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Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates *cdc2*/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells

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When BHK21 cells synchronized in early S phase were exposed to okadaic acid (OA), an inhibitor of protein phosphatases 1 and 2A, mitosis specific events such as premature chromosome condensation, the production of MPM-2 antigens, dispersion of nuclear lamins and the appearance of mitotic asters were induced, and then disappeared upon further incubation. These mitosis specific events occurred even in the presence of cycloheximide. Within 1 h of exposure to OA, *cdc2*/histone H1 kinase activity rose 10-fold compared with untreated controls, but returned to the control level upon further incubation. Using antibodies against either p34^{cdc2} or cyclin B it was found that p34^{cdc2} complexed with cyclin B was dephosphorylated after OA treatment concomitant with the activation of *cdc2* kinase, and that cyclin B was subsequently degraded concomitant with a decrease in *cdc2* kinase activity, as in normal mitosis. In contrast, when cells in G_1 phase were treated with OA no increase in *cdc2* kinase activity was observed. Moreover when cells in pseudo-metaphase induced by nocodazole were treated with OA, *cdc2* kinase was inactivated. These results suggest that OA sensitive protein phosphatases control both the activation and inactivation of the p34^{cdc2} kinase.

Key words: BHK21/*cdc2* kinase/cyclin B/okadaic acid

Introduction

Phosphorylation and dephosphorylation of proteins is one of the major regulatory processes in the eukaryotic cell. The mitotic cycle in somatic cells is thought to be controlled by reversible protein phosphorylation. The *cdc2* protein, p34^{cdc2}, has been revealed as the key protein kinase component of M phase promoting factor (MPF) and M phase specific histone H1 kinase (for reviews see Lee and Nurse, 1988; Dorée *et al.*, 1989; Maller *et al.*, 1989; Norbury and Nurse, 1989; Nurse, 1990). The activity of p34^{cdc2} is clearly regulated by phosphorylation. p34^{cdc2} is highly phosphorylated on tyrosine and threonine residues at the end of S phase (Draetta and Beach, 1988; Lee *et al.*, 1988; Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Labbé *et al.*, 1989a; Morla *et al.*, 1989). This phosphorylation correlates with the formation of a complex between p34^{cdc2} and a set of cell cycle regulated proteins, the cyclins (Evans *et al.*, 1983; Draetta *et al.*, 1989; Meijer *et al.*, 1989; Minshull *et al.*, 1989; Murray and Kirschner, 1989; Pines and Hunters, 1989; Gautier *et al.*, 1990, Pondaven *et al.*, 1990). In G_2-arrested oocytes, the inactive hyperphosphorylated form of p34^{cdc2} is found to be associated with cyclin B. This complex is activated by the dephosphorylation of p34^{cdc2} on both tyrosine and threonine residues coupled with phosphorylation of cyclin B at the G_2-M phase boundary (Meijer *et al.*, 1989; Pondaven *et al.*, 1990). Following the degradation of cyclin B, p34^{cdc2} is inactivated and cells exit from mitosis. Thus, the activation of the p34^{cdc2} histone H1 kinase is normally transient and its activity oscillates during the progression of the cell cycle, depending on its phosphorylation state and association with cyclin. Thus, both kinases and phosphatases regulate the activities of the p34^{cdc2} kinase.

Okadaic acid (OA) is a potent protein phosphatase (PP) inhibitor (Takai *et al.*, 1987; Bialojan and Takai, 1988; Haystead *et al.*, 1989) and a tumor promoter (Suganuma *et al.*, 1988). Microinjection of OA into oocytes of *Xenopus* and starfish induces meiotic maturation and MPF activation (Goris *et al.*, 1989; Picard *et al.*, 1989). In the starfish oocyte the histone H1 kinase activity induced by OA was reported to remain high. Contrary to this report, Félix *et al.* (1990) showed that in a cell-free extract of *Xenopus* eggs p34^{cdc2} histone H1 kinase was transiently activated by OA, in a manner similar to the normal cell cycle. Since dephosphorylation of p34^{cdc2} occurred concomitantly with its activation as a protein kinase, these results indicate that at least two kinds of protein phosphatases are involved in the regulation of p34^{cdc2} kinase. The phosphatases that activate p34^{cdc2} by dephosphorylating key tyrosine and threonine residues are clearly insensitive to OA, whereas the premature induction of p34^{cdc2} histone H1 kinase activity by OA suggests that phosphatases which are sensitive to OA, such as PP1 and PP2A, regulate entry into mitosis.

In this paper we show that OA transiently induces the activation of p34^{cdc2} and various mitotic phenotypes such as premature chromosome condensation (PCC) and formation of mitotic asters in intact BHK21 cells, a fibroblast cell line derived from the Golden hamster.

Results

OA induces mitotic phenotypes in BHK21 cells by a post-translational mechanism

BHK21 cells were synchronized in early S phase by sequential isoleucine deprivation and hydroxyurea (HU) treatment (Nishimot *et al.*, 1981) and treated with 0.5 μM OA. One hour later, the OA-treated cells rounded up like mitotic cells and showed chromosome condensation with pulverized

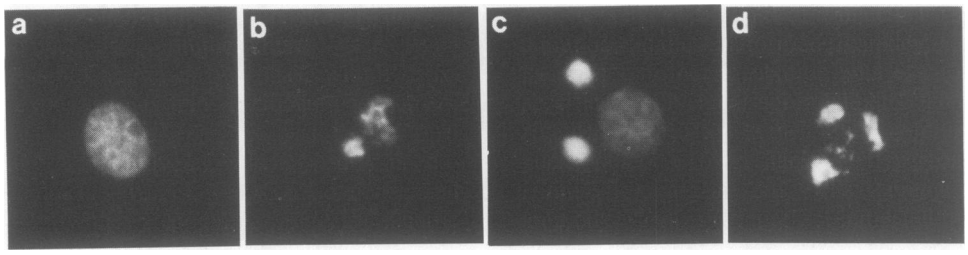

Fig. 1. Figures of PCC induced by OA. Cells synchronized at early S phase by HU were treated with 0.5 μM OA for 0 h (**a**), 1 h (**b**) or 3 h (**c**) or 5 mM caffeine for 3 h (**d**), fixed with methanol and DNA was stained with 1 μg/ml of Hoechst 33258. Optical magnification was × 400.

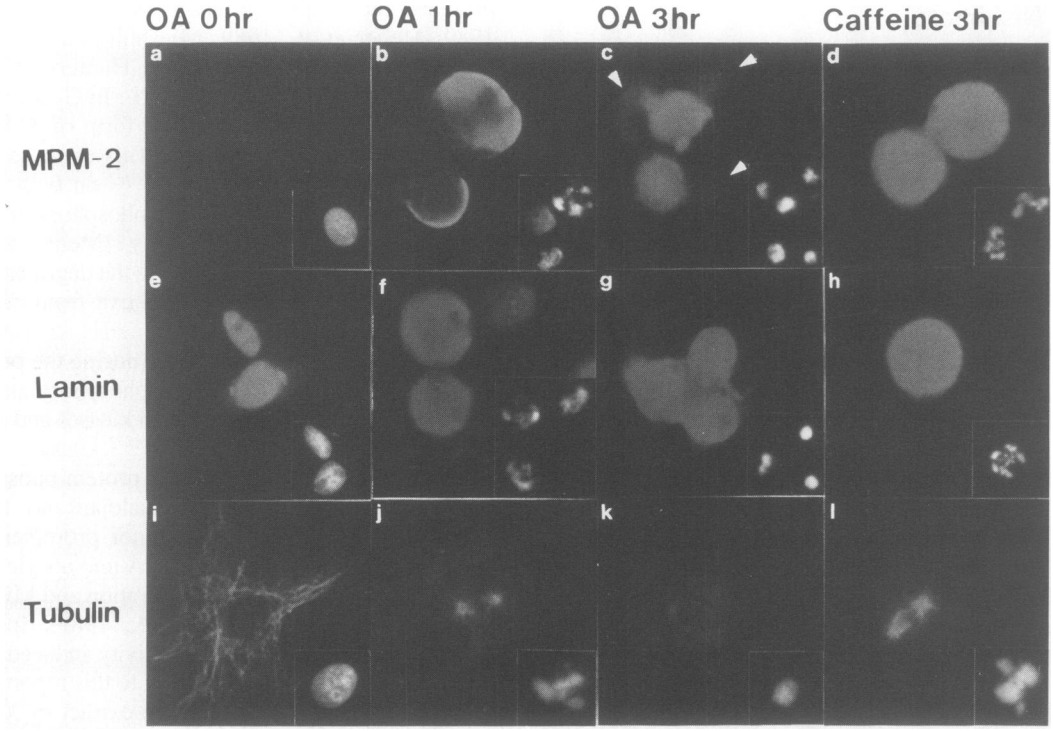

Fig. 2. Indirect immunofluoresence with MPM-2, anti-lamin antibody or anti-β-tubulin antibody. Cells in early S phase were treated with 0.5 μM OA for 0 h (**a**, **e**, **i**), 1 h (**b**, **f**, **j**) or 3 h (**c**, **g**, **k**) or 5 mM caffeine for 3 h (**d**, **h**, **l**), fixed and stained with MPM-2 (**a**, **b**, **c**, **d**), anti-lamin antibody (**e**, **f**, **g**, **h**) or anti-β-tubulin antibody (**i**, **j**, **k**, **l**). Insert in each figure represents DNA stained with Hoechst 33258 of the same cell. Arrowheads indicate hypercondensed nuclei without MPM-2 antigens. Optical magnification was × 400.

nuclei, corresponding to a typical S phase PCC (Figure 1b). Similar phenomena occurred in the *ts*BN2 cell line, which was isolated from BHK21 cells as a temperature sensitive mutant defective in cell growth, after shifting to the non-permissive temperature (Nishimoto *et al.*, 1978, 1981) and in BHK21 cells blocked in early S phase with hydroxyurea (HU) and exposed to caffeine (Schlegel and Pardee, 1986; Figure 1d).

To confirm that OA was inducing mitosis-specific phenomena, the rounded up cells were stained with the monoclonal antibody MPM-2, which recognizes mitosis specific phosphoproteins (Davis *et al.*, 1983; Figure 2a–d), and with antibodies to determine the distribution of nuclear lamins (Figure 2e–h) and β-tubulin (Figure 2i–l). Cells with caffeine-induced PCC were used as the control (Figure 2d, h and l). MPM-2 antigens appeared in a similar manner in both OA-treated cells and in cells blocked with HU and treated with caffeine (Figure 2b and d). The nuclear lamins, which localize specifically in nuclei in untreated cells (Figure 2e), were dispersed into the cytoplasm in cells treated with OA for 1 h (Figure 2f). In OA-treated cells the interphase specific microtubule network (shown in Figure 2i) was disrupted and mitotic asters were observed (Figure 2j), but mitotic spindles did not form. In cells treated with caffeine, a mitotic spindle was formed (Brinkley *et al.*, 1988; Figure 2l). After further incubation with OA (3 h) these mitosis specific phenomena disappeared, although the cells were still rounded and floated in the culture medium; the pulverized nuclei took on a round shape with hypercondensed chromatin (Figure 1c), and in some cells staining with the MPM-2 antibody was lost (Figure 2c, indicated by arrowhead) and the mitotic asters disappeared (Figure 2k). Thus, the OA-induced mitosis specific phenotype in BHK21 cells was transient. Interestingly, the typical interphase microtubule array did not reform but tubulins remained solubilized in the cytoplasm in all the OA-treated cells, in contrast to caffeine-induced PCC or PCC induced in *ts*BN2 cells at the non-permissive temperature (Brinkley *et al.*, 1988; H.Nishitani, in preparation).

The characteristics of PCC induction by OA were

Table I. Independence of PCC induction by OA on DNA synthesis

Cell line	OA (0.5 μM)	Temp. (°C)	Caffeine (5 mM)	Aphidicolin (5 μM)	PCC index[a] (%)
BHK21	+	37.5	−	+	43.4
	+	37.5	−	−	47.7
*ts*BN2	−	40.0	−	+	38.7
	−	40.0	−	−	45.5
BHK21	−	37.5	+	+	40.1
	−	37.5	+	−	6.2

[a]PCC index was determined 3 h after the treatment.

Table II. Cycloheximide resistance of PCC induction by OA

Cell line	OA (0.5 μM)	Temp. (°C)	Caffeine (5 mM)	CH (10 μg/ml)	PCC index[a] (%)
BHK21	+	37.5	−	−	55.9
	+	37.5	−	+	40.4
*ts*BN2	−	40.0	−	−	57.8
	−	40.0	−	+	0.4
BHK21	−	37.5	+	−	53.7
	−	37.5	+	+	6.2

[a]PCC index was determined 3 h after the treatment.

compared with the induction of PCC in *ts*BN2 cells at the non-permissive temperature and in BHK21 cells treated with caffeine (Nishimoto *et al.*, 1981; Schlegel and Pardee, 1986). As shown in Tables I and II, PCC induction by OA or in *ts*BN2 cells did not require the inhibition of DNA synthesis, which is required for caffeine-induced PCC. Remarkably, PCC induction by OA occurred in the presence of cycloheximide, which completely inhibits PCC induction both by caffeine and by temperature shift of the *ts*BN2 cell line. Therefore new protein synthesis is not necessary for PCC induction by OA, and OA-induced chromosome condensation occurs in a purely post-translational fashion.

Transient activation of histone H1 kinase in OA-treated cells

The histone H1 kinase activity of $p34^{cdc2}$ increases dramatically upon entry into M phase (Gautier *et al.*, 1989; Morla *et al.*, 1989). We measured H1 kinase activity in BHK21 cells in response to OA. A 5- to 10-fold increase in histone H1 kinase activity was detected in crude extracts of cells treated with OA at a dose of 0.25 or 0.5 μM for 1 h. After a 2 h incubation, this activity reverted to the control level (Figure 3a). This reduction in histone H1 kinase activity occurred even in the presence of a higher dose (3 μM) of OA, and in these extracts the maximum specific activity of histone H1 kinase was the same as that of cells treated with 0.5 μM OA (~10 pmol phosphate/mg protein/min in crude extracts) (data not shown). This reduction in histone H1 kinase activity was not due to the inactivation of OA since the addition of fresh OA after 1 or 2 h incubation did not prevent reduction of H1 kinase activity (data not shown). As a control no significant activation of H1 kinase was observed when cells were treated with okadaic acid tetramethyl ether, a synthetic inactive derivative of OA (Suganuma *et al.*, 1988). Thus the activation of histone H1 kinase, along with the other mitosis specific phenomena, occurred transiently in BHK21 cells treated with OA (Figures 1 and 2). This is in contrast to what has been shown for starfish oocytes (Picard *et al.*, 1989).

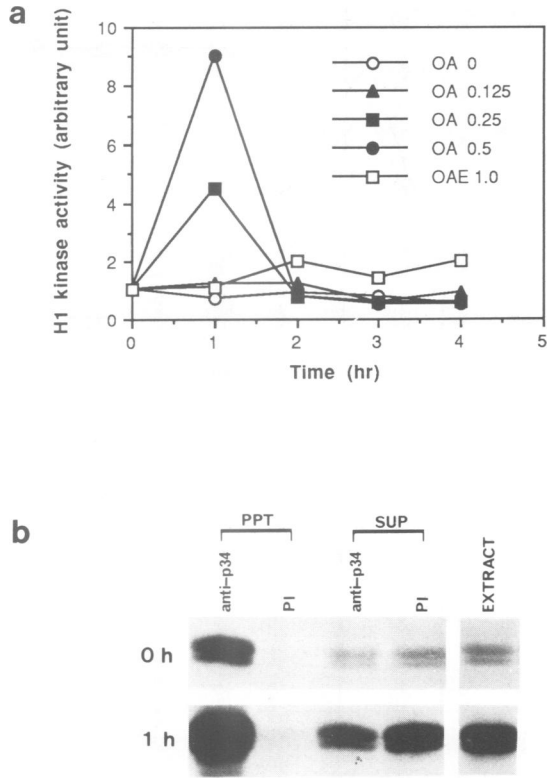

Fig. 3. Histone H1 kinase activity in OA-treated BHK21 cells. (**a**) Plots of the H1 kinase activity in crude extract. Increase in activity over that at time 0 is plotted against the time of treatment (h). The insert indicates the concentration (μM) of OA or OAE (OAE, okadaic acid tetramethyl either; Suganuma *et al.*, 1988) The specific activity of H1 kinase at time 0 was ~1.0 pmol/mg protein/min. (**b**) H1 kinase activities of immunoprecipitates (PPT) by antibody against a 17 amino acid C-terminal peptide of $p34^{cdc2Hs}$ (anti-$p34^{cdc2}$; Lee and Nurse, 1987), preimmune serum (PI), the residual supernatants after immunoprecipitation (SUP) and the crude extract (EXTRACT). The same amount of protein equivalent to that in crude extract (2.5 μg/assay) was used in SUP and EXTRACT. In b, only regions of autoradiograms corresponding to histone H1 are shown.

The histone H1 kinase induced by OA is the cdc2 kinase

The histone H1 kinase activity detected in BHK21 cells treated with OA could be attributed to the activation of $p34^{cdc2}$ by the following experiments. Firstly, we used an antibody raised against the C-terminal peptide (17 amino acids) of the human *cdc2* protein (Lee and Nurse, 1987), which recognizes three forms of the hamster *cdc2* protein (data not shown) and precipitates the *cdc2*−cyclin B complex (Figure 5a). Immunoprecipitates made with this antibody from OA-treated cells had 8-fold higher histone H1 kinase activity than that from untreated cells (Figure 3b), comparable with the difference in histone H1 kinase activity in the crude extracts. The same anti-$p34^{cdc2}$ antibody removed two-thirds of the H1 kinase activity in the crude extract (Figure 3b). Secondly, the histone H1 kinase activity induced by OA could be bound to p13−Sepharose beads and was precipitated by an anti-human cyclin B antibody (data not shown). Finally, the addition of 0.5 μM OA to crude extracts of control cells failed to activate H1 kinase significantly (data not shown). Thus, we concluded that the increase in H1 kinase was due to the change in $p34^{cdc2}$ kinase activity *in vivo*.

The activation of H1 kinase did not require new protein

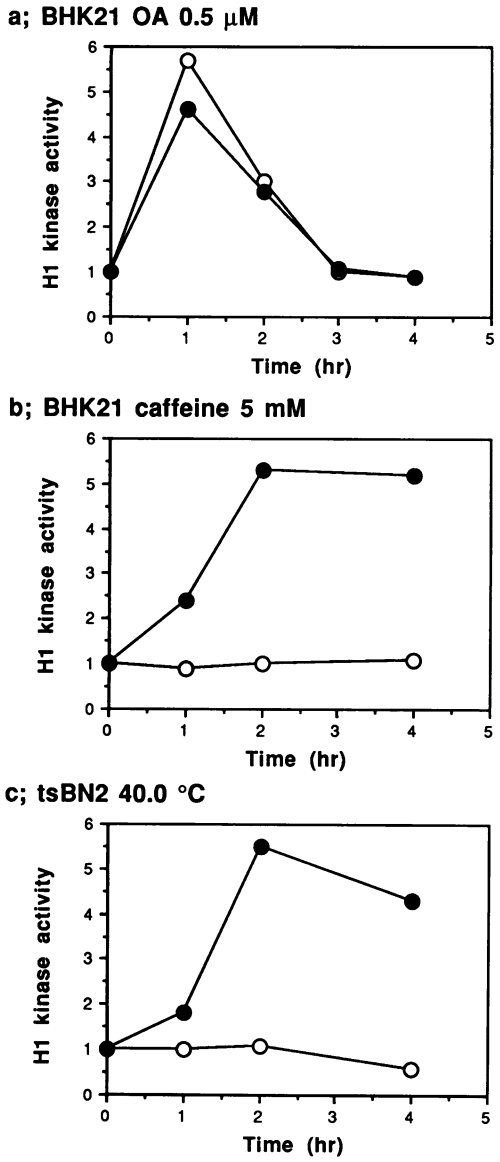

Fig. 4. Cycloheximide resistance of PCC induction and H1 kinase activation in OA-treated BHK21 cells. Relative increases of H1 kinase activity in crude extract (arbitrary unit), of OA—treated (0.5 μM) (**a**) and caffeine-treated (5 mM) (**b**) BHK21 cells or temperature-treated (40.0°C) *ts*BN2 cells (**c**) were shown. Filled circle and open circle indicate the treatment of OA, caffeine or temperature in the absence and presence of 20 μg/ml CH, respectively.

synthesis and occurred in the presence of up to 300 μg/ml of cycloheximide (Figure 4a). By contrast, H1 kinase activation in *ts*BN2 cells at the non-permissive temperature and in BHK21 cells by caffeine (Figure 4b and c) were both inhibited by cycloheximide. Thus, OA activation of the *cdc2* kinase occurred post-translationally.

Complex formation of p34cdc2 with cyclin B

A prerequisite for the activation of $p34^{cdc2}$ is the formation of a complex between $p34^{cdc2}$ and cyclin B. As cells enter mitosis $p34^{cdc2}$ complexed with cyclin B is dephosphorylated by the action of uncharacterized mitotic phosphatases to activate its H1 kinase (Hunt, 1989). In the post-meiotic starfish egg, it is likely that OA can only activate the $p34^{cdc2}$ if cyclin B is present (Picard *et al.*, 1989), since emetine inhibits the OA-induced activation of histone H1 kinase in these eggs. In our experiments, cycloheximide did

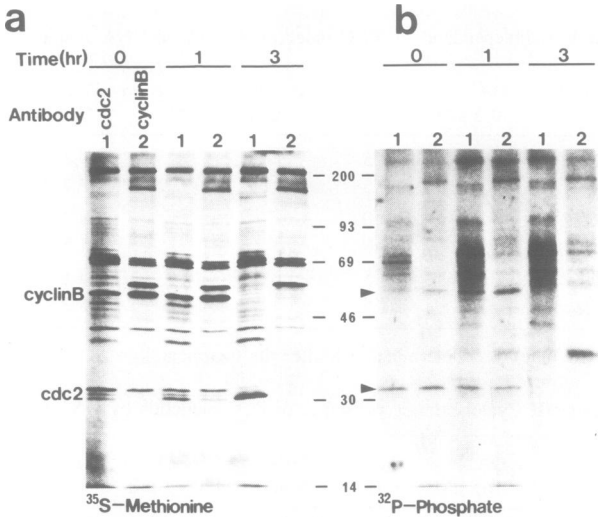

Fig. 5. Immunoprecipitation of extracts from OA—treated BHK21 cells with anti-*cdc2* or anti-cyclin B antibody. Proteins labeled with [^{35}S]methionine (**a**) or [^{32}P]orthophosphate (**b**) were extracted from cells treated with OA for 0, 1 and 3 h indicated in figures as Time (h) and immunoprecipitated with anti-human *cdc2* (lane 1) or anti-human cyclin B (lane 2) and the precipitates were analyzed by SDS–PAGE on 12% gel and fluorography (a; [^{35}S]methionine) or autoradiography (b; [^{32}P]phosphate) with intensifying screen. Films were exposed for 48 h (^{35}S) or 72 h (^{32}P). The same amounts of acid precipitable radioactivity (1×10^7 c.p.m. for [^{35}S]methionine or 1×10^6 c.p.m. for [^{32}P]phosphate) were used for immunoprecipitation.

not inhibit the activation of $p34^{cdc2}$ by OA, indicating that OA acts after cyclin synthesis. To confirm this point, we assayed for a $p34^{cdc2}$–cyclin B complex in BHK21 cells during incubation with OA, by immunoprecipitation using antibodies against human $p34^{cdc2}$ and against human cyclin B (Pines and Hunter, 1989). We also assayed for the incorporation of $^{32}PO_4$ into cyclin B and $p34^{cdc2}$.

We found that cyclin B (apparent M_r 60 000) was already complexed with $p34^{cdc2}$ in BHK21 cells synchronized in early S phase with HU. Cyclin B was associated with the two upper bands of $p34^{cdc2}$, which are its more highly phosphorylated forms (Figure 5a and b). The desphosphorylated $p34^{cdc2}$ began to appear after 1 h and some of the hyperphosphorylated forms of $p34^{cdc2}$ complexed with cyclin B shifted to the fastest migrating position, corresponding to its least phosphorylated state (Figure 5a and b). At the same time we noted an increase in the phosphorylation of cyclin B over that in untreated cells (Figure 5b), consistent with the observation that cyclin B is phosphorylated concomitantly with the dephosphorylation of $p34^{cdc2}$ in the activation of sea urchin M phase specific histone H1 kinase (Meijer *et al.*, 1989). Thus, the activation of $p34^{cdc2}$ as the histone H1 kinase in OA-treated cells is correlated with the presence of a complex between cyclin B and the fastest migrating, underphosphorylated form of $p34^{cdc2}$.

After incubation with OA for 3 h cyclin B was completely undetectable and almost all $p34^{cdc2}$ was dephosphorylated (Figure 5a). Thus, the normal transition from M phase to G_1 phase in terms of the protein kinase activity of $p34^{cdc2}$ and degradation of cyclin B took place during treatment of BHK21 cells with OA.

Effects of OA on cells during the transition from M to G_1 and S phase

The data presented so far indicate that BHK21 cells synchronized in early S phase with HU contain cyclin B

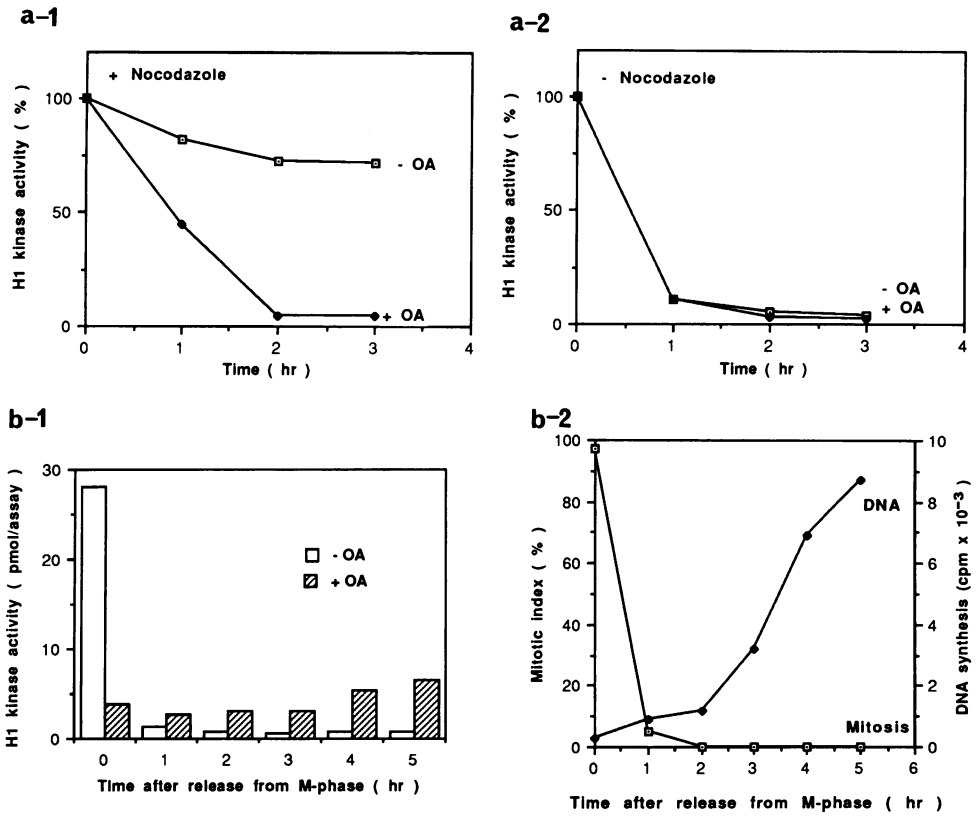

Fig. 6. Effects of OA on H1 kinase activity in cells in M, G_1 or S phases. BHK21 cells synchronized with nocodazole were incubated in the presence (a-1) or absence (a-2) of nocodazole. b-1; H1 kinase activity of anti-*cdc2* immunoprecipitates from cells released from nocodazole block and treated with OA was measured at the indicated times. The H1 kinase activity induced by OA was determined as follows; cells received OA at the indicated times and the extract was prepared 1 h later. B-2; Mitotic index and incorporation of [^{3}H]thymidine. For mitotic index, cells were collected at the indicated times and for the measurement of DNA synthesis cells received [^{3}H]thymidine (0.5 μCi/ml) at the indicated times and were harvested 1 h later.

complexed with p34^{cdc2}, which can be activated by OA as a histone H1 kinase, but it is not clear whether the presence of cyclin B is required for the activation of p34^{cdc2} by OA. To answer this question, we investigated the effect on BHK21 cells of OA added at other phases of the cell cycle.

Nocodazole blocks cells in pseudo-metaphase and stabilizes histone H1 kinase. Cultures of BHK21 cells were blocked in M phase with nocodazole and then incubated in the presence or absence of the drug, with or without the addition of OA. Histone H1 kinase activity was measured after 1, 2 or 3 h. High histone H1 kinase activity persisted in the presence of nocodazole, but rapidly decreased upon addition of OA (Figure 6a-1). Immunoblotting with anti-cyclin B antibody revealed that the addition of OA caused the destruction of cyclin B (data not shown). After the removal of nocodazole, histone H1 kinase activity decreased regardless of the presence or absence of OA (Figure 6a-2). Thus in BHK21 cells, OA did not stabilize histone H1 kinase in M phase, contrary to the previous observation that OA microinjected into starfish oocytes stabilized H1 kinase activity in meiosis (Picard *et al.*, 1989).

After release from a nocodazole block, cells entered G_1 phase within 1 h and DNA synthesis began 3 h later (Figure 6b-2). Every hour, OA was added and the activity of H1 kinase was measured 1 h later (Figure 6b−1). We found that histone H1 kinase activity induced by OA was low in G_1 phase and increased from the beginning of S phase (Figure 6b-1 and b-2). These results correlate with the amount of cyclin B through the cell cycle (Pines and Hunter, 1989; Nishitani, *et al.*, in preparation), supporting our conclusion that cyclin B is required for OA to activate p34^{cdc2}.

Changes in phosphoprotein levels and MPM-2 antigens in OA-treated cells

The inhibition of protein phosphatases by OA was expected to increase phosphorylation of cellular proteins (Haystead *et al.*, 1989). Figure 7a shows that the level of phosphoproteins in cells treated with OA for 2 h was >2.5 times that in control cells, and >4 times that at 0 h, but decreased to the same level as control cells during the next 1 h incubation. The appearance of MPM-2 antigens in OA-treated cells exhibited the same profile as the overall phosphoprotein level; they appeared 1 h after OA addition, remained present for another 1 h and much of the MPM-2 antigens have disappeared at 3 h (Figure 7b and c). Thus, MPM-2 antigens did not remain for very long in OA-treated BHK21 cells.

Discussion

In this study, we demonstrated that p34^{cdc2} was activated in BHK21 cells treated with OA. This agrees with observations in other systems such as *Xenopus* or starfish oocytes, where microinjection of OA induces meiotic maturation and the activation of MPF (Goris *et al.*, 1989; Picard *et al.*, 1989). In a *Xenopus* egg cell-free system, OA activated the *cdc2* kinase (Félix *et al.*, 1990). Recently, Kipreos and Wang (1990) reported that 1 μM OA treatment of interphase

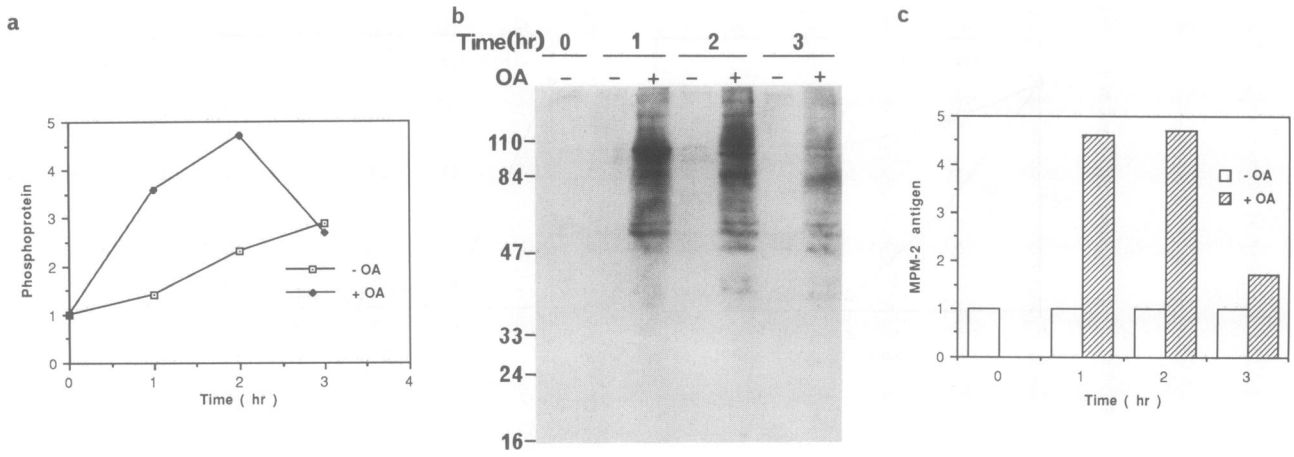

Fig. 7. Effects of OA on the level of phosphoproteins and MPM-2 antigens in cells arrested at the early S phase. (**a**) Cellular phosphoprotein levels determined as described in Materials and methods. Vertical axis represents relative phosphoprotein level. The specific count at time 0 was 1.5×10^6 c.p.m./mg protein. (**b**) Western blotting of proteins from cells treated with (+) or without (−) OA probed with MPM-2 (1: 1,000) followed by ^{125}I-labeled anti-mouse Ig (10 μCi/ml) (Amersham, UK). The film was exposed for 4 h at room temperature. (**c**) the level of MPM-2 antigens. Each lane in b was cut and counted by gamma-counter. Vertical axes represent the relative amounts of MPM-2 antigens measured as above.

NIH3T3 cells induced a rounded phenotype and mitotic phosphorylation of the c-*abl* protein. Lamb *et al.* (1990) reported that the microinjection of active p34^{cdc2} into interphase REF-52 cells induced partial chromosome condensation and reversible and transient reorganization of microtubules. In our results, OA treatment of BHK21 cells induced a number of mitosis specific events such as chromosome condensation, the dispersal of nuclear lamins, the appearance of MPM-2 antigens and the formation of mitotic asters. Of these phenomena, the disappearance of the interphase microtubule network and the formation of mitotic asters have been shown to be closely correlated with the activity of p34^{cdc2} (Verde *et al.*, 1989; Lamb *et al.*, 1990). Recently, nuclear lamins have been shown to be phosphorylated by p34^{cdc2} and disassembled by phosphorylation (Heald and Mckeon, 1990; Peter *et al.*, 1990a; Ward and Kirschner, 1990). Moreover, nucleolin, one of the major nucleolar proteins and a substrate for p34^{cdc2} (Peter *et al.*, 1990b), is phosphorylated in cells treated with OA (Issinger *et al.*, 1988). These results are consistent with our finding that OA activates p34^{cdc2}, thereby inducing mitotic phenomena in BHK21 cells.

Upon addition of OA, p34^{cdc2} was activated, concomitantly with its dephosphorylation and phosphorylation of its associated cyclin B. Cyclins have been suggested to be essential for p34^{cdc2} activation by OA (Picard *et al.*, 1989; Felix *et al.*, 1990). Our results also suggest that the induction of histone H1 kinase activity by OA depends on the amount of cyclin B present in cells, since only a low histone H1 kinase activity could be induced by OA in G_1 phase cells when cyclin B was barely detectable. In BHK21 cells, cyclin B was present in early S phase and its amount increased through S phase (H.Nishitani, in preparation). The activity of p34^{cdc2} kinase induced by OA increased through S phase correlating with the accumulation of cyclin B, supporting the conclusion that the activity of p34^{cdc2} induced by OA depends on the amount of cyclin B. Furthermore, we found that OA treatment of cells synchronized in early S phase induced a level of p34^{cdc2} kinase as high as two-thirds of that detected in M phase. It should be noted that synchronization of BHK21 cells in early S phase with HU or aphidicolin caused the accumulation of cyclin B (H.Nishitani, in preparation), which differs from the situation in HeLa cells (Pines and Hunter, 1989). This difference is probably reflected in our observation that caffeine can induce premature chromosome condensation in BHK21 cells but not in HeLa cells synchronized in early S phase (unpublished results; R.Schlegel, personal communication).

OA is thought to act by inhibiting protein phosphatases, especially PP1 and PP2A, which are two of the major protein phosphatases in eukaryotic cells (Bialojan and Takai, 1988; Cohen, 1989; Cohen and Cohen, 1989; Haystead *et al.*, 1989). In this study, we have no direct evidence that OA inhibited intracellular phosphatases. However, considering the results in Figure 7 that OA caused a rapid rise in cellular phosphoprotein level and increased the intensity of background bands in the ^{32}P-labeled anti-p34^{cdc2} or anti-cyclin B immunoprecipitates in Figure 5, as well as others (Haystead *et al.*, 1989), it is reasonable to suppose that OA inhibited protein phosphatase activities *in vivo* in BHK21 cells. Taking into account the inhibitory dose of OA for several kinds of phosphatases *in vitro* (Bialojan and Takai, 1988; Haystead *et al.*, 1989) and the concentration of OA needed to activate p34^{cdc2} kinase, both PP1 and PP2A could be targets of OA. It has previously been shown that a mutation in a PP1 gene (*bim*G) of *Aspergillus* caused the hyperphosphorylation of MPM-2 antigens (Doonan and Morris, 1989), indicating that this PP1/*bim*G is required for removal of phosphate groups from MPM-2 antigens. The disappearance of MPM-2 antigens in cells incubated for a long time with OA in our experiments might indicate that this type of PP1/*bim*G is active under our experimental conditions. In addition, in a *Xenopus* egg cell-free system 0.5 μM OA mainly inactivated PP2A but not PP1 (Félix *et al.*, 1990) and INH, a negative regulator of MPF activation in *Xenopus* oocytes (Cyert and Kirschner, 1988), probably corresponds to PP2A (Lee *et al.*, 1990). These observations suggest that PP2A is a negative regulatory

component in the activation of p34^{cdc2}, but from our results we cannot exclude the possibility that both PP2A and another type of PP1 different from *bim*G are involved in the activation of p34^{cdc2}.

p34^{cdc2} activated by OA in BHK21 cells was dephosphorylated even in the presence of OA. In addition, vanadate, an inhibitor of phosphotyrosine phosphatases, inhibited the activation of p34^{cdc2} by OA (unpublished results). Thus, at least three kinds of protein phosphatases appear to be involved in the activation of p34^{cdc2}. One is an OA sensitive phosphatase (probably PP2A, a negative regulator) and the other two phosphatases are uncharacterized OA insensitive phosphatases acting as phosphotyrosine and phosphothreonine phosphatases to activate p34^{cdc2} directly (Dorée *et al.*, 1989; Hunt, 1989; Félix *et al.*, 1990).

There are some discrepancies between our results with BHK21 cells and previous observations on starfish oocytes (Picard *et al.*, 1989). Firstly, we found OA treatment induced the destruction of cyclin B and thus the destabilization of p34^{cdc2} kinase activity in BHK21 cells arrested by nocodazole in M phase, whereas OA microinjected into oocytes stabilized histone H1 kinase activity. In a *Xenopus* egg cell-free system undergoing mitosis, OA also did not stabilize the *cdc2* kinase activity nor cyclin B, rather it accelerated cyclin degradation (Félix *et al.*, 1990). These results imply that mitosis differs from meiotic maturation, in that OA sensitive phosphatases are involved not only in the activation but also the inactivation of p34^{cdc} kinase activity. In mitosis, OA sensitive phosphatases may suppress the cyclin proteolysis pathway. Secondly, OA induced microtubule reorganization and the formation of mitotic asters in BHK21 cells, whereas in starfish oocytes OA stabilized the interphase microtubule network (Picard *et al.*, 1989). Microtubule dynamics are influenced by p34^{cdc2} and the balance between p34^{cdc2} activity and phosphatases, especially PP1, is thought to regulate microtubule stability (Verde *et al.*, 1990). Thus far we are unable to explain the contradictory results from starfish oocytes and BHK21 cells. If OA induces a state in which both activities of another type of PP1 than *bim*G and PP2A are inactivated and p34^{cdc2} is active, the formation of asters could be observed in the presence of OA. In addition, there may be a stockpile of maternally provided cellular components such as for microtubule organization in oocytes and eggs but not in somatic cells. Thus these discrepancies are probably caused by the differences between meiosis and mitosis in early development, and mitosis in somatic cells. An *in vitro* system is needed to understand the precise control mechanisms for microtubule dynamics in somatic cells.

The activation of p34^{cdc2} by OA is clearly a post-translational event, whereas in the case of the *ts*BN2 mutant or caffeine, protein synthesis is necessary to activate p34^{cdc2} (Nishimoto *et al.*, 1981; Schlegel and Pardee, 1986; results in this report). Thus, in effect the *ts*BN2 mutation or caffeine seems to produce a protein(s) that has an activity equivalent to OA. In all three cases, mitotic phenotypes are induced without the completion of DNA synthesis. The clarification of the relationship between the newly synthesized protein(s) and the activity of PP2A will provide clues to understanding not only the role of the new factor(s) in the activation of p34^{cdc2}, but also the signal(s) from the completion of DNA replication which initiates mitosis.

Materials and methods

Cell synchronization and labeling

BHK21 cells and its temperature sensitive mutant, *ts*BN2, were cultured and synchronized by isoleucine deprivation and HU as described (Nishimoto *et al.*, 1981). For PCC induction, BHK21 cells were treated with OA or 5 mM caffeine at 37.5°C or *ts*BN2 cells at 40.0°C. Mitotic cells were obtained by nocodazole block (0.2 μg/ml) for 18 h after isoleucine deprivation at 37.5°C and by gentle pipetting. The mitotic index of cells treated in such a way was usually >95%. Mitotic or PCC index was determined as described (Nishimoto *et al.*, 1981). For *in vivo* labeling of proteins with [^{35}S]methionine (100 μCi/ml) or [^{32}P]orthophosphate (1 mCi/ml) (both from Amersham, UK), cells were washed twice with methionine- or phosphate-free medium and labeled for 3 h with the medium as described (Pines and Hunter, 1989).

Indirect immunofluoresence

Cells grown on coverslips were washed with PBS and fixed with 3.7% formaldehyde in PBS and processed for indirect immunofluorescence as described (Riabowol *et al.*, 1989) using monoclonal MPM-2 (a gift from Dr Rao) and rabbit anti-lamin (a gift from Dr Gerace) as primary antibodies and rhodamine-labeled goat anti-mouse or anti-rabbit antibodies (TAGO, USA), respectively, as second antibodies. Cells floating in medium or rounded cells loosely attached to dishes were collected by pipetting and then attached to coverslips coated with poly-L-lysine (Sigma, USA) (Harlow and Lane, 1988) and treated as described above. For examination of microtubules, cells were treated with microtubule stabilizing buffer and fixed with methanol at −20°C (Pachter *et al.*, 1987) and stained with monoclonal anti-β-tubulin (purchased from Amersham, UK). DNA was visualized with 1 μg/ml of Hoechst 33258 (Calbiochem, USA). Photomicroscopy was done using Axiophot (Zeiss, FRG).

Preparation of cell extract and immunoprecipitation

Cells collected by scraping or by pipetting were washed with ice-cold Tris-buffered saline, lysed by adding 100 μl per 2 × 10^5 cells of the buffer containing 40 mM HEPES−NaOH pH 7.5, 60 mM 2-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.5 mM Na_3VO_4, 250 mM NaCl, 15 mM $MgCl_2$, 1% Triton X-100, 10 μg/ml each of aprotinin, leupeptin, pepstatin and antipain−HCl and 0.5 mM (*p*-amidinophenol) methanesulfonylfluoride−HCl, kept on ice for 10 min then centrifuged at 12 000 *g* for 15 min at −2°C. A sample of 100 μg protein (determined with Bio-Rad protein assay kit) or 1 × 10^7 c.p.m. of acid precipitable counts of [^{35}S]methionine-labeled materials in 100 μl were treated with rabbit anti-human p34^{cdc2} C-terminal peptide (17mer) (Lee and Nurse, 1987) or anti-human cyclin B (Pines and Hunter, 1989) antiserum, washed as described (Pines and Hunter, 1989) after addition of 20 μl protein A−agarose (50% v/v) (Boehringer Mannheim, FRG) and analyzed for H1 kinase activity or for 10% SDS−PAGE followed by autoradiography.

Histone H1 kinase activity

Histone H1 kinase activity was determined by incubation of 10 μg of protein in crude extracts or immunoprecipitates with 40 μl of a buffer containing 20 mM HEPES−NaOH pH 7.5, 15 mM EGTA, 20 mM $MgCl_2$, 1 mM dithiothreitol, 500 nM A-kinase inhibitory peptide (Sigma, USA), 20 μg of histone H1 (Boehringer Mannheim, FRG) and 50 μM ATP (1000−2000 c.p.m./pmol) for 10 min at 30°C. The reaction was terminated by addition of 20 μl of 3 × SDS sample buffer and boiled for 3 min. The samples were further processed for gel electrophoresis and autoradiography. The bands of histone H1 were excised and the radioactivity was determined by scintillation counting.

Incorporation of $^{32}PO_4$ into proteins

BHK21 cells were synchronized in early S phase. After 14 h of HU treatment, cells were washed twice with phosphate-free medium and labeled with [^{32}P]orthophosphate (200 μCi/ml) for 4 h in the presence of HU, then cells received 1 μM OA and were incubated further. For the measurement of the incorporation of $^{32}PO_4$ into proteins, cells were lysed and cytoplasmic fraction was collected as described in H1 kinase assay. The lysate was treated with 100 μg/ml each of DNase I and RNase A and 10 000 U/ml of RNase T1 (Worthington Biochemicals, USA) and kept on ice for 30 min and at 37°C for 10 min. After centrifugation at 12 000 *g* for 30 min, TCA was added to the supernatant at the final concentration of 10% and the sample was kept on ice for 30 min. The precipitate collected on glass filter (GF/C, Whatman, USA) was scintillation counted.

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