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Inhibition of cAMP-dependent protein kinase plays a key role in the induction of mitosis and nuclear envelope breakdown in mammalian cells

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Inhibiting cAMP-dependent protein kinase (A-kinase) in mammalian fibroblasts through microinjection of a modified specific inhibitor peptide, PKi(m) or the purified inhibitor protein, PKI, resulted in rapid and pronounced chromatin condensation at all phases of the cell cycle. Together with these changes in chromatin, a marked reorganization of microtubule network occurred, accompanied in G_2 cells by extensive alterations in cell shape which have many similarities to the premitotic phenotype previously observed after activation of $p34^{cdc2}$ kinase, including the lack of spindle formation and the persistence of a nuclear envelope. In order to examine whether A-kinase inhibition and $p34^{cdc2}$ kinase form part of the same or different inductive pathways, PKI and $p34^{cdc2}$ kinase were injected together. Co-injection of both components resulted in nuclear envelope disassembly, an event not observed with injection of either component alone. This result implies that $p34^{cdc2}$ and A-kinase inhibition have complementary and additive effects on the process of nuclear envelope breakdown in living fibroblasts, a conclusion further supported by our observation of a pronounced dephosphorylation of lamins A and C in cells after injection of PKi(m). Taken together, these data suggest that down-regulation of A-kinase is a distinct and essential event in the induction of mammalian cell mitosis which co-operates with the $p34^{cdc2}$ pathway.

Key words: cAMP-dependent protein kinase/lamins/mitosis/nuclear envelope breakdown

Introduction

Whilst the mechanisms which regulate precise and co-ordinate duplication of cells at mitosis still remain to be completely determined, there is ample recent evidence that reversible phosphorylation and dephosphorylation of proteins play an implicit role. The identification of a ubiquitous protein kinase $p34^{cdc2}$ and its two potential regulatory subunits cyclins A and B in a wide variety of organisms has led to the suggestion that it acts as a key regulator in the inductive events of mitosis (reviewed by Pines and Hunter, 1990; Draetta, 1990).

In mammalian cells a number of other reports have implied that $p34^{cdc2}$ is involved in mitotic induction (Lee and Nurse, 1987; Draetta *et al.*, 1987). Its activation in HeLa cells could be correlated with prophase entry (Draetta and Beach, 1988). We have recently described the effects of elevating the levels of this kinase in synchronized mammalian fibroblasts following microinjection of the purified active catalytic subunit with or without cyclin B (Lamb *et al.*, 1990). In both G_2 and G_1 cells, we observed marked changes in cell shape, chromatin organization, microtubule and actin cytoskeletal networks which closely resembled the changes occurring in cells at prophase. However, cells could not be induced to pass further into mitosis and remained stationary with an intact nuclear envelope, partially condensed chromatin and the absence of a mitotic spindle. These effects were independent of the presence of intact cyclin B, suggesting that for a mammalian cell to enter mitosis, a number of other pathways or intracellular signals must be set prior to mitotic induction. In this respect the mammalian cell differs markedly from an oocyte since microinjection of the same purified catalytic subunit rapidly induces a quiescent oocyte to enter mitosis, perhaps implying that in the occyte system many of these potential steps have already been made.

Amongst other potential mediators of mitosis, A-kinase has previously been implicated as a negative regulator in the early mitotic cycles of a variety of organisms. Indeed inhibition of A-kinase could be directly correlated with induction of quiescent *Xenopus* oocytes into the mitotic cycle (Maller and Krebs, 1977). However, this could be differentiated from the inductive effect of MPF in its absolute requirements for protein synthesis which is not the case for injection of MPF. Other studies have identified that a reduction in basal levels of cAMP occurs during oocyte maturation in a variety of organisms including starfish (Meijer *et al.*, 1989), fish (Jalabert and Finet, 1986; DeManno and Goetz, 1987) amphibians (Maller *et al.*, 1979; Schorderet-Slatkine *et al.*, 1982; Schorderet-Slatkine and Baulieu, 1982) and mammals (Schultz *et al.*, 1983). Maturation can also be induced in most species through injection of phosphodiesterases (Foerder *et al.*, 1982, Bornslaeger *et al.*, 1986) (although not in starfish: Meijer and Zarutskie, 1987; Meijer *et al.*, 1989). Finally, microinjection of either PKI, the inhibitor protein of A-kinase, or R subunits induces maturation in amphibians (Maller and Krebs, 1977; Huchon *et al.*, 1981; Doree *et al.*, 1981). Similarly, in mammalian oocytes, PKI induces maturation whereas A-kinase activation (Urner *et al.*, 1983) or injection of purified A-kinase catalytic subunit ('C') is inhibitory (Bornslaeger *et al.*, 1986), whilst in starfish, although 'C' remains inhibitory, PKI does not seem to be competent to induce maturation (Doree *et al.*, 1981). Taken together, these data have led to the suggestion that in oocyte maturation, cAMP forms part of a negative pathway which participates in maintenance of prophase arrest and is released by hormonal stimulation (Maller, 1985; Meijer *et al.*, 1989). We have been interested in determining if A-kinase plays an equivalent role in maintaining somatic mammalian cells in an interphase state. We have recently

synthesized a modified form of PKi, the specific inhibitory peptide derived from the protein PKI, and originally described by Cheng *et al.* (1986). The modifications we have made on the PKi peptide, whilst preventing its proteolytic degradation following its injection into cells, did not alter its activity or specificity, therefore providing a useful tool for the study of A-kinase dependent processes *in vivo* (Fernandez,A., Mery,J., Vandromme,M., Basset,M., Cavadore,J-C. and Lamb,N.J.C., submitted). Using both the purified inhibitor protein (derived from an *E.coli* expression system) or the modified inhibitory peptide, we have examined the involvement of A-kinase in the events leading to mitosis to determine if its inhibition plays a role in mitotic induction, and furthermore, whether this is related to the action of $p34^{cdc2}$ kinase or operates through an independent pathway. In the present paper we describe the close similarities between the effects of inhibition of A-kinase and $p34^{cdc2}$ kinase and the co-operative effect of co-injection of the two in producing a further step towards mitotic induction: nuclear envelope breakdown. The implications of these results are discussed with respect to a pathway complementary to $p34^{cdc2}$ activation, requiring A-kinase down-regulation for the entry of mammalian cells into mitosis.

Results

Initial experiments were performed to assess the morphological consequences of inactivating cAMP-dependent protein kinase (A-kinase) in mammalian fibroblasts. We have previously observed that artificially activating endogenous A-kinase through treatment of cells with cell soluble analogues of cAMP produced differential reorganization of cytoskeletal networks in a manner which implied that there were cell cycle changes in A-kinase activity. In light of this, we assessed the consequences of inhibiting A-kinase activity in rat embryo fibroblasts synchronized through serum deprivation. As described previously, serum starvation of this cell line allows us to define four separate stages of the cell cycle in addition to mitosis, quiescent G_0 cells which are still serum starved, G_1 cells: 0–15 h after re-feeding, S phase cells: 15–20 h after re-feeding and G_2 cells: from 20 h to mitosis (occurring 25–28 h post serum stimulation).

Chromatin condensation

Injecting a 200 μM solution of PKi(m) [a peptide corresponding to amino acids 6–24 of the specific inhibitor protein PKI, modified to increase its *in vivo* stability as described elsewhere (Fernandez,A., Mery,J., Vandromme,M., Basset,M., Cavadore,J-C. and Lamb,N.J.C., submitted)] into synchronized REF 52 cells revealed a dramatic condensation of the chromatin which occurred in all injected cells regardless of the stage in the cell cycle. As shown in Figure 1 whilst microinjection of the marker antibody solution containing, as a control, peptide fragment PKi(m) (15–24) in injection buffer alone, has no effect on the organization of the chromatin (panels A–C), cells injected in G_1 (panels D–F) or G_2 (panels G–I) with PKi(m) showed pronounced and marked condensation of the chromatin. This effect occurred within 10 min of injection and could be reproduced at all periods of the cell cycle including S phase and G_0 (data not shown). In addition, as shown in the phase contrast panel (Figure 1, G) in G_2 cells, chromatin condensation was accompanied by changes in cell shape; this is discussed further below. This effect on chromatin here observed in cells fixed 10 min after injection occurs as soon as 1 min after microinjection of the inhibitor and is stable for 4–10 h [which reflects the half-life of the PKi(m) peptide inhibitor in cells under these conditions]. Chromatin condensation can be elicited with concentrations of peptide inhibitor ranging from 50 μM to 1 mM, but we have routinely used a 100 μM solution of the inhibitor peptide in the needle. Similar induction of chromatin condensation by PKi(m) was also observed in all cell lines so far tested including HeLa, human fibroblasts, Swiss 3T3 and CV-1 (data not shown).

To ensure that these effects were truly related to the inhibition of an A-kinase dependent pathway, cells were injected with a gene construct encoding the complete inhibitor protein under the regulation of a SV40 enhancer (Day *et al.*, 1989). As shown in Figure 2 panels A–C, a similar if not equivalent effect could be observed on chromatin organization following injection of this gene construct (although in this case the chromatin condensation takes place after 45–60 min, reflecting the period required for the protein to be synthesized). Furthermore, injection of the purified inhibitor protein at 1.25×10^{-5}M (in the needle) (Figure 2 panels D and E), also produced identical changes in chromatin organization as observed with either the peptide or the PKI gene. However, unlike the injection of the peptide, which effected changes in chromatin organization if injected in the cytoplasm or nucleus, chromatin condensation only took place if the protein was injected into the nucleus (note that the marker antibody is present in both the cytoplasm and nucleus of the injected cell). Since the protein can form different conformational isomers (McPherson *et al.*, 1979), this could account for the necessity for nuclear injection. Alternatively, the protein may become rapidly inactivated through post-translational modifications when injected into the cytoplasm. To confirm further that this effect on chromatin structure relates to A-kinase, we examined if it be specifically reversed through injection of the purified catalytic subunit of A-kinase into cells in which the chromatin had been condensed by previous injection of PKi(m) or PKI. As shown in Figure 2 panels F and G, when cells are subsequently injected with A-kinase (1.2×10^{-5} M in the needle), little or no evidence of PKi(m)-induced chromatin condensation remains after as little as 10 min. No such reversal was observed following injection of other kinases including casein kinases I and II and calcium phospholipid dependent protein kinase (data not shown). The finding that A-kinase (and not a number of other kinases tested) could reverse the effect of both PKI and PKi(m) strongly implies that the effects on chromatin were specifically related to the inactivation of A-kinase dependent pathways and not to the inhibition of other kinase pathways in these cells.

Altered cell morphology

When cells were followed for longer periods of time after the injection of the PKi(m) peptide, we noted that some changes in cell shape could be discerned in cells in late G_1 or S phases and were highly pronounced in cells during the G_2 phase. Figure 3 shows phase contrast images of a group of cells in G_2 phase, microinjected with the inhibitor peptide and photographed at different times after microinjection. Figure 3 panel A, shows cells immediately following

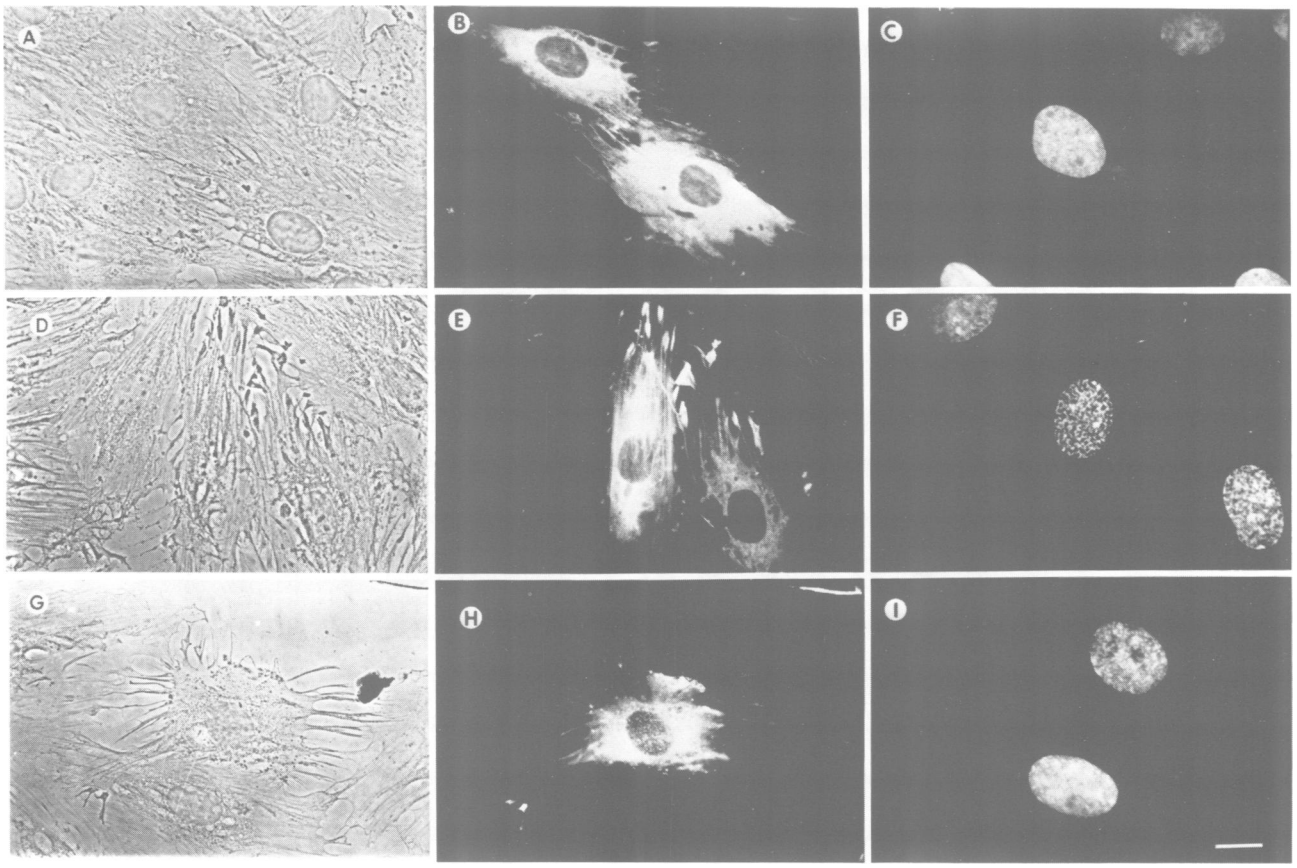

Fig. 1. Rapid chromatin condensation accompanies cAMP-dependent protein kinase inhibition by the inhibitor peptide, PKi(m). Synchronized rat embryo fibroblasts grown and subcultured onto glass were microinjected with either PKi(m) (6–24), the modified inhibitor peptide for the cAMP-dependent protein kinase or the inactive PKi(m)-fragment (15–24) (both peptides are described in Fernandez,A., Mery,J., Vandromme,M., Basset,M., Cavadore,J.-C. and Lamb,N.J.C., submitted). After a 30 min incubation, cells were fixed and stained for the presence of a co-injected marker antibody or the organization of the DNA. Shown are phase contrast (A,D and G) or fluorescence micrographs of cells stained for the marker antibody (B, E and H) or the DNA (C, F and I). Panels **A–C**, cells injected with inactive peptide, panels **D–F**, cells injected in G_1 with PKi(m), panels **G–I**, cells injected in G_2 with PKi(m). Panels A, B, D, E, G and H were shot with a 63× lens; panels C, F and I (Hoechst staining) were shot with a 100× lens.

injection; there are clearly no signs of morphological changes, and the injected cells appear similar to the surrounding uninjected cells. In contrast, within 20 min of microinjecting PKi(m), the injected cells (marked by arrows in Figure 3B) show the beginning of phase-dense, thickened regions forming dark foot-like structures at the periphery of the cells. By 60 min, cells have begun to lose their contact with the substratum (they also contain visibly condensed chromatin) and show long foot-like protrusions (Figure 3C). These alterations in cell shape following A-kinase inhibition are very similar to those observed previously in cells injected with the purified catalytic subunit of $p34^{cdc2}$ kinase (Lamb *et al.*, 1990) and are also reminiscent of the shape changes in these cells during prophase, in the early stages of division (cf. Lamb *et al.*, 1990). These effects of G_2 cells are stable for 4 or 6 h, after which the cells enter mitosis. Higher concentrations of PKi(m) whilst eliciting little or no difference in the extent of chromatin condensation induce the changes in cell shape more quickly, whilst lower concentrations simply induce less pronounced effects. We also observed similar effects on cell shape of the protein PKI, and in cells microinjected with a gene construct encoding PKI protein (Figure 3D–E). Interestingly, unlike the effects of PKI protein on chromatin organization which were dependent on the locale of microinjection, the same effects on the alterations in cell shape in G_2 cells were observed in nuclear or cytoplasmic injected cells (data not shown).

Microtubule reorganization

Since clear correlation exists between effects on cell morphology and the reorganization of the underlying cytoskeletal networks, we have assessed possible changes in cytoskeletal organization accompanying these effects of PKi(m) on cell shape by examining if microinjection of PKi(m) leads to any change in microtubule organization. We have previously observed that microinjection of the catalytic subunit of A-kinase did not induce any significant changes in microtubule reorganization in REF-52 cells (A.Fernandez, N.Lamb, unpublished observation). In contrast, cells injected with PKi(m) showed marked reorganization of the microtubule networks in all phases of the cell cycle. As shown in Figure 4, unlike cells injected with buffer alone (Figure 4A–B) which show essentially no changes in microtubule organization, cells in G_1 microinjected with the A-kinase inhibitor have a very disturbed organization of microtubules, making it difficult to observe microtubule staining in injected cells (Figure 4D). Compared with the surrounding uninjected cells, there are relatively few

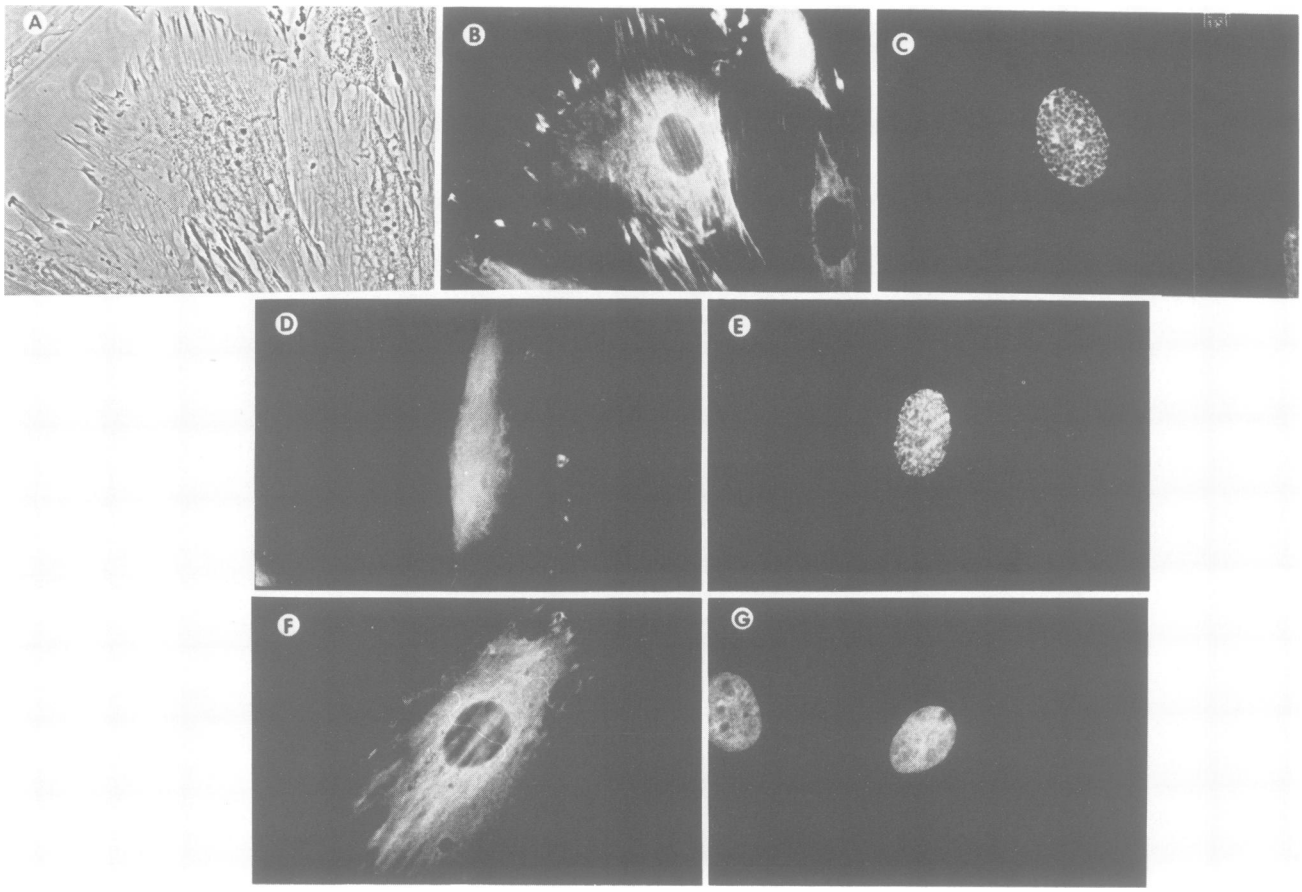

Fig. 2. Chromatin condensation is induced by the inhibitor protein for A-kinase and reversed by subsequent injection of A-kinase. In an experiment similar to that described in Figure 1, synchronized fibroblasts in G_2 were microinjected with the purified inhibitor protein PKI or a gene construct encoding it. After 60 min, cells were fixed and stained for the presence of an inert marker antibody co-injected with the gene or protein and for the organization of the chromatin. Alternatively cells were injected with PKi(m) peptide and 15 min thereafter re-injected with the purified catalytic subunit of the cAMP-dependent protein kinase. 30 min later these cells were fixed and stained as described above. Shown is a phase contrast micrograph (panel A), or staining for injected antibody (panels B, D and F) or DNA organization (panels C, E and G). Panels **A–C**, G_1 cells (12 h after refeeding) injected with pSV-40 PKI (a gene construct encoding the entire rabbit skeletal muscle gene for PKI), panels **D** and **E**, cells in G_1 injected in the nucleus with the purified inhibitor protein and panels **F** and **G**, cells injected in G_1 with PKi(m) and subsequently re-injected with A-kinase catalytic subunit.

microtubule fibres left in cells injected with PKi(m). We also observed similar changes in microtubule organization in cells injected with the gene construct overexpressing PKI in G_1 cells (Figure 4E–F) and in G_2 cells injected with either PKi(m) or the PKI expressing gene (data not shown). To ensure that these changes in microtubules did not result from artefactual loss of the epitope for the antibodies, the cells in panels 4B and D were stained with anti-alpha-tubulin DMA-1A whilst the cells in panel 4F were stained with anti-alpha-tubulin YL1/2. Since these antibodies recognize different epitopes but show similar results, it suggests that changes in microtubule organization truly occur. Moreover, we have also observed similar effects when cells were fixed in glutaraldehyde with prior extraction showing that the apparent loss of microtubule staining did not result from the methanol fixation protocol (data not shown). These data strongly suggest that in living fibroblasts, an intact microtubule network is dependent upon the presence of active A-kinase. It should be noted, however, that this disassembly of the microtubule network induced by inhibiting A-kinase, like that previously observed with $p34^{cdc2}$ injection, is not accompanied by the formation spindle.

Nuclear envelope breakdown

Since there seem to be a number of similarities between the effects of microinjecting PKi(m) and the effects observed with prematurely activated $p34^{cdc2}$ kinase, i.e. a prophase phenotype, we have assessed if changes other than those observed with $p34^{cdc2}$ kinase occur with PKi(m). Figure 5 shows the distribution of lamins A and C as determined by indirect immunofluorescence in cells injected in G_2 with active or inactive PKi(m) and fixed 60 min afterwards. In cells injected with inactive peptide (as determined by its inability to reverse the effects of A-kinase activation by drugs), the organization of the nuclear lamins appears very similar in the injected cells (marked by antibody Figure 5A) and surrounding uninjected cells (Figure 5B). When cells are injected with the active peptide in G_1 (Figure 5C and D) there is also little or no evidence of a loss of lamin staining (Figure 5D). A similar finding is also observed in cells injected with the active $p34^{cdc2}$ kinase. As shown in Figure 5G and H, cells injected with $p34^{cdc2}$ kinase in G_2, whilst showing extensive reorganization in cell shape (shown in Figure 5G), still show a bright perinuclear staining of the nuclear lamins, corresponding to the nuclear envelope

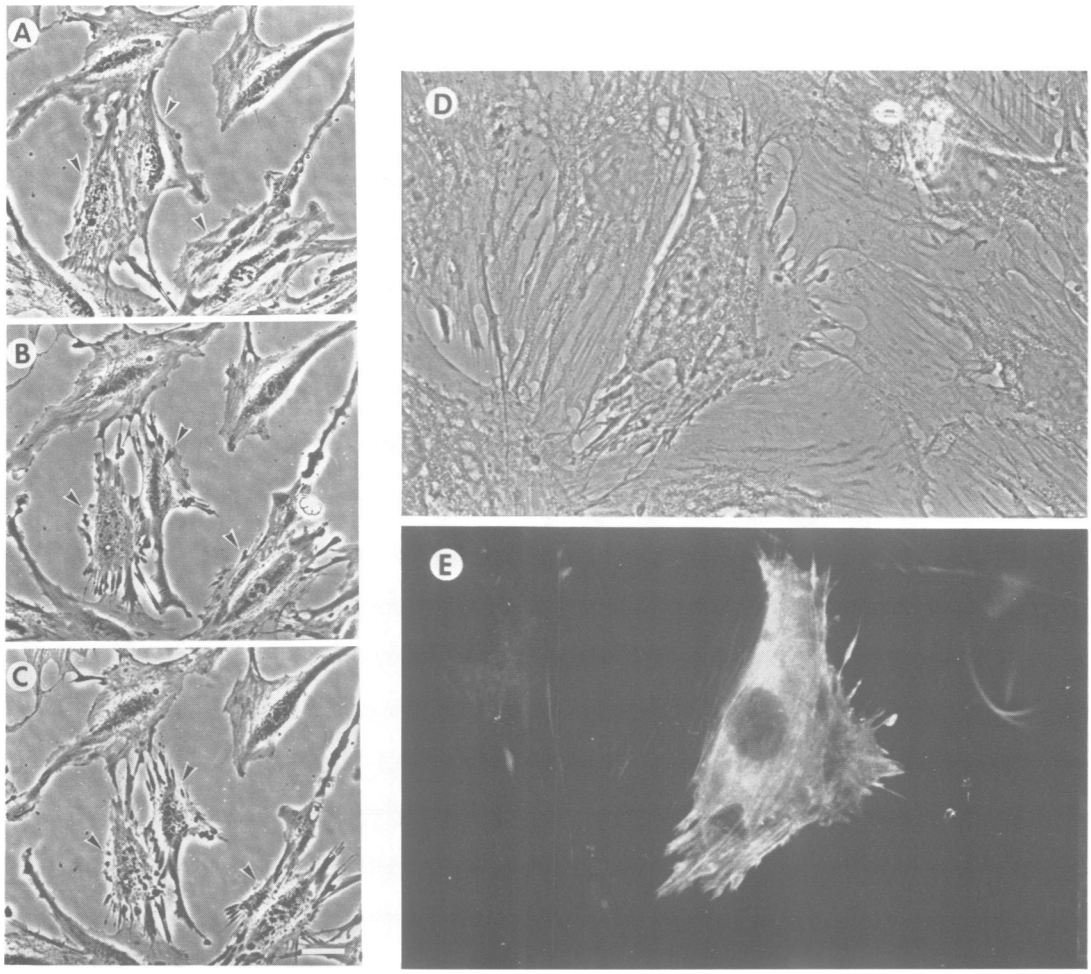

Fig. 3. Changes in cell morphology accompany inhibition of the cAMP-dependent protein kinase in G_2 cells. Rat embryo fibroblasts, growing on glass coverslips and synchronized through serum starvation were injected after S phase with PKi(m) peptide. Changes in cell morphology were followed by phase contrast microscopy before and 15 or 30 min after injection. Alternatively, cells in the same period of the cell cycle were injected with a plasmid construct encoding for PKI protein inhibitor. Cells were subsequently fixed 2 h after injection and photographed for the changes in morphology and distribution of marker antibody. Panels **A**–**C**: phase micrographs of cells prior (A) and 15(B) and 30(C) minutes after injection of PKi(m). Panels **D** and **E** are phase contrast (D) and fluorescence micrographs (E) of cells injected with the gene and stained for the marker antisera.

(Figure 5H). We have previously reported that $p34^{cdc2}$ kinase did not result in nuclear envelope breakdown in G_1 cells as assessed by electron microscopy (Lamb *et al.*, 1990). In G_2 cells (Figure 5E and F), whilst microinjection of PKi(m) results in a rapid loss of cell shape as described above, Figure 5F shows that again, injected cells keep a functionally intact image of their nuclear envelope. In contrast, cells co-injected with the kinase $p34^{cdc2}$ and the inhibitor peptide show little or no staining for the nuclear envelope (Figure 5I and J). Instead, the cytoplasm is filled with a bright diffuse fluorescence staining pattern. An identical effect on nuclear envelope was observed in G_1 cells and was confirmed through thin section electron microscopy and immunofluorescence for lamin B (data not shown). However, cells co-injected with PKi(m) and $p34^{cdc2}$ in late G_2 could still pass through mitosis, suggesting that this loss of lamin staining reflected a physiological effect on nuclear envelope organization. These effects of $p34^{cdc2}$ kinase and PKi(m) can be abolished by incubating the $p34^{cdc2}$ kinase with antibodies directed against it or p13 suc beads (data not shown) indicating that they require the active $p34^{cdc2}$ kinase activity as well as active PKi(m).

Finally, we have examined if PKI induces change in lamin phosphorylation through *in vivo* metabolic labelling of injected cells. As shown in Figure 6A, whereas the injection of an inactive PKi(m) peptide fragment (15–24) has no effect on the levels of lamin phosphorylation, injection of PKi(m) (6–24) (the same numbers of cells are injected in both cases) led to a pronounced decrease in phosphorylation of lamins A and C. Notable in these effects is the almost complete loss of lamin C phosphorylation (arrowed in Figure 6B). On the contrary, injection of $p34^{cdc2}$ kinase induces a rapid phosphorylation of all three lamin proteins under the same circumstances (Figure 6C), further supporting the idea that the two components (PKI and $p34^{cdc2}$) operate through separate and distinct pathways in the induction of mitosis.

Taken together, these data suggest that a combination of inhibition of A-kinase and activation of $p34^{cdc2}$ kinase is required for the process of nuclear envelope breakdown to occur *in vivo* and for the progression of mammalian cells further into mitosis, an observation which is supported by the finding that entry into mitosis can indeed be delayed through microinjection of purified A-kinase (A.Fernandez and N.Lamb, unpublished observation).

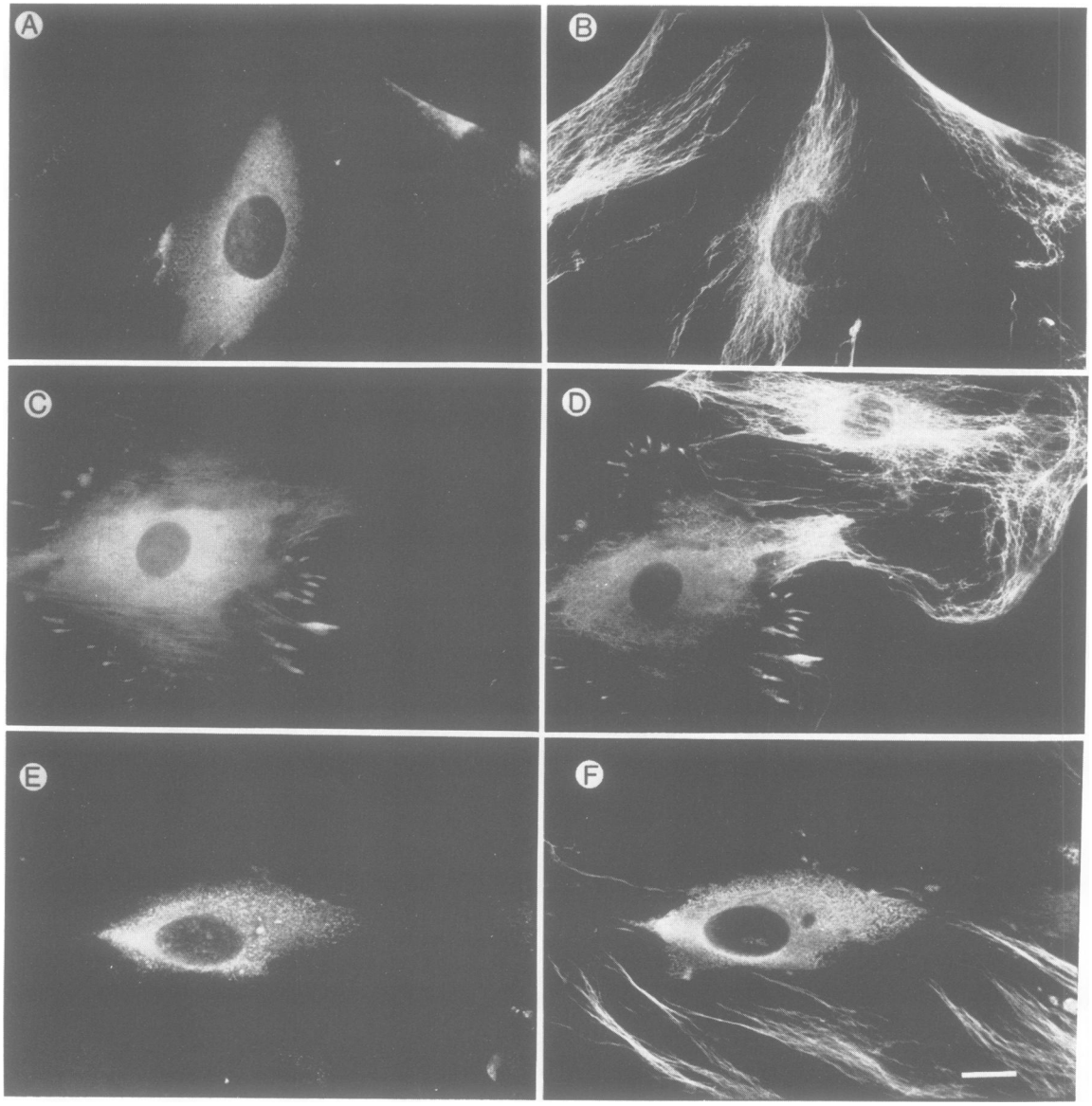

Fig. 4. Microtubule reorganization results from inhibition of the cAMP-dependent protein kinase in living cells. Changes in microtubule organization following cAMP-dependent protein kinase inhibition were followed in synchronized REF-52 cells after injection of PKi(m) or the purified protein kinase inhibitor protein. Fluorescence micrographs of injected cells stained for the co-injected marker antibody (panels A, C and E) or the distribution of the microtubules stained with anti-alpha-tubulin DMA 1A (panels B and D) or anti-alpha-tubulin YL-1/2 (panel F). Panels **A–B** show a cell injected with the microinjection buffer alone in G_1, panels **C–D** show a cell injected with PKi(m) in G_1 and panels **E–F** a cell injected with the inhibitor protein.

Discussion

Inhibiting cAMP-dependent protein kinase in living cells leads to profound changes in cellular organization and chromatin structure in a manner which appears to be cell cycle dependent. At all stages in the cell cycle, inhibition of A-kinase leads to the rapid condensation of the chromatin and disassembly of the microtubule network. In addition to these effects, cells injected in G_2 round up and collapse around the nucleus in a fashion similar to that seen following microinjection of active $p34^{cdc2}$ kinase. However, inhibiting A-kinase together with microinjecting active $p34^{cdc2}$ kinase leads cells to proceed further into mitosis than was observed with $p34^{cdc2}$ or PKI alone. Our results imply that inhibition of A-kinase constitutes an important second regulatory pathway in the regulation of mammalian cell mitotic entry which involves crucial dual phosphorylation/dephosphorylation events.

Chromatin condensation

One of the most dramatic and immediate effects to follow A-kinase inhibition is the condensation of the chromatin. Striking in this effect are its speed and apparent cell cycle independence. That this effect on chromatin organization is specific to inhibition of A-kinase and physiological is supported by our observations that it is readily reversed through microinjection of purified A-kinase (Figure 1) and that injection of catalytic subunit is sufficient to prevent cells from entering prophase (unpublished observations). These features of the effect of A-kinase inhibition on chromatin condensation imply that A-kinase performs a continuous

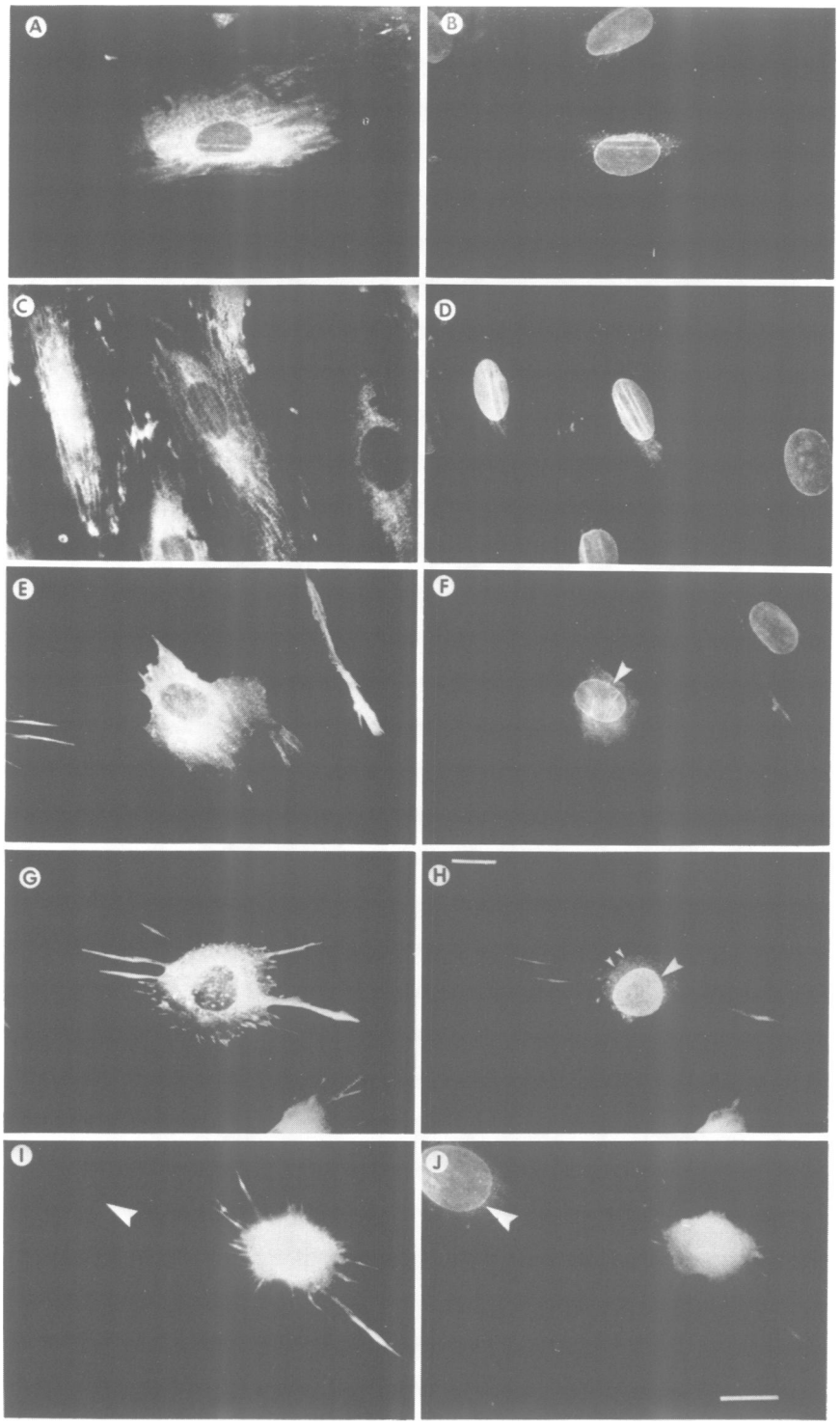

Fig. 5. Inhibition of the cAMP-dependent protein kinase is accompanied by nuclear envelope breakdown in the presence of active $p34^{cdc2}$ kinase. REF-52 cells growing synchronously on glass coverslips were microinjected with PKi(m) in G_1 or G_2. Alternatively, cells in G_2 were injected with a solution of purified $p34^{cdc2}$ kinase containing cyclin alone or together with PKi(m). After 60 min cells were fixed and stained for the distribution of lamins A and C and co-injected inert marker antiserum. Shown are fluorescence micrographs of cells stained for the marker antibody (panels **A**, **C**, **E**, **G** and **I**) or lamins (**B**, **D**, **F**, **H** and **J**). Shown are cells injected with buffer (panels **A** and **B**), PKi(m) in G_1 (panels **C** and **D**) or G_2 (panels **E** and **F**), $p34^{cdc2}$ kinase alone (panels **G** and **H**) or both PKi(m) and $p34^{cdc2}$ kinase (**I** and **J**).

function in the maintenance of chromatin higher order structure throughout interphase. Although demonstration of such a role for A-kinase is original, various lines of evidence would support it. For example the catalytic subunit of A-kinase has been associated with transcriptionally active chromatin during changes in transcriptional activity (Johnson, 1977; Sikorska *et al.*, 1988; Mellon *et al.*, 1989) and it has recently been shown that the free catalytic subunit tends to localize mainly in the nucleus (Meinkoth *et al.*, 1990). Changes in chromatin structure may result from the inac-

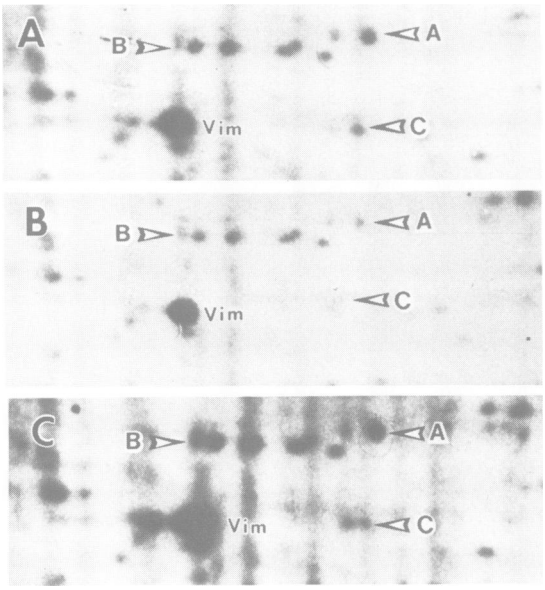

Fig. 6. Changes in lamin phosphorylation following either inhibition of A-kinase or injection of $p34^{cdc2}$ kinase. Cells growing on 2 mm^2 glass coverslips were injected with injection buffer containing inactive peptide, PKi(m) peptide or $p34^{cdc2}$ kinase. Immediately after, cells were transferred to 5 μl of phosphate free medium and labelled for 30 min. Phosphoproteins were subsequently analysed by two dimensional electrophoresis as described before (Lamb *et al.*, 1988). Shown are autoradiograms from regions of the gels containing the lamins from cells injected with control peptide (panel **A**), PKi(m) (panel **B**) or $p34^{cdc2}$ kinase (panel **C**). Arrowed are the relative positions of lamins, A, B, C and vimentin (vim) as determined by immunoblotting. Gels are shown with the acidic side to the left.

tivation of an A-kinase dependent phosphorylation of either histones (West *et al.*, 1985; reviewed by Wu *et al.*, 1986), or other DNA related structural regulators such as topoisomerases (Roberge *et al.*, 1990). Moreover, it is now clear that a number of genes are under the transcriptional regulation of cAMP responsive factors (CREB) which have been linked to the activity of the catalytic subunit (Riabowol *et al.*, 1988; Day *et al.*, 1989; Grove *et al.*, 1987; Maurer, 1989; Mellon *et al.*, 1989; Buchler *et al.*, 1990). In these models, increased catalytic subunit activity induces transient gene expression and modulates the overall levels of gene transcription. Again this latter function would be highly amenable to the implication that A-kinase activity is diminished at mitosis, since this would also ensure that gene transcription was no longer taking place in the dividing cell. Indeed we have observed a marked reduction in RNA synthesis resulting from inhibition of A-kinase (A.Fernandez and N.Lamb, unpublished observations) and we are currently examining these possibilities in more detail.

Although apparently contradictory to the dogma linking H1 phosphorylation (presumably by $p34^{cdc2}$) to chromatin condensation, the effect of inhibiting A-kinase on chromatin is consistent with a number of previous observations and whilst $p34^{cdc2}$ periodic activation coincides with the entry of cells into prophase (reviewed by Pines and Hunter, 1990; Draetta, 1990), different lines of evidence argue against it being solely responsible for chromatin condensation. For instance, whilst there is an overall increase in phosphorylation during the G_2/M transition (Gurley *et al.*, 1974, 1978; Langan, 1982; Woodford and Pardee, 1986), specific decreases in histone phosphorylation have been reported including the cAMP-dependent phosphorylation sites on H3 (reviewed by Wu *et al.*, 1986). Furthermore, in mammals both H1 phosphorylation and chromatin condensation increase continuously from late G_1 to late G_2 at a time when $p34^{cdc2}$ is still apparently inactive (Gurley *et al.*, 1974). *Tetrahymena* macronucleus which undergoes only amitotic division is an example where hyperphosphorylation of histone H1 occurs during changes in gene expression and is never associated with chromosome condensation (Roth *et al.*, 1988), despite the presence of a $p34^{cdc2}$ like kinase in those nuclei (D.Allis, personal communication). These data suggest that H1 phosphorylation by $p34^{cdc2}$ kinase is insufficient to induce chromatin condensation alone and, moreover, the phosphorylation state of H1 may have important functions other than chromatin condensation as has been implied before (Lake and Salzman, 1972; Gurley *et al.*, 1974; Woodford and Pardee, 1986). Indeed, the precise role of histone phosphorylation still awaits unravelling (Wu *et al.*, 1986) since it has been related to both transcriptional activation [for example the increased nuclease sensitivity following histone 2A phosphorylation (West *et al.*, 1985)] and chromosome condensation at mitosis (Langan, 1982). One possibility is that the effects of A-kinase inhibition and $p34^{cdc2}$ on chromatin condensation reflect part of a co-operative pathway, such that the extent of H1 phosphorylation by $p34^{cdc2}$ is related to prior inhibition of A-kinase dependent histone phosphorylation or other chromatin bound proteins. In support of such a possibility, we had observed that the extent of chromatin condensation induced by microinjection of active $p34^{cdc2}$ in synchronized fibroblasts varies with the phase of the cell cycle, being very high in G_0, low in G_1, zero in S, and high in late G_2 (Lamb *et al.*, 1990). These fluctuations reflect well the variations in cAMP levels described during the cell cycle (Burger *et al.*, 1972; Costa *et al.*, 1976). Such a requirement for both $p34^{cdc2}$ activation and A-kinase inactivation to produce heavy chromatin condensation would also explain previous observations by others (Peter *et al.*, 1990) that active cdc2 kinase produced little or no chromatin condensation when added to isolated nuclei. Finally, the observation that injection of both PKI and $p34^{cdc2}$ produces highly condensed chromatin similar to a prometaphase phenotype implies that cells have not relied solely on the action of one kinase for a commitment as vital as chromatin to chromosome transition.

In addition to the effect of inhibiting A-kinase on chromatin, further support for the hypothesis that A-kinase is involved in maintaining the interphase state comes from the observed effects of PKI on microtubules. There has long been a controversial implication for A-kinase in the regulation of microtubules. Both classes of heavy molecular weight MAP proteins are *in vitro* substrates for A-kinase (Theurkauf and Vallee, 1982; Vallee *et al.*, 1984; Lohmann *et al.*, 1984). Moreover catalytic and regulatory subunits of the kinase have been localized onto some microtubule networks (Browne *et al.*, 1980; Murtaugh *et al.*, 1982; Lohmann *et al.*, 1984) and centrosomal structures (Nigg *et al.*, 1985; Meinkoth *et al.*, 1990). However, despite this apparent association, we have never observed changes in the organization of the microtubules after injection of A-kinase, or elevation of A-kinase levels through drugs. Instead, our observation that inhibiting A-kinase leads to microtubule disassembly raises the possibility that in growing cells, A-kinase dependent phosphorylation of MAPS occurs continuously, and its inhibition leads to microtubule disassembly.

Together, these effects of inhibiting A-kinase on chromatin and microtubules organization suggest that a basal level of A-kinase activity is continuously required to maintain the interphase organization of these two structures, it would imply A-kinase as a vital 'house-keeping' enzyme activity. This hypothesis is further supported by the fact that long-term transfectants with PKI-SV40 (leading to continuous overexpression of PKI and suppression of A-kinase activity), are impossible to obtain (Day *et al.*, 1989; R.Maurer, personal communication), most likely because of the apparent lethality of such recombinants.

Nuclear envelope breakdown

A second striking observation was that, whilst separate injection of PKI or $p34^{cdc2}$ induced similar alterations in cell morphology (in G_2 cells), co-injection of both led cells further into mitosis in that we observed for the first time in G_1 and G_2 cells, the disassembly of the nuclear envelope. This latter observation is of particular interest with respect to the potential mechanism of nuclear envelope assembly/disassembly. Three recent reports established a correlation between the phosphorylation of specific sites on nuclear lamins at mitosis and the dissassembly of the nuclear envelope. Essentially, these reported that elimination of these sites prevented correct nuclear envelope disassembly (Heald and McKeon, 1990), that MPF-induced phosphorylation of these sites leads to the disassembly of human lamin C filaments in oocyte extracts (Gard and Kirschner, 1990) and that *in vitro*, $p34^{cdc2}$ kinase phosphorylates mitosis-specific sites leading to lamin depolymerization on isolated chicken nuclei (Peter *et al.*, 1990). However, we were unable to observe the envelope disassembly in cells microinjected with the purified $p34^{cdc2}$ kinase, although we observed an increased phosphorylation of all three lamins (Figure 6C), suggesting that phosphorylation of the lamins by this kinase (and/or other kinases activated downstream $p34^{cdc2}$, such as possibly S6 kinase II), was insufficient to induce nuclear envelope breakdown *in vivo*.

The observation that co-injection of PKI and $p34^{cdc2}$ made cells competent to undergo disassembly of the envelope implies that in addition to increased phosphorylation at $p34^{cdc2}$ related sites, nuclear lamin disassembly involves a dephosphorylation event at A-kinase site(s). Indeed, we were able to observe a reduction in lamin phosphorylation (especially for lamins A and C) following injection of PKi(m) (Figure 6B). Moreover, this suggestion is supported by at least one of the reports mentioned above in which Peter *et al.* (1990) showed that in both chicken lamins B1 and B2, in addition to the sites which showed increased phosphorylation after $p34^{cdc2}$ kinase treatment and during mitosis, several sites phosphorylated *in vivo* during interphase became clearly dephosphorylated during mitosis (see Peter *et al.*, Figure 5). Furthermore, these sites appear to be the same which show increased phosphorylation following Forskolin treatment (Figure 7 in Peter *et al.*, 1990) thus corresponding to A-kinase phosphorylation sites. Since we have identified the site on vimentin which is heavily phosphorylated *in vivo* by A-kinase (a site also present on all three lamins) we are currently examining if a similar site becomes dephosphorylated during the disassembly of the lamins *in vivo* and at mitosis.

Down-regulation of A-kinase and mitotic induction

The implication of A-kinase as a negative regulator of oocyte maturation has been previously well documented. Ample evidence exists in *Xenopus* (Maller and Krebs, 1977; Cicirelli and Smith, 1985), starfish (Meijer *et al.*, 1989) and mammals (Schorderet-Slatkine and Baulieu, 1982; Schorderet-Slatkine *et al.*, 1982; Bornslaeger *et al.*, 1986) that a reduction in cAMP levels (and reduced associated kinase activity) occurs upon induction of maturation. Moreover, this can be correlated to decreased A-kinase levels since injection of purified catalytic subunits is in many systems (but not all) inhibitory (see Meijer *et al.*, 1989). Finally inactivation of intracellular A-kinase through injection of purified inhibitor protein PKI could be shown to induce maturation in quiescent *Xenopus* and mouse oocytes (Maller and Krebs, 1977; Bornslaeger *et al.*, 1986). In mammalian somatic cell mitosis, however, a similar effect did not seem to be the case, since both RII and catalytic subunits could be cytolocalized to the mitotic spindle (Browne *et al.*, 1980) and PKI had been reported to act as a mitotic inhibitor or retarding agent in Poteroo epithelial cells (Browne *et al.*, 1987) We have observed the contradictory result that PKI injected into late G_2/early prophase cells substantially accelerates the entry of these cells into mitosis, further supporting our conclusion that at least one of the final events in mitotic entry in mammalian cells is the inactivation of A-kinase. Whilst the reason for these differences is unclear it may result from the extreme proteolytic susceptibility of protein PKI or impurities in the PKI preparation [the preparation described by Browne *et al.* (1987) was 60% pure]. Our observations that PKI injection induces pronounced changes in shape in G_2 cells and not at other stages of the cell cycle, suggests that other factors synthesized or activated late in the cycle are required for these morphological effects to take place. Whilst the nature of such factors is unknown, there are clearly a number of proteins which are synthesized in G_2 (Westwood *et al.*, 1985) or become activated only at mitosis, for example $p34^{cdc2}$ kinase associated cyclin proteins (reviewed in Pines and Hunter 1990; Draetta, 1990). Alternatively, this effect of PKI may require cell cycle dependent activation of other kinases or phosphatases for example there are similarities between the effects on cell shape of c-src activation and PKI (A.Fernandez and N.Lamb unpublished observations).

Since the present data imply down-regulation of A-kinase as a key event in mitotic induction and little is known of the *in vivo* biology of PKI, we must question how this is brought about in cells under normal conditions. That PKI and not for example RI or RII carry out this process at mitosis is supported by a number of lines of evidence. Firstly, the inactivation of A-kinase at mitosis must be cAMP-independent since there is a marked peak of cAMP accumulation at this time (Burger *et al.*, 1972; Costa *et al.*, 1976). Furthermore, recent microinjection experiments have revealed that RI subunits are constitutively localized in the cytoplasm, postulating that they may serve as a cytoplasmic anchor sequestering the catalytic subunit which, upon dissociation from RI (by elevated cAMP) seems to migrate into the nucleus (Meinkoth *et al.*, 1990). In agreement with this, a previous study had observed the localization of RII mainly in the cytoplasm (Nigg *et al.*, 1985). In this respect the regulatory subunits would be poorly located to play a role in chromatin condensation which commences prior to nuclear envelope breakdown. In contrast, there are a number of features of KPI which make it an attractive candidate for A-kinase down-regulation. The effects of PKI appear to be focused on prophase entry, implying that PKI becomes inac-

tivated during the later periods of mitosis. In this respect at least two potential regulatory mechanisms may exist to down-regulate PKI. Firstly, it is a highly unstable protein, making it an ideal substrate for the process of protease activation that has already been invoked in the regulation of cyclin proteins (reviewed by Draetta, 1990). Alternatively, PKI can be apparently inactivated *in vitro* by tyrosine phosphorylation (Van Patten *et al.*, 1987). Although there is no evidence for such a process *in vivo*, since $p34^{cdc2}$ dependent mitotic induction correlates with the activation of a tyrosine kinase (reviewed by Pines and Hunter 1990), particularly during prometaphase, it is tempting to speculate that such an activation could potentially play a role in PKI down-regulation. However, any firm evidence for a possible cell cycle fluctuation of PKI will require the use of anti-PKI antibodies, which we are in the process of producing and characterizing.

Previous work on oocyte maturation had shown that dephosphorylation of specific substrates was occurring simultaneously with increased phosphorylation of other proteins (Maller and Smith, 1985). Together with the inhibitory effect of A-kinase on progesterone induced oocyte maturation, these dephosphorylation events had been potentially linked to A-kinase inactivation. The present report demonstrates that A-kinase inhibition acts as a crucial second component in somatic cell mitotic entry as supported by a number of lines of evidence. Firstly, injection of PKI induced more convincing chromatin condensation than ever observed with $p34^{cdc2}$ alone and this resembled prometaphase when injected in the presence of $p34^{cdc2}$. Secondly, co-injection of $p34^{cdc2}$ and PKI induced cells to disassemble the nuclear envelope an effect which could not be obtained with $p34^{cdc2}$ or PKI alone and represent a further step toward induction of an interphase cell into mitosis. Together, these results imply that A-kinase dependent protein phosphorylation acts as part of a negative cross-talk pathway against $p34^{cdc2}$ and/or other kinases and that differential protein phosphorylation pathways constitute an essential mechanism to regulate mitotic induction in somatic mammalian cells.

Materials and methods

Cell culture

Rat embryo fibroblasts cells (REF-52) were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 6% (v/v) fetal calf serum in a humidified atmosphere containing 5% (v/v) CO_2 as described elsewhere (Lamb *et al.*, 1990). Cells were subcultured onto glass coverslips, made quiescent through serum deprivation and synchronized essentially as described before (Lamb *et al.*, 1990). To obtain cells in late G_2, cells made quiescent by serum starvation were resynchronized at 12 h after serum addition by the additions of 2 mM hydroxyurea (HU). Cells were cultured in HU for 10 h, washed twice with PBS, twice with fresh media without HU but containing serum and used 4–8 h afterwards. Under these conditions the majority of cells passed through mitosis 9–10 h after the HU removal.

Peptide and kinase preparation

PKi(m) a dual modified PKi(6–24) peptide was synthesized and assayed *in vitro* and *in vivo* as described elsewhere (Fernandez,A., Mery,J., Vandromme,M., Basset,M., Cavadore,J.-C. and Lamb,N.J.C., submitted). PKI protein was expressed in a bacterial expression system and purified as described elsewhere (Thomas,J., Van Patten,S.M., Howard,P., Day,K.H., Richardson,J., Mitchell,R.D., Sosnick,T., Trewhella,J., Walsh,D.A. and Maurer,R.A., submitted). A similar gene construct under the regulation of an SV40 transcriptional enhancer (Day *et al.*, 1989) was used to express the protein in cells after microinjection. $p34^{cdc2}$ kinase was purified from starfish oocytes as described before (Lamb *et al.*, 1990), both cyclin free and cyclin B containing preparations gave the same results.

Microinjection and immunofluorescence

Cells synchronized as described above were microinjected with either purified PKI protein, PKi(m) peptide, purified kinases or both, essentially as described before (Lamb *et al.*, 1990). In all cases, injection solutions contained 0.5 mg/ml inert mouse or rabbit antisera to allow identification of injected cells. One of three injection solutions was used either 0.5 × PBS, 100 mM K^+ HEPES (pH 7.20), 1 mM $MgSO_4$ or 100 mM K^+ glutamate, 40 mM K^+ citrate, 1 mM $MgSO_4$. No differences were seen using these solutions. Kinase preparations (5 μl) were dialysed (using colloidoin dialysis membranes, Sartorius Inc.) into injection buffers prior to use after storage at either 4 or −70°C. PKi peptides were desalted into water on a Sephadex G25 resin and lyophilized four times prior to use (to ensure removal of residual acid), resuspended at 5–10 × stock solutions (~10 mg/ml) in injection buffer and stored at −70°C. The peptide is completely stable to repeated freeze–thaw cycles or storage at 4°C after sterile filtration.

Cells for immunofluorescence were fixed and stained for tubulin as described before (Lamb *et al.*, 1990). Cells stained for lamins were fixed either in −20°C methanol or 3.7% (v/v) formalin in PBS followed by extraction in −20°C acetone. Cells were stained for lamins A/C with a human auto-antiserum as described elsewhere (Heald and McKeon, 1990). Cells were subsequently counter-stained for the mouse marker antibodies using biotinylated anti-mouse antisera visualized with Texas red streptavidin and for the distribution of the lamins with fluorescein anti-human antisera. In all cases, cells were stained with Hoechst, mounted in Airvol 205 and photographed as described before (Lamb *et al.*, 1990). Hoechst images were recorded with either a Zeiss plan-Neofluar 63× lens (1.4 N/A) or a Zeiss plan-Neofluar 100× lens (NA 1.3) on either Kodak Plus-X pan film (developed in Kodak D-76:water 1:1) or Technical pan films. Other fluorescence images were photographed as described before (Lamb *et al.*, 1990).

In vivo metabolic labelling

Changes in phosphorylation in injected cells were followed by *in vivo* metabolic labelling of the cells following microinjection essentially as described before (Lamb *et al.*, 1988). Cells were injected with either inactive PKi fragments (15–24), PKi(m) or the $p34^{cdc2}$ kinase. Following injection cells were transferred to humidified chambers and labelled for 30 min with [^{32}P]orthophosphate. Changes were followed by two-dimensional electrophoresis and autoradiography as described before (Lamb *et al.*, 1988).

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