

# Involvement of p21 in the PKC-induced regulation of the G2/M cell cycle transition

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**Abstract** Activation of protein kinase C (PKC) inhibits cell cycle progression at the G1/S and G2/M transitions. We found that phorbol 12-myristate 13-acetate (PMA) induced upregulation of p21, not only in MCF-7 cells arrested in the G1 phase as previously shown, but also in cells delayed in the G2 phase. This increase in p21 in cells accumulated in the G1 and G2/M phases of the cell cycle after PMA treatment was inhibited by the PKC inhibitor GF109203X. This indicates that PKC activity is required for PMA-induced p21 upregulation and cell cycle arrest in the G1 and G2/M phases of the cell cycle. To further assess the role of p21 in the PKC-induced G2/M cell cycle arrest independently of its G1 arrest, we used aphidicolin-synchronised MCF-7 cells. Our results show that, in parallel with the inhibition of cdc2 activity, PMA addition enhanced the associations between p21 and either cyclin B or cdc2. Furthermore, we found that after PMA treatment p21 was able to associate with the active Tyr-15 dephosphorylated form of cdc2, but this complex was devoid of kinase activity indicating that p21 may play a role in inhibition of cdc2 induced by PMA. Taken together, these observations provide evidence that p21 is involved in integrating the PKC signaling pathway to the cell cycle machinery at the G2/M cell cycle checkpoint.

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**Key words:** Protein kinase C; p21; Cell cycle; cdc2 kinase activity; G2/M transition

## 1. Introduction

Cell cycle progression requires the activation of different cyclin-dependent kinases (CDKs), which are positively regulated by cyclins and negatively regulated by CDK inhibitors [1]. Two families of CDK inhibitors have been identified, one family includes p16, p15, p18 and p19, which are specific for CDK4 and CDK6, and the other family contains p21, p27 and p57, with a broader specificity for CDKs [2]. Although p21 is an universal inhibitor of CDKs, its growth inhibitory effect is largely exerted during the G1 phase of the cell cycle by binding to CDK4 and CDK2 [3,4]. p21 also directly inhibits DNA replication in a PCNA-dependent manner [5]. Transient overexpression of p21 in tumour cells [6] and in normal fibroblasts [7] leads to an accumulation of cells in the G1 phase of the cell cycle, whereas treatment with p21

antisense RNA promotes entry of quiescent cells into S phase [8]. The p21 gene is induced by DNA damaging agents through p53-dependent pathways [6,9]. The p21 gene can also be up-regulated via p53-independent pathways by serum stimulation [10] as well as in cellular senescence [11] and differentiation [12,13]. In addition to this well characterised role of p21 as an inhibitor of cell cycle progression in G1, recent findings are in favour of a role for p21 in G2 and mitotic checkpoints. Firstly, during the cell cycle, p21 mRNA expression peaks in both G1 and G2/M phases [14]. In addition, p21 accumulates in the nucleus late in G2, concomitantly with nuclear translocation of cyclin B, and is associated with cyclin B/cdc2 and cyclin A/CDK2 [15]. Secondly, p21-inducible or uninducible overexpression [16–19] results in an arrest of cells in both G1 and G2/M phases of the cell cycle. Activation of the mitotic checkpoint by damage to microtubules is also associated with induction of p21 [20]. We have recently shown that after microtubular damage, the association of p21 with cyclin B/cdc2 complexes occurs in parallel with an inhibition of cdc2 activity and mitotic exit [21].

Protein kinase C (PKC) isoenzymes are involved in growth control at the G1/S and G2/M transitions as regulators linking signal transduction pathways to the cell cycle machinery review in [22]. PKC-mediated inhibition of growth results from both a block of the cell cycle in G1 and a delay in the transit through the G2 phase of the cell cycle [23,24]. PKC-mediated cell cycle arrest in G1 is accompanied by accumulation of the hypophosphorylated form of retinoblastoma protein due to inhibition of CDK2 activity [25,26]. Furthermore, the two CDK inhibitors, p21 and p27, have been shown to be mediators of the inhibition of CDK2 activity induced by phorbol 12-myristate 13-acetate (PMA) [27,28]. p21 and p27 are also involved in the inhibition of CDK2 activity after ectopic expression of PKC- $\eta$  in NIH-3T3 cells [29]. The fact that resistance of cancer cells to PMA has been associated in some cases with a failure of this compound to increase p21 [30] is a further argument for a role of p21 in growth arrest after PKC activation. Induction of p21 by PMA depends on Raf-1 kinase and MAPK signaling pathways [30], but is mediated by p53-independent pathways at the transcriptional level [27]. The G2/M transition is also regulated by the PKC signaling pathway. The delay in the G2/M transition induced by PMA results from an inhibition in the activity of cdc2 [31,32]. Dephosphorylation of cdc2 on tyrosine residues by cdc25 phosphatases allows cdc2 activation and entry of the cell into mitosis. Inhibition of cdc2 activity after PMA treatment has been found to be associated with down-regulation of cdc25 phosphatase [32,33]. In the present study, we found that in addition to its role in the G1 phase of the cell cycle, p21 contributes to the inhibition of cdc2 activity induced by PKC activation in the G2/M phase of the cell cycle.

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**Abbreviations:** PKC, protein kinase C; CDKs, cyclin-dependent kinases; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; BSA, bovine serum albumin

## 2. Materials and methods

### 2.1. Cell culture and cell synchronisation

MCF-7 cells were obtained from the American Type Culture Collection. Cells were grown in humidified 5% CO<sub>2</sub>/95% air at 37°C in RPMI 1640 medium supplemented with sodium bicarbonate (2 g/l), 2 mM glutamine, 1 µM insulin and 5% foetal calf serum. For cell cycle synchronisation, MCF-7 cells were treated with aphidicolin (1 µg/ml) (Sigma) for 24 h to arrest the cells in the G1/S phase. Cells were released from the G1/S cell cycle block by washing the cells three times with phosphate-buffered saline (PBS) and adding culture medium containing 5% foetal calf serum in the presence or absence of PMA (Sigma). At the indicated times, cells were either fixed and prepared for flow cytometric analysis of cell cycle parameters or harvested for determination of cdc2 activity and immunoprecipitations.

### 2.2. Flow cytometry

Aphidicolin-synchronised MCF-7 cells were treated with PMA. At the indicated times, cells were washed in PBS then treated with 0.25% trypsin-0.02% EDTA and harvested after removal of trypsin. After centrifugation (300×g, 10 min), the pelleted cells were fixed in 80% ethanol overnight at −20°C. The samples were centrifuged, washed with PBS and resuspended in 10 µg/ml of propidium iodide (Sigma) and 0.1% RNase A (Sigma) in PBS. After a 15 min incubation at 37°C, the distribution of cells in the different phases of the cell cycle was determined by analytical flow cytometry using LYSIS II software (Becton Dickinson). For bivariate analysis of p21 and DNA contents, cells were rinsed with PBS and suspended in 80% ethanol overnight at −20°C as described by Darzynkiewicz et al. [34]. The samples were then centrifuged, washed with PBS and treated with 0.25% Triton in PBS for 5 min on ice. After addition of PBS and centrifugation, the cells were incubated overnight at 4°C in the presence of the mouse monoclonal antibody to human p21 (Calbiochem) diluted 1:20 in PBS containing 1% bovine serum albumin (BSA). Cells were washed and incubated with a fluorescein 5-isothiocyanate FITC-conjugated goat anti-mouse IgG antibody (Sigma) diluted 1:100 in PBS containing 1% BSA. Cells were washed and resuspended in 10 µg/ml of propidium iodide and 0.1% RNase A in PBS and incubated for 15 min prior to FACScan analysis. The control was prepared in an identical way except that an isotype-specific antibody (mouse IgG1 from Sigma) was used instead of the p21 antibody. Cellular fluorescence was measured in a FACScan flow cytometer (Becton Dickinson). The red (propidium iodide) and green (FITC) fluorescences from each cell were separated and quantitated by the FACScan using the standard optics and LYSIS II software (Becton Dickinson).

### 2.3. Western blotting, immunoprecipitation and kinase activity

Cells were washed with PBS and lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.1% Triton, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 100 µg/ml PMSF and TPCK, 50 µg/ml TLCK, 1 µg/ml leupeptin, pepstatin and aprotinin). Lysates were clarified by centrifugation. For Western blot analysis, 100 µg of proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes by semi-dry blotting. The membranes were hybridised with p21 (Calbiochem) antibodies. The blots were probed with actin antibody (Chemicon International Inc.) for equal loading control. For immunoprecipitation as for kinase activity determination, rabbit polyclonal cdc2 antibody was added to 1 mg of precleared cell lysates and incubated at 4°C for 2 h on a rotating wheel. Immune complexes were recovered with protein A-agarose (Pharmacia) and washed three times with lysis buffer. For immunoprecipitation, immune complexes were separated on 15% SDS-polyacrylamide gels as described above and a mouse monoclonal p21 antibody (Calbiochem) or a mouse monoclonal cdc2 (Transduction Laboratories) were used for immunoblot analysis. For cdc2 kinase activity, beads were washed again with kinase assay buffer (50 mM Tris-HCl pH 8, 10 mM MgCl<sub>2</sub>, 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 30 µl of a reaction mixture containing 100 µM ATP (Boehringer Mannheim), 5 µCi [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; New England Nuclear) and 2 µg histone H1 (Boehringer Mannheim) in kinase assay buffer for 10 min at 30°C. The reaction was stopped by boiling the samples in Laemmli SDS sample buffer for 5 min, and samples were resolved by 12% SDS-PAGE. The gels were dried and subjected to autoradiography.

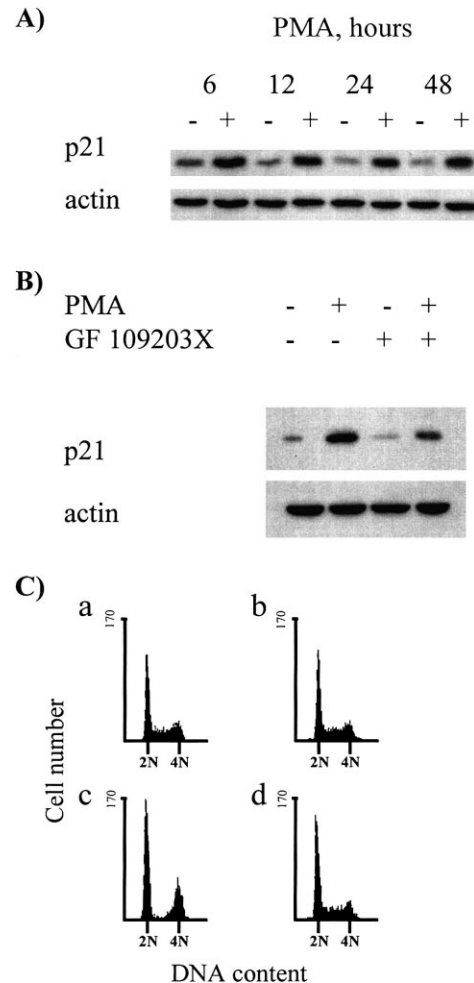


Fig. 1. Effect of PKC activation on MCF-7 cells. A: Western blot analysis of p21 protein after PMA treatment in MCF-7 cells. Total cell lysates were prepared at various times after addition of PMA (100 ng/ml). B: Effect of GF109203X on PMA-induced upregulation of p21. MCF-7 cells were treated or not with PMA (100 ng/ml) for 24 h in the presence or absence of GF109203X (0.5 µM) added 2 h before PMA. Total cell proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blotting was carried out using an antibody against p21. To control for the amount of proteins in each line, the same blot was probed with anti-actin mAb. C: Effect of GF109203X on modifications of cell cycle parameters induced by PMA. Cell cycle parameters were analysed by flow cytometry (a) control MCF-7 cells; (b) cells treated with GF109203X (0.5 µM) for 26 h; (c) MCF-7 cells treated with PMA (100 ng/ml) for 24 h; (d) cells pretreated with GF109203X (0.5 µM) for 2 h and then treated with PMA (100 ng/ml) and GF109203X for 24 h.

## 3. Results

### 3.1. p21 is induced after PMA treatment by PKC activation in the G1 and G2/M phases of the cell cycle

In line with the findings of several groups [12,28,35], we found that PMA induced a time- and a dose-dependent increase in p21 protein level in MCF-7 cells (Fig. 1A). Six hours after the addition of PMA (100 ng/ml) we observed a significant increase in p21 protein level which remained high until 48 h. We found that preincubation of MCF-7 cells with 0.5 µM GF109203X, a compound which inhibits conventional PKCs by interacting with the ATP binding site of this enzyme [36], prevented the elevation of p21 protein induced by PMA (Fig. 1B). In parallel, we observed that GF109203X inhibited the

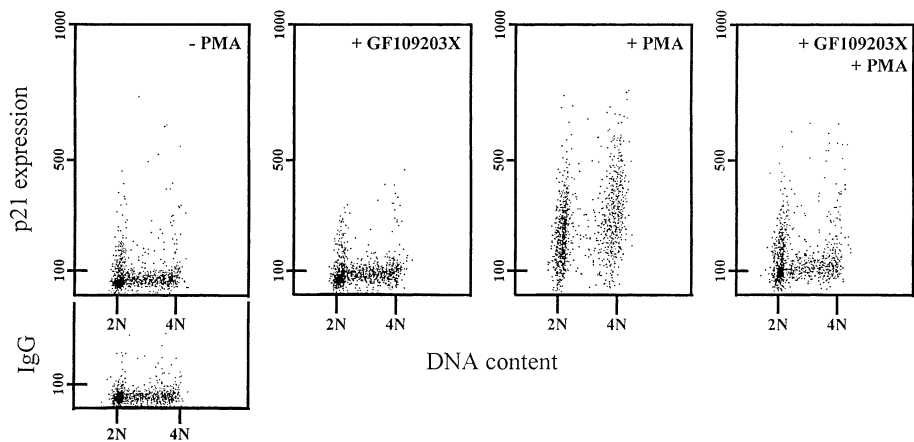


Fig. 2. Bivariate analysis of p21 expression versus DNA content of either control MCF-7 cells, cells treated with GF109203X (0.5  $\mu$ M) for 26 h, cells treated with PMA (100 ng/ml) for 24 h, cells treated with GF109203X (0.5  $\mu$ M) and PMA for 24 h (GF109203X was added 2 h before PMA). Isotypic control (IgG) done in parallel makes it possible to discriminate between specific and non-specific p21 fluorescence. Cells under FITC channel 100 were considered p21-negative, cells above this channel were considered p21-positive.

PMA-induced accumulation of MCF-7 cells in the G1 and G2/M phases of the cell cycle (Fig. 1C).

Since it is now well established that p21 overexpression regulates cell cycle progression in the G2/M phase of the cell cycle [18,19], we investigated if p21 could be involved in the PKC-induced regulation of the G2/M transition. Cells were thus analysed by flow cytometry for both DNA and p21 content. A 24 h PMA treatment resulted in an elevation of p21 in cells accumulated with G1 and G2/M DNA contents (Fig. 2). The PKC inhibitor GF109203X (0.5  $\mu$ M) inhibited the upregulation of p21 protein in MCF-7 cells accumulated not only in G1 but also in the G2/M phase of the cell cycle after PMA addition (Fig. 2) indicating that PKC activation is involved in regulation of p21 expression in G1 and G2/M phases of the cell cycle.

3.2. Inhibition of *cdc2* kinase activity in MCF-7 cells after PMA treatment

To assess the PKC-induced G2/M cell cycle checkpoint independently of its G1 effect, we synchronised MCF-7 cells at the G1/S transition by treatment with aphidicolin (1  $\mu$ g/ml). The cell cycle block induced by aphidicolin was released by washing the cell monolayers and incubating them in fresh medium. Analysis of the DNA content of MCF-7 cells after release from the block indicated that MCF-7 cells entered S phase within 3 h; most cells were in G2 after 6–9 h and had returned to G1 between 12 and 15 h (Fig. 3A). In the presence of PMA, applied at the time of aphidicolin release, we observed a 6–9 h lag in the progression through the G2/M phase. Most of the PMA-treated cells were in the G2/M phase between 6 h and 15 h (Fig. 3A) and entered G1 of the subse-

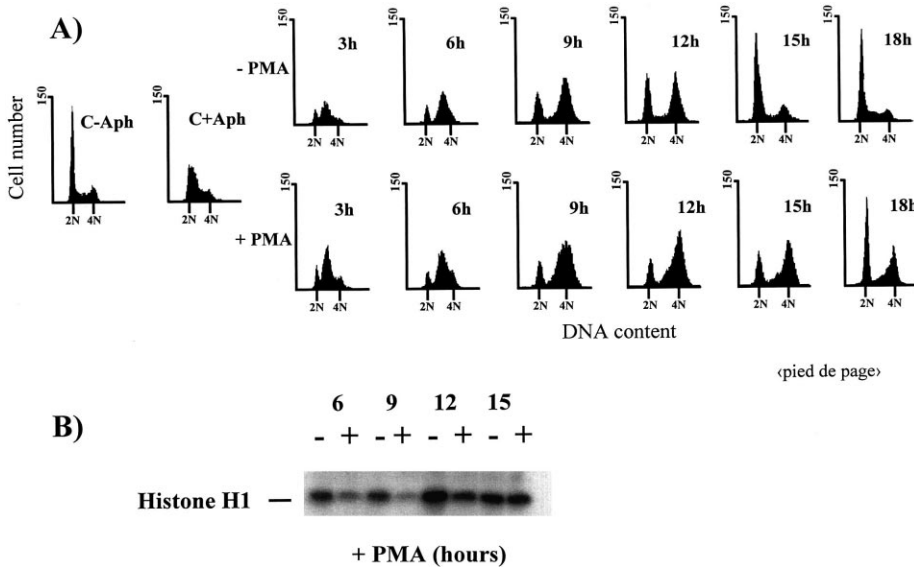


Fig. 3. The effect of PMA on aphidicolin-synchronised MCF-7 cells. MCF-7 cells were arrested at the G1/S transition by treatment with aphidicolin (Aph) (1  $\mu$ g/ml) for 24 h. Cells were released from the aphidicolin block by washing and incubation in fresh medium. MCF-7 cells were treated or untreated with PMA (100 ng/ml) at the time of aphidicolin release. Control and PMA-treated cells were harvested at various times after aphidicolin release. A: FACS analysis of cell cycle parameters. B: *cdc2* kinase activity associated with the immunoprecipitate of *cdc2* was assayed using histone H1 as a substrate.

quent cell cycle within 18 h. This confirms that PMA transiently inhibits cell cycle progression in the G2/M phase of the cell cycle. Since active cdc2 is required for the G2/M transition, we investigated the activity of this kinase in MCF-7 cells synchronised at the G1/S transition by treatment with aphidicolin. In control cultures, after aphidicolin release, kinase activity rose to a peak at 12 h and declined between 12 h and 15 h when the majority of MCF-7 cells were in the G1 phase of the subsequent cell cycle (Fig. 3B). In the presence of PMA (100 ng/ml), the kinase activity detected 6 h and 9 h after aphidicolin release was much lower than that observed in the control cells. cdc2 kinase activity subsequently increased indicating that this phenomenon was transient (Fig. 3B). We observed that after PMA treatment the delay in the activation of cdc2 paralleled the delay in the progression of cells into the G2/M phase of the cell cycle.

### 3.3. p21 is involved in the regulation of cdc2 activity after PMA treatment

p21 is an universal inhibitor of CDK. It inhibits G1 CDK with a  $K_i$  of 0.5–15 nM, although it is less effective towards the cyclin B/cdc2 complex with a  $K_i$  of 400 nM [3]. Since PMA led to an elevation in p21 in the G1 and G2/M phases of the cell cycle (Fig. 2), p21 may participate not only in the PMA-induced inhibition of CDK2, as previously shown [37], but also in the inhibition of cdc2 activity. We therefore assayed cyclin B and cdc2 immunoprecipitates from PMA-treated MCF-7 cells for the presence of p21. We found that PMA led to an elevation of p21 associated with cdc2 and cyclin B immunoprecipitates (Fig. 4). The increase in p21 binding to cyclin B/cdc2 complexes was apparent at 6 h, reaching a peak 9 h after PMA addition, indicating that this increase preceded the lag in G2/M. Eighteen hours after aphidicolin release, whereas PMA-treated cells were in the subsequent G1 phase, the association of p21 with cdc2 immunoprecipitates decreased (data not shown).

In order to determine if the increasing associations of p21

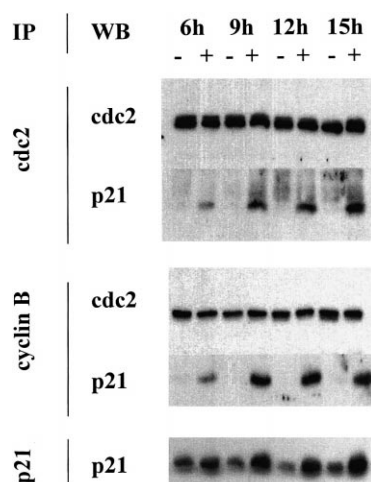


Fig. 4. Time course analysis of in vivo association of p21 with cyclin B and cdc2 immunoprecipitates after PMA treatment. Cell lysates were prepared from aphidicolin-synchronised MCF-7 cells treated or not with PMA (100 ng/ml). 1 mg of total protein was immunoprecipitated (IP) with antibodies against cdc2, cyclin B and p21. After immunoprecipitation, immune complexes were resolved on SDS-polyacrylamide gels, and associated proteins were analyzed by Western blotting (WB).

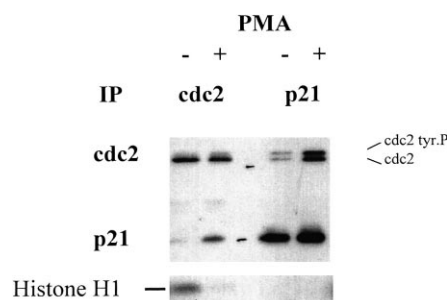


Fig. 5. p21-associated complexes are inactivated. Extracts were prepared from control (–) and PMA-treated cells (+), 12 h after release from the aphidicolin block. 1 mg of total protein was immunoprecipitated (IP) with antibodies against cdc2 and p21. After immunoprecipitation, immune complexes were either resolved on SDS-polyacrylamide gels, and associated proteins were analysed by Western blotting (WB), or tested for histone H1 kinase activity using histone H1 as a substrate.

with cyclin B/cdc2 complexes were responsible for the kinase activity inhibition, we analysed further the nature of the cdc2 complexes, 12 h after aphidicolin release of control and PMA-treated MCF-7 cells (Fig. 5). Despite the fact that cdc2 immune complexes from control and PMA-treated cells contain the same high proportion of the active tyrosine-dephosphorylated form, the kinase activity of cdc2 immunoprecipitates was strongly reduced after PMA treatment; the inhibition of histone kinase activity was correlated with the presence of a large amount of p21 bound to cdc2. In parallel we showed that p21 immunoprecipitates from control PMA-treated cells were devoid of histone kinase activity even though these complexes contain the active form of cdc2 (Fig. 5). Western blot analysis of p21 immunoprecipitates indicated that p21 was also associated with the inactive tyrosine phosphorylated form of cdc2 kinase. Binding of p21 to the active and inactive forms of cdc2 was higher after PMA treatment (Fig. 5). This result argues in favour of a role of p21 in the PMA-induced inhibition of cdc2 activity and G2/M delay.

## 4. Discussion

p21 has been shown to be implicated in the regulation of G1/S transition in response to various inhibitors of cell proliferation including PMA. The results presented here indicate that p21 also plays a role in integrating the PKC signaling pathway in the cell cycle machinery at the G2/M cell checkpoint. First, we found that PKC activation in MCF-7 cells results in an increase of p21 not only in cells arrested in G1 but also in cells accumulated in the G2/M phase of the cell cycle. Second, PMA inhibits cdc2 kinase activity and stimulates binding of p21 to cyclin B/cdc2 complexes. Third, after PMA treatment, p21-containing complexes were devoid of histone H1 kinase activity.

A role of p21 in the cell cycle block in G1 induced by PMA has been previously shown [12,35,37]. Furthermore it has been shown that overexpression of PKC- $\eta$  in NIH-3T3 cells induces an increase in the expression of both p21 and p27 and their association with cyclin E/CDK2 [29]. PMA-induced inhibition of cell cycle progression in the G2 phase of the cell cycle is known to be associated with inhibition of cdc2 kinase activity [31,32]. Several molecular mechanisms come to mind for the inhibition of cdc2 activity. The cyclin B/cdc2 complex

is inactivated as long as tyrosine 15 is phosphorylated, and dephosphorylation of this residue by cdc25 phosphatases enables cdc2 activation and entry into mitosis. It has been reported that the G2/M arrest induced by PMA results from the prevention of dephosphorylation of tyrosine residues in cdc2 due to a down-regulation of cdc25 expression [32,33,38]. In the present study we did not observe a significant variation in the proportion of phosphorylated inactive form of cdc2 in PMA-treated MCF-7 cells compared to control cells indicating that, depending on the cell type, different mechanisms are responsible for PKC-induced inhibition of cdc2 kinase activity. Another possibility is that a CDK inhibitor may be responsible for cdc2 inhibition. Indeed, a role for inhibitors of CDK in the regulation of cell cycle progression in the G2/M phase have been shown in several recent studies. Indeed, UV-C irradiation of HeLa cells results in an accumulation of p16, which correlates with S and G2 delays [39]. However, the mechanism by which p16 regulates the progression in S and G2 phases is not known. p21 is a universal inhibitor of CDKs and it inhibits G1 CDK in vitro with a  $K_i$  value of 0.5–15 nM, although it is less effective toward the cyclin B/cdc2 complex with a  $K_i$  of 400 nM [3]. Several observations argue in favour of a role of p21 in other phases of the cell cycle than G1. p21 mRNA peaks in G1 and G2 phases of the cell cycle and p21 protein is found associated with cyclin B complexes during G2 [14]. At the G2/M boundary, p21 is bound to inactivated cyclin A/CDK2 complexes and to a lesser extent to cyclin B1/cdc2 complexes and these associations correlate with a nuclear reaccumulation of p21 protein in late G2 [15]. We have recently shown that p21 participates in the regulation of mitotic exit after microtubule damage induced by paclitaxel via an action on the cyclin B/cdc2 complex [21]. In the present study, we found that PMA induced an increase in p21 native protein level in MCF-7 cells transiently arrested in the G2/M phase of the cell cycle. This upregulation of p21 was inhibited by an inhibitor of conventional PKCs (GF 109203X) indicating that PKC activation is involved in p21 induction at the G2/M transition in MCF-7 cells. It has recently been reported that in Calu-1 and A549 cells which are insensitive to PMA-induced G1 arrest, a G2/M cell cycle block is associated with the appearance of a truncated form of p21 that lacks the C-terminal epitope [40]. A similar truncated version of p21, which was unable to associate with CDK2, has been also observed in control and UV-irradiated transformed cell lines [41]. In MCF-7 cells we did not find any evidence of the appearance of a truncated form of p21 after PMA treatment. The results of the present study argue in favour of a role for p21 in the transient inhibition of the kinase activity of cyclin B/cdc2 complexes induced by PMA in MCF-7 cells. However, the increase in p21 could be a cause or a consequence of the delay induced by PMA at this particular phase of the cell cycle. The increase in p21 in immunoprecipitated cyclin B/cdc2 complexes was parallel to the inhibition of cdc2 activity and occurred before the delay induced by PMA on the G2/M transition, suggesting that the increase in the association between p21 and cyclin B/cdc2 complexes was a cause rather than a consequence of the cell cycle delay. p21-containing cdk exist in both active and inactive states [42]. At a low concentration p21 promotes the assembly of active cdk complexes whereas at high concentrations it inhibits cdk activity [42,43]. We observed here that whereas p21 was found associated with the active dephosphorylated form of either cdc2 or

cyclin B, these complexes were devoid of histone kinase activity. This result argue in favour of a role of p21 in inhibiting cdc2 kinase activity after PMA treatment. However, we cannot exclude the possibility that some other mechanism participates in the PKC-induced inhibition of cdc2 activity. Indeed, it has recently been shown that irradiation and expression of p53 in H1299 cells released from aphidicolin block result in a very significant increase in p21 and an inhibition of cdc2 activity, but, in this case, only a small proportion of total cdc2 is complexed with p21 [44]. Furthermore, PMA may activate a cdc2-independent signaling pathway in the regulation of the G2/M checkpoint in different cell types. Indeed, in this respect, inhibition of the PKC $\beta$ II isoenzyme by chelerythrine has been found to lead to a block of the cell cycle in G2, which was due to an inhibition of PKC-mediated lamin B phosphorylation and mitotic nuclear lamina disassembly [45]. These observations show that the regulation of G2/M transition involves a number of PKC-mediated pathways.

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