1 h by rotation. The beads were spun down. Precipitates were washed three times with ATR-lysis buffer, and then with incomplete kinase buffer (20 mM HEPES (pH 7.4), 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 50 mM NaCl and 1 mM DTT) (Zhao & Piwnicka-Worms 2001). Kinase assays (40 µL) were performed in incomplete kinase buffer supplemented with 10 µM ATP, 1 µM microcystin, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>, 10 µCi of [ $\gamma$ -<sup>32</sup>P] ATP and 25 µg/mL of PHAS-1 peptide as a substrate (Guo *et al.* 2000). After incubation at 30 °C for 25 min, kinase reaction was stopped by boiling in SDS-sample buffer, and then resolved by SDS-PAGE. Radiolabelled proteins were visualized and analysed by using BAS 2000.

### Immunoblotting

Total cell lysates (25 µg) were subjected to SDS-PAGE, optimized to resolve modified forms of Chk1 as described (Feijoo *et al.* 2001), transferred to nitrocellulose, and detected with the anti-Chk1 antibodies using HRP-conjugated rabbit anti-sheep antibody (ZYMED) and enhanced chemiluminescence (ECL; Amersham Biosciences).

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