p21^{WAF1} expression by an activator of protein kinase C is regulated mainly at the post-transcriptional level in cells lacking p53: important role of RNA stabilization

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p21^{WAF1} inhibits cyclin–cyclin-dependent kinase (Cdk) complexes, causing cell cycle arrest. p21^{WAF1} contains p53-binding sites in its promoter and expression of p21^{WAF1} is induced by functional p53. In the present work, we have studied the role of protein kinase C (PKC) in the induction of p21^{WAF1} and show that induction of p21^{WAF1} expression can occur by activation of PKC in cells having no p53. Human ovarian carcinoma cells, SKOV-3, lack p53 protein and PMA, a potent activator of PKC, did not induce p53. PMA increased the expression of p21^{WAF1} mRNA both in these cells and in other cells which do not contain p53 (THP-1 and U937). Treatment of human embryonic fibroblasts, WI38, with PMA also induced the accumulation of p21^{WAF1} without affecting p53 levels. However, PMA did not increase levels of p21^{WAF1} mRNA in cells where either the PKC or the mitogen-activated protein kinase pathway was blocked. Furthermore, treatment of cells with various phorbol ester

derivatives which activate PKC resulted in the induction of p21^{WAF1} in SKOV-3 cells. In contrast, phorbol esters which do not activate PKC failed to induce p21^{WAF1} expression. PMA increased the transcriptional rate of p21^{WAF1} and activated the transcription of a luciferase reporter gene, controlled by the p21 promoter, in SKOV-3 cells with or without a p53 consensus-binding sequence. By contrast, PMA markedly stabilized p21^{WAF1} mRNA; the half-life ($t_{1/2}$) of p21^{WAF1} in PMA-treated cells was > 8 h compared with < 1 h in untreated cells. These findings provide evidence that the PKC pathway induces expression of p21^{WAF1} independently of p53. Our present study also suggests that the accumulation of p21^{WAF1} transcripts by PMA occurs mainly at post-transcriptional level.

Key words: protein kinase C activation, RNA stabilization, cyclin–cyclin-dependent kinase complex.

INTRODUCTION

Cell proliferation is mediated through various factors such as cytokines or other biological substances. When cells are stimulated through cell-surface receptors, diacylglycerol is initially produced by the hydrolysis of inositol phospholipids; diacylglycerol links extracellular signals and intracellular responses through activation of protein kinase C (PKC) [1]. PKC phosphorylates and activates Raf-1, which leads to the activation of mitogen-activated protein kinase (MAPK) [2]. By contrast, other studies have shown that proliferation of cells is also inhibited by PKC activation; phorbol esters which can activate PKC have been shown to inhibit the phosphorylation of retinoblastoma-susceptibility gene product (Rb) [3]. Moreover, increased levels of diacylglycerol are essential for long-term cellular responses, such as differentiation and a potent activator of PKC, PMA, induces cell differentiation, suggesting that PKC plays crucial roles in signal transduction leading to cell differentiation, a terminal cell cycle arrest [1]. Thus activation of PKC is a physiological event and PKC plays a pivotal role in cellular regulation.

The cell cycle machinery is tightly controlled by a complex molecular mechanism; deregulation results in neoplastic transformation. Studies have shown that regulation of the cell cycle is governed by a series of structurally related serine/threonine protein kinases, which consist of catalytic subunits known as

cyclin-dependent kinases (Cdks) and regulatory subunits, cyclins [4]. Several types of cyclins are made; the expression of cyclins and the activation of the different cyclin–Cdk complexes at different points of the cell cycle are required for cell cycling to occur [4]. Progression of the cell cycle is thus regulated by activating or inhibiting phosphorylation of the Cdk subunits. At different stages of the cell cycle this programme has internal check points [5]. The activated Cdk can be inactivated through several pathways; levels of cyclins may be reduced by turning down transcription or by degradation of the protein itself. Recently, investigators have found additional mechanisms for inactivation of Cdk complexes, including additional regulatory subunits for Cdks [6–8]. These proteins bind to the cyclin–Cdk complex and inhibit its activity. When cells are exposed to external insults, such as DNA-damaging agents, negative regulation of the cell cycle occurs; Cdk activities are inhibited by these proteins and the cell cycle is arrested in either G₁ or G₂ phase, thus preventing the cells from prematurely entering into the next stage of the cell cycle before their DNA is repaired [9,10]. One of these regulatory proteins, named p21^{WAF1}, Sdi1 or Cip1 (a protein of 21 kDa), also blocks proliferating-cell nuclear antigen-dependent DNA replication [8,11]. More recently, other members of this family of inhibitory proteins, including p27^{kip-1} and p57^{kip-2}, have been identified, which can also block the phosphorylation of retinoblastoma by cyclin A–Cdk2, cyclin E–Cdk2 and cyclin D1–Cdk4 [12,13].

Abbreviations used: PKC, protein kinase C; Cdk, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MEK, MAPK kinase; PDD, phorbol 12,13-didecanoate; 4 α -PDD, 4 α -phorbol 12,13-didecanoate; PDB, phorbol 12,13-dibutyrate; 3'UTR, 3'-untranslated region.

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The *p21^{WAF1}* gene contains p53-binding sites in its promoter and studies have shown that the transcription of *p21^{WAF1}* is directly regulated by p53 [12,14] and cells where p53 activity had been lost due to mutation were unable to induce *p21^{WAF1}* in response to DNA damage [8,15,16]. Other reports have shown that *p21^{WAF1}* can be induced by p53-independent mechanisms [17,18]. However, few studies have analysed the signal transduction pathways in the p53-independent expression of *p21^{WAF1}*. In the present study, we have examined the role of PKC in the regulation of the *p21^{WAF1}* gene in cells lacking p53, including the human ovarian cancer cell line (SKOV-3) as well as several other cell lines and the possible mechanisms by which PKC regulates expression of *p21^{WAF1}* also have been explored. Our data show that phorbol esters activate PKC to induce *p21^{WAF1}* gene expression independently of p53 activity and that the induction occurs mainly at the post-transcriptional level.

MATERIALS AND METHODS

Cells and culture

All cell lines used in the present study were obtained from the American Type Tissue Culture Collection. Cells were cultured in α -medium (Cosmo Bio Co. Ltd., Tokyo, Japan) supplemented with 7% (v/v) fetal-calf serum (Mitsubishi Kasei Co., Tokyo, Japan) in a humidified atmosphere containing 5% CO₂. Non-adherent cell lines were cultured at an initial concentration of 2×10^5 cells/ml and flasks containing 1×10^6 cells/ml were used for experiments. For experiments where adherent cell lines were used, subconfluent cultures were employed.

Reagents

The p53 monoclonal antibody PAb1801 (Ab-2) and the *p21^{WAF1}* monoclonal antibody EA10 (Ab-1) were purchased from Oncogene Science (Cambridge, MA, U.S.A.). The anti-human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) polyclonal antibody was from Trevigen (Gaithersburg, MD, U.S.A.). A selective PKC inhibitor, GF109203X [19] and a selective inhibitor of MAPK kinase (MEK), PD-98059 [20], were purchased from Research Biochemicals International (Natick, MA, U.S.A.) and Biomol (Plymouth Meeting, PA, U.S.A.) respectively. Cycloheximide, PMA, 4-O-methyl PMA, phorbol 12,13-didecanoate (PDD), 4 α -phorbol 12,13-didecanoate (4 α PDD) and phorbol 12,13-dibutyrate (PDB) were from Sigma Chemicals (St. Louis, MO, U.S.A.); these reagents were dissolved in acetone and stored at -20°C . Actinomycin D was purchased from Serva (Heidelberg, Germany).

Western-blot analysis

Cells were lysed in buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.02% NaN₃, 0.1% (w/v) SDS, 100 mg/ml PMSF, 1 mg/ml aprotinin, 1% (v/v) Nonidet P-40 and 0.5% (w/v) sodium deoxycholate. After centrifugation, the protein concentrations in each sample were determined by the method of Bradford [21]. Samples, each containing 50 μg of cell lysates in loading buffer were subjected to SDS/PAGE (12% gel) and proteins were transferred to a PVDF membrane (Immobilon; Millipore, Bedford, MA, U.S.A.). Immunoblotting was performed using anti-human p53 or anti-human *p21^{WAF1}* antibodies (10 $\mu\text{g}/\text{ml}$ and 1 $\mu\text{g}/\text{ml}$ final concentration respectively). After washing the blots, alkaline-phosphatase-conjugated goat anti-(human) mouse IgG, diluted 1:2000, was added to the blots. Immunoreactivity was detected by Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Gibco-BRL, Gaithersburg,

MD, U.S.A.). The G3PDH band (30 kDa) was used to confirm that similar amounts of protein were loaded on to each lane.

Cell-cycle analysis

For the flow cytometric analysis of DNA content, cells were cultured, after serum starvation for 48 h, in serum-containing medium with or without 20 nM PMA and then were harvested and fixed with 70% ethanol. Cells were incubated for 30 min at 37°C with 1 $\mu\text{g}/\text{ml}$ RNase A. After washing, cells were resuspended in 500 μl PBS containing 25 μg propidium iodide and stained for 1 h, on ice. Cells were filtered through 50–70- μm pore size nylon mesh and the cell cycle distribution was analysed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

DNA probes

The plasmid containing human *p21^{WAF1}* cDNA (2.1 kb, *NotI*) was kindly provided by Professor B. Vogelstein (Johns Hopkins University, Baltimore, MD, U.S.A.) [8] and p53 cDNA was purified from the pR4-2 plasmid (0.5 kb, *NcoI*). The cyclophilin cDNA probe (0.7 kb, *BamHI*) [14] was a gift from Dr H. Namba (Nagasaki University, Nagasaki, Japan). These probes were ³²P-labelled by a random priming method for Northern blotting [22]. The specific radioactivity was approximately 2×10^8 c.p.m./ μg of DNA.

Isolation and blotting of RNA

Total RNA from cells was obtained by the guanidinium/hot phenol method [23]. Cells were lysed in 4 M guanidinium isothiocyanate/50 mM Tris/HCl, pH 7.6/20 mM EDTA/2% (v/v) sodium lauryl sarcosinate/140 mM 2-mercaptoethanol. The lysed cells were treated with proteinase K and total RNA was extracted with phenol/chloroform (1:1, v/v). For cytoplasmic RNA from adherent cells, cells were lysed with hypotonic buffer containing 10 mM Tris/HCl (pH 7.4), 1 mM KCl, 3 mM MgCl₂ and 0.3% Nonidet P-40 [24]. After denaturation at 65°C , RNA was electrophoresed in agarose/formaldehyde (1%) and transferred to a nylon membrane filter (Amersham). Filters were hybridized with a ³²P-labelled probe for 16–24 h at 42°C in 50% formamide, $2 \times \text{SSC}$ ($1 \times \text{SSC} = 150 \text{ mM NaCl}/15 \text{ mM sodium citrate}$), $5 \times \text{Denhardt's solution}$, 0.1% (w/v) SDS, 10% (w/v) dextran sulphate and 100 $\mu\text{g}/\text{ml}$ of salmon sperm DNA. Filters were washed to a stringency of $0.1 \times \text{SSC}$ for 10 min at 65°C and exposed to X-ray film (RX, Fuji Photo Film Co. Ltd., Kanagawa, Japan). Autoradiograms were developed at different exposures. For quantitative analysis, the relative densities of the hybridization bands of *p21^{WAF1}* were scanned by laser densitometry (LKB UltroScan XL).

Plasmids

A luciferase reporter plasmid controlled by the 2.4 kb *p21^{WAF1}* promoter with (WVP-Luc) or without (DM-Luc) the p53-binding site was constructed as described previously [8]. For the expression of p53, the cDNA of wild-type p53 was cloned into the pcDNA1 containing the cytomegalovirus (CMV) promoter as reported previously [25].

Luciferase assay

Reporter plasmid (2 μg) was transfected to confluent cultures of SKOV-3 by the FuGENE 6 (Boehringer-Mannheim) [26]. Cells were cultured for 24 h after transfection and 2 nM PMA was

added. After incubation for 24 h, cells were rinsed with cold PBS and harvested using a rubber policeman. Cells were lysed with 200 μ l of LC β buffer (Toyo Ink, Tokyo, Japan) and debris was removed by centrifugation. Supernatant (20 μ l) was added to 100 μ l of reconstituted luciferase substrate (Toyo Inc.). Luciferase activity in lysates was assayed by integrating total light emission over 10 s (Lumat LB 9507 luminometer; Berthold, Pforzheim, Germany).

Transcriptional run-on assay

SKOV-3 cells were exposed to PMA, the nuclei were isolated by suspension in hypotonic buffer (10 mM Tris/HCl, pH 7.4/10 mM KCl/3 mM MgCl₂) and lysis was accomplished in 0.5% (v/v) Nonidet P-40. Nuclei were harvested by centrifugation (500 g, 5 min), washed in hypotonic buffer and resuspended in nuclear storage buffer [40% (v/v) glycerol/50 mM Tris/HCl, pH 8.3/5 mM MgCl₂/0.1 mM EDTA]. Nuclei were incubated for 30 min at 30 °C in a reaction buffer containing 150 mM KCl, 3 mM MgCl₂, 0.25 mM ATP, 0.25 mM GTP, 0.25 mM CTP and 200 μ Ci of [α -³²P]UTP (3000 Ci/mmol). The reaction was terminated by adding DNase I (10 min at 30 °C). The reaction products were digested with 40 μ g/ml of proteinase K in a solution containing 10 mM EDTA and 1% (w/v) SDS, followed by phenol/chloroform (1:1, v/v) extraction. The aqueous phase was precipitated with 70% ethanol at -70 °C in the presence of 0.3 M sodium acetate and the precipitate was collected by centrifugation and dissolved in TE buffer (10 mM Tris/HCl, pH 8.0/1 mM EDTA). After denaturation in ice-cold 0.2 M NaOH and neutralization in 0.2 M Hepes, nuclear RNA was passed through a Sephadex G50 spun column to remove unincorporated [³²P]UTP. Plasmid DNA containing the cDNA coding inserts was denatured by heat and alkalization (0.3 M NaOH). Denatured plasmids (5 μ g for p21^{WAF1} and p53 and 2 μ g for cyclophilin) were bonded to nylon membranes (Hybond-N) using Bio-Dot SF (Bio-Rad) and immobilized by UV cross-linker. Newly elongated nuclear RNA was hybridized to the filters containing plasmids. Hybridizations were performed with ³²P-labelled RNA (10⁷ c.p.m./ml) in 3 $\times$ SSC containing 5 mM EDTA, 0.1% (w/v) SDS, 10 $\times$ Denhardt's solution, 50% (v/v) formamide, 10 mM NaH₂PO₄ (pH 7.0), 200 μ g/ml of yeast tRNA and 100 μ g/ml of salmon sperm DNA for 3 days at 42 °C. After hybridization, the filters were rinsed in 2 $\times$ SSC at room temperature and then in 2 $\times$ SSC and finally in 0.1 $\times$ SSC at 42 °C. The relative density of the hybridization bands of p21^{WAF1} and cyclophilin in untreated and radioactive lanes was scanned by laser densitometry (LKB Ultrascan XL).

RESULTS

Induction of p21^{WAF1} protein by PMA in SKOV-3 cells

We determined whether PMA affected the expression of p21^{WAF1} in SKOV-3 cells, which lack p53, by Western blotting using a p21^{WAF1} monoclonal antibody (Figure 1). SKOV-3 cells were cultured with 0, 0.2, 2 or 20 nM PMA for 8 h, harvested and the levels of p21^{WAF1} in total cell protein was examined. The level of p21^{WAF1} was significantly increased in cells treated with 2 nM of PMA and accumulation of p21^{WAF1} increased little above this concentration. At 20 nM of PMA, the level of p21^{WAF1} was approx. six times greater than that from untreated cells.

Dose-dependent effect of PMA on levels of p21^{WAF1} mRNA in SKOV-3 cells

The SKOV-3 cells were cultured with 0.2, 2 or 20 nM PMA for 4 h and gels were subjected to Northern-blot analysis using ³²P-

Figure 1 Expression of p21^{WAF1} in SKOV-3 cells cultured with PMA

Cells were cultured with PMA (0.2–20 nM) for 8 h. After cell lysis, whole cell protein (50 μ g) was subjected to SDS/PAGE (12% gel), transferred to PVDF membranes and analysed for p21^{WAF1} protein (WAF1) as described in the Materials and methods section. SK-HEP-1, a hepatoma cell line, was used as a positive control and the G3PDH protein as a loading control.

labelled p21^{WAF1} and p53 cDNA probes (Figure 2). PMA at 2 nM increased p21^{WAF1} gene expression to a detectable level; the induction of p21^{WAF1} RNA was dependent on the dose of PMA. In contrast, transcripts of p53 were not detected in these cells.

Figure 2 Dose-dependent effect of PMA on levels of p21^{WAF1} mRNA in SKOV-3 cells

Cells were cultured with PMA for 4 h. Upper panel: total RNA (15 μ g/lane) was prepared and analysed by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane, as described in the Materials and methods section. Hybridization was with ³²P-labelled p21^{WAF1} cDNA (WAF1) (2.1 kb band) and p53 cDNA (2.8 kb band). Lower panel: ethidium bromide-stained formaldehyde gel before Northern blotting; levels of 28 S and 18 S rRNA were comparable in each lane.

Figure 3 Effect of phorbol ester derivatives on induction of $p21^{WAF1}$ in SKOV-3 cells

Upper panels: cells were cultured with various phorbol compounds (20 nM) for 8 h. The levels of $p21^{WAF1}$ protein (WAF1) and p53 (indicated with arrows) were determined by Western blotting of total cell lysate (50 μ g/lane) and immunostaining using anti- $p21^{WAF1}$ antibodies and anti-p53 antibodies. Bottom panel: cells were exposed to various phorbol compounds (50 nM) for 4 h. Northern-blot analysis of $p21^{WAF1}$ mRNA (WAF1) was performed using total RNA. The levels of 28 S and 18 S mRNA are shown in an ethidium bromide-stained formaldehyde gel, before Northern blotting.

Effect of phorbol derivatives on levels of $p21^{WAF1}$ protein and mRNA in SKOV-3 cells and WI38 fibroblasts

The potent phorbol diester, PMA, activates PKC in a variety of cells [27]. Various phorbol derivatives were examined for their ability to stimulate accumulation of $p21^{WAF1}$ protein (Figure 3, upper) and mRNA (Figure 3, bottom panel). PMA, PDD and PDB are potent activators of PKC [27]; their derivatives 4-*O*-methyl PMA and 4 α -PDD are poor stimulators of PKC. Cells were cultured with 20 nM of each derivative for either 8 h (for protein) or 4 h (for mRNA). The potency of each of the phorbol diesters to stimulate the accumulation of $p21^{WAF1}$ protein and mRNA paralleled their known abilities to activate PKC [27].

Figure 4 Effect of phorbol ester derivatives on $p21^{WAF1}$ expression in human fibroblasts WI38

Upper panel: WI38 cells were untreated or cultured with PMA (2 or 20 nM) for 8 h and levels of $p21^{WAF1}$ (WAF1) and p53 proteins were determined by Western-blot analysis. Lower panel: WI38 cells were treated with various phorbol compounds (20 nM) for 8 h as described for Figure 3. The levels of $p21^{WAF1}$ protein (WAF1) were determined by Western blotting. G3PDH was used as a loading control.

These derivatives did not induce p53 expression in these cells. Similar results for levels of $p21^{WAF1}$ expression were obtained in human embryonic lung fibroblasts WI38 (Figure 4). The PKC-activators did not affect the levels of p53 protein in these fibroblasts.

Induction of the $p21^{WAF1}$ gene by PMA in other cells lacking p53

In order to investigate whether PMA was capable of inducing $p21^{WAF1}$ mRNA in other cells having no p53, human monocytic

Figure 5 Induction of p21^{WAF1} mRNA expression by PMA in myeloid cells

Human histiocytic cells U937 (left) or monocytic THP-1 cells (right) were cultured with 0.2, 2 or 20 nM of PMA for 4 h. Total RNA was extracted from each cell line and analysed by formaldehyde-agarose gel electrophoresis (15 µg/lane) as described in the Materials and methods section. Hybridization was with ³²P-labelled p21^{WAF1} (WAF1) and p53 cDNA. Bottom panel: ethidium bromide stained formaldehyde gel before Northern blotting showing positions of 28 S and 18 S mRNA.

Table 1 Cell-cycle distribution in SKOV-3 cells treated with or without PMA

Cells were cultured in serum-free medium for 48 h and then stimulated with 7% (v/v) fetal-calf serum in the presence or absence of 20 nM PMA. Cells were harvested at various times, fixed in 70% ethanol and the DNA content of the cells was estimated by propidium iodide staining.

		Distribution of cells in each stage (%)				
		0 h	6 h	12 h	24 h	48 h
Untreated	G ₁	62.4	46.5	44.4	46.3	79.4
	S	27.3	26.0	42.2	43.9	12.2
	G ₁ /S ratio ...	2.3	1.8	1.1	1.1	6.5
	G ₂ /M	10.3	27.5	13.4	9.8	8.4
PMA	G ₁		56.8	54.3	51.3	59.0
	S		25.1	21.8	43.2	31.8
	G ₁ /S ratio ...		2.3	2.5	1.2	1.9
	G ₂ /M		18.1	23.9	5.5	9.9

cells (THP-1) and human histiocytic cells (U937) were examined (Figure 5). The THP-1 and U937 cells did not express p53 mRNA, which is consistent with the results of previous studies [28,29]. The cells were treated with 0.2, 2 or 20 nM PMA for 4 h, harvested and examined by Northern blotting. Exposure to PMA clearly induced expression of p21^{WAF1} mRNA in the p53-negative cells.

Table 2 Cell-cycle analysis of WI38 human fibroblasts treated with or without PMA

Cells were cultured in serum-free medium for 48 h and then stimulated with 7% (v/v) fetal-calf serum in the presence or absence of 20 nM PMA. After 12 h, cells were harvested, fixed in 70% ethanol and the DNA content of the cells was estimated by propidium iodide staining.

		Distribution of cells in each stage (%)	
		0 h	12 h
Untreated	G ₁	82.9	49.2
	S	6.3	46.6
	G ₁ /S ratio ...	14.2	1.1
	G ₂ /M	10.7	4.2
PMA	G ₁		59.2
	S		21.1
	G ₁ /S ratio ...		2.8
	G ₂ /M		19.2

Effects of PMA on cell cycle progression in SKOV-3 and WI38 cells

To determine the effect of PMA on the cell cycle progression in SKOV-3 and WI38 fibroblasts, flow cytometric analysis of cells stained with propidium iodide was performed. Analyses of the cell cycle of SKOV-3 and WI38 cells after exposure to PMA are shown in Tables 1 and 2 respectively. Cell cycles were synchronized by serum deprivation for 48 h, the medium was then replaced with fresh medium containing 7% (v/v) fetal-calf serum. When SKOV-3 cells were released from the block caused by serum deprivation, the cell cycle continued and more than 40% of the cells had progressed into S phase by 12 h. Thereafter, cells gradually re-entered G₁ phase. In the presence of PMA, cell-cycle progression was delayed when compared with control cells; the percentage of cells in S-phase increased from 27.3% to 43.2% by 24 h with a concomitant decrease in the G₁/S ratio. Moreover, in WI38 fibroblasts, treatment with PMA failed to induce cell cycle arrest and cell cycle progression was delayed when compared with cells cultured with serum alone.

Effect of inhibition of the PKC pathway on expression of p21^{WAF1} mRNA

Cells subjected to prolonged exposure to PMA had reduced PKC activity, thus making them resistant to repeated exposure to PMA [30]. SKOV-3 cells cultured with PMA (50 nM, 4 h) had markedly increased accumulation of p21^{WAF1} mRNA compared with untreated cells and prolonged exposure (24 h) to PMA (100 nM) resulted in a return in the level of p21^{WAF1} mRNA to baseline (Figure 6, upper panels). Re-exposure of these cells to PMA (50 nM) for 4 h failed to increase the baseline level of p21^{WAF1} transcripts. These experiments were repeated twice with similar results. Experiments were also performed using a specific inhibitor of PKC, GF-109203X (1 or 10 µM) [19], which almost completely inhibited the PMA-induction of expression of p21^{WAF1} mRNA in SKOV-3 cells (Figure 6, lower panels).

Effects of MEK inhibition on expression of p21^{WAF1} mRNA by PMA in SKOV-3 cells

Many extracellular stimuli, including growth factors and stress, result in activation of phosphorylation cascades through MAPK.

Figure 6 Effect of inhibition of the PKC pathway on expression of p21^{WAF1} mRNA

Upper panels: SKOV-3 cells were untreated, treated with PMA (100 nM, 24 h), or pre-treated with PMA (100 nM, 24 h), washed and re-treated with PMA (50 nM). Total RNA was extracted after 4 h and Northern blotting was performed. As a control, cells were cultured with PMA alone (50 nM, 4 h). Lower panels: SKOV-3 cells were cultured with either 1 μ M or 10 μ M of GF-109203X (GF), a specific inhibitor of PKC, for 1 h and then 20 nM of PMA was added. As controls, cells were treated with either PMA (20 nM) alone or GF-109203 (10 μ M). WAF1, p21^{WAF1} mRNA. The levels of 28 S and 18 S RNA are shown in an ethidium bromide-stained formaldehyde gel, before Northern blotting.

To examine the role of the MAPK cascade in the induction of p21^{WAF1} mRNA by PMA, a specific inhibitor of MEK (also known as extracellular signal-related kinase, ERK), PD-98059

Figure 7 Effects of MEK inhibition on expression of p21^{WAF1} mRNA by PMA in SKOV-3 cells

SKOV-3 cells were untreated, pre-treated with PD-98059 (PD) (10 or 100 μ M), a specific inhibitor of MEK, for 1 h and then PMA was added (20 nM). After 4 h, total RNA was extracted. As controls, cells were treated with either PMA (20 nM) alone or PD-98059 (100 μ M) alone. Bottom panel: the levels of 28 S and 18 S RNA are shown in an ethidium bromide-stained formaldehyde gel, before Northern blotting.

was used (Figure 7). This compound inhibits both the phosphorylation and activation MAPKs but has no inhibitory activity against a number of other serine/threonine and tyrosine kinases [20]. SKOV-3 cells were cultured for 1 h with either 10 or 100 μ M of PD-98059 and were then treated with 20 nM of PMA for 4 h (Figure 7). Pretreatment with PD-98059 attenuated the induction of expression of p21^{WAF1} mRNA by PMA by more than 80%.

Transcriptional regulation of p21^{WAF1} in PMA-treated SKOV-3 cells

Transcriptional run-on assays were performed to help to determine the mechanisms responsible for the accumulation of p21^{WAF1} transcripts by PMA (Figure 8). p21^{WAF1} was constitutively transcribed in untreated SKOV-3 cells. Exposure of the cells to PMA (2 h) increased the transcriptional rate in a dose-dependent manner; treatment with 2 nM PMA stimulated the rate about 2-fold.

In order to determine whether the increase in transcriptional rate of p21^{WAF1} by PMA required the p53-responsive element, a reporter plasmid controlled by the p21^{WAF1} promoter was constructed; the 2.4 kb promoter of p21^{WAF1} with (WWP-Luc) or without (DM-Luc) the p53-responsive element was placed upstream of promoterless luciferase reporter gene. Treatment with PMA increased the luciferase activity in SKOV-3 cells transfected with the reporter gene DM-Luc as well as those with the WWP-Luc (Figure 9). In contrast, co-transfection with wild-type p53 expression vector markedly enhanced luciferase activity (15-fold).

Stability of steady-state p21^{WAF1} mRNA in SKOV-3 cells treated with PMA

To determine if PMA affected post-transcriptional regulation of p21^{WAF1} mRNA, SKOV-3 cells were either untreated or treated with PMA (20 nM for 4 h) and then actinomycin D (5 μ g/ml)

Figure 8 Transcriptional regulation of p21^{WAF1} in PMA-treated SKOV-3 cells

Cells were either untreated or treated with PMA (2 or 20 nM) for 2 h and the nuclei were isolated as described in the Materials and methods section. Upper panel: newly elongated ³²P-labelled transcripts were hybridized to the linearized plasmid containing inserts of either p21^{WAF1} (WAF1), cyclophilin or the control plasmid, pUC19. Lower panel: quantitative analysis of the relative density of the bands of hybridized p21^{WAF1} and cyclophilin in untreated and PMA-treated lanes, scanned by laser densitometry.

Figure 9 Effects of PMA on luciferase activity from reporter gene controlled by the p21 promoter

SKOV-3 cells were transfected with luciferase reporter gene (2 µg) having the 2.4 kb natural promoter elements of p21 with (WWP-Luc) or without (DM-Luc) a p53 binding sequence. As positive control, the p53 expression vector was co-transfected with the WWP-Luc. After 24 h of culture, PMA (2 nM) was added to selected cultures for an additional 24 h, the cells were harvested and luciferase assay was performed. Luciferase activity was measured by integrating total light emission using a Berthold luminometer. Results are expressed as a fold-increase in luciferase activity relative to control (no p53 transfected/no PMA added to plates).

Figure 10 Stability of steady-state p21^{WAF1} mRNA in SKOV-3 cells exposed to PMA

Upper panel: untreated cells or cells exposed to PMA (20 nM for 4 h) were cultured with actinomycin D (5 µg/ml) for 1–8 h. Total RNA (30 µg/lane for untreated cells and 15 µg/lane for PMA-treated cells) was extracted and analysed by Northern blotting, as described in the Materials and methods section. The levels of 28 S and 18 S RNA are shown in an ethidium bromide-stained formaldehyde gel, before Northern blotting. Lower panel: the intensity of hybridization was determined by densitometry of several different exposures of the autoradiograms. p21^{WAF1} RNA (WAF1) stability was expressed as a percentage of control; untreated cells were assumed to have 100% activity.

was added to cultures. Cells were sequentially harvested (1–4 h) and examined for levels of p21^{WAF1} mRNA (Figure 10). In order to make clear the constitutive levels of p21^{WAF1} mRNA in untreated cells, the blot probed with the ³²P-labelled p21^{WAF1} cDNA was exposed to X-ray film for longer than usual (96 h). The half-life ($t_{1/2}$) of steady-state p21^{WAF1} mRNA in untreated SKOV-3 cells was < 1 h, whereas the $t_{1/2}$ of p21^{WAF1} mRNA was > 8 h when cells were exposed to PMA. Treatment with PMA also stabilized p21^{WAF1} mRNA in THP-1 cells (results not shown).

Stabilization of p21^{WAF1} by PMA in SKOV-3 cells

The stability of the p21^{WAF1} protein was examined in PMA-treated SKOV-3 cells (Figure 11). After treatment of cells with 2 nM PMA for 8 h, the cells were cultured with cycloheximide (20 µg/ml) to inhibit *de novo* protein synthesis. At the indicated times, steady-state levels of p21^{WAF1} were determined. The half-life of p21^{WAF1} in control cells was < 2 h. In contrast, the half-life of the p21^{WAF1} protein was extended to almost 4 h in cells cultured with PMA.

Figure 11 Stabilization of p21^{WAF1} protein by PMA in SKOV-3 cells

Cells were untreated or cultured with 2 nM PMA for 4 h, cycloheximide was then added to a final concentration of 20 μ g/ml. Upper panel: at the indicated times, cells were harvested and whole cell protein (150 μ g/lane for untreated cells and 50 μ g/lane for PMA-treated cells) was analysed by Western blotting as described in the Materials and methods section. G3PDH was used as a loading control. Lower panel: p21^{WAF1}, determined as shown in the upper panel, were quantified by densitometry and the relative density of the bands were compared. Untreated cells were assumed to have 100% activity

DISCUSSION

Mutations of the p53 gene are commonly found in various human cancers [31] and loss of normal p53 activity leads to uncontrolled cell growth [32]. Although the mechanism of control by p53 is unclear, p53 is known to bind to DNA in a sequence-specific manner and stimulate the transcription of genes downstream of the binding sites [33]. p21^{WAF1} is an inhibitor of the kinase activity of the cyclin–Cdk complex. The upstream region of the p21^{WAF1} gene contains p53 binding sites, which allow p53 to bind to the p21^{WAF1} promoter region and enhance transcription of the gene [8,14]. When exposed to external insults, cells activate p53 to induce expression of p21^{WAF1}, which causes arrest of the cell cycle, suggesting that p53 plays an important role as a checkpoint of the cell cycle [15,16]. Recent studies, including ours, have demonstrated that p21^{WAF1} could be induced in cells lacking a functional p53 [34,35]. The pathways of signal transduction inducing expression of p21^{WAF1} in p53-deficient cells are not fully understood. A recent study has shown that activation of MAPK induces p21^{WAF1} expression [36] and also studies suggest the possible involvement of PKC in modulating p21^{WAF1} expression [15,17,34]. In the present study, we provide evidence that p21^{WAF1} expression is induced by activation of PKC through a pathway which does not require p53. This increased expression of p21^{WAF1} by exposure to PMA occurs mainly through stabilization of p21^{WAF1} mRNA and protein, while stimulation of transcription of p21^{WAF1} was observed.

PKC is involved in signal transduction by coupling receptor-mediated inositol phospholipid turnover with a variety of cellular functions [1]. Our results show that an activator of PKC, PMA,

induced p21^{WAF1} mRNA expression in a dose-dependent manner. We also found that phorbol compounds which activate PKC (PMA, PDD, PDB) markedly increased levels of p21^{WAF1} mRNA and protein in SKOV-3 cells. In contrast, phorbol esters (4-*O*-methyl PMA, 4 α -PDD), which are incapable of activating PKC, were unable to increase levels of p21^{WAF1} protein and mRNA in these cells. Prolonged exposure of cells to PMA is known to cause inactivation of PKC by a feedback inhibition pathway [30]. Taking advantage of this fact, we found that prolonged exposure to PMA (100 nM for 24 h) blocked the accumulation of p21^{WAF1} mRNA after re-exposure of cells to PMA. Moreover, an inhibitor of PKC, GF-109203X, almost completely prevented the induction of p21^{WAF1} by PMA. The SKOV-3 cells have a rearrangement of the p53 gene and no transcripts or protein of p53 are detected [37]. Consistent with this finding, our Western blot data using monoclonal anti-p53 antibody, which reacts with both wild-type and mutated p53 [38], detected no p53 in SKOV-3 cells. The two myeloid cell lines used also lacked p53; the THP-1 cells have a 26-base deletion beginning at codon 174 of the p53 coding sequence and the U937 cells also have a deletion of 46 bases beginning at codon 172 [28,29]. Treatment with PMA induced the accumulation of p21^{WAF1} transcripts in the absence of p53 in these cells. Moreover, expression of the p21^{WAF1} gene was induced by activation of PKC in fibroblasts with a normal p53, without affecting p53 levels. We have also shown that inhibition of MEK with PD-98059 blocked PMA-induced p21^{WAF1} expression. Recent studies reported that activation of the MAPK signalling pathway via PKC is an important mechanism for several biological events, such as apoptosis and PKC regulates the MAPK pathway alone or with other mechanisms [39,40]. Taken together, our studies indicate that activation of PKC leads to the accumulation of p21^{WAF1} transcripts through a p53-independent pathway and that activation of the MAPK signalling cascade is required for the induction of p21^{WAF1} by PMA.

Other important findings of this study are that the transcriptional rate of p21^{WAF1} was increased in response to PMA in the SKOV-3 cells and PMA induced the transcription of a luciferase reporter gene regulated by the p21 promoter without and with a p53 binding site. These results suggest that activation of PKC may induce a factor(s) which increases transcription of p21^{WAF1} through a pathway other than p53. Recently, phorbol ester and okadaic acid have been shown to activate the p21^{WAF1} promoter through Sp1 binding sites [41]. Moreover, 1 α ,25-dihydroxycholecalciferol (vitamin D₃), an inducer of myeloid cells to differentiate into monocytes/macrophages, increased the transcriptional rate of p21^{WAF1} in U937 cells that lack p53 and a nuclear vitamin D₃ receptor-binding site was identified 770 bp upstream of the start site of p21^{WAF1} transcription (–778 to –756) [42]. In myoblasts, the skeletal muscle-specific transcriptional regulator, MyoD, induces the p21^{WAF1} transcription in a p53-independent manner [43]. Furthermore, a serum-response element was identified between positions –2581 and –1871 in the murine p21^{WAF1} promoter [44]. A more recent study has shown that either interferon- γ (IFN- γ) or epidermal growth factor induces p21^{WAF1} transcription through signal transducers and activators of transcription (STAT) proteins; sis-inducible elements (SIE, –640 nt, –2540 nt and –4183 nt from the TATA promoter site) were identified in the p21^{WAF1} promoter and induction of p21^{WAF1} through these DNA sequences did not involve p53 [45]. Moreover, studies have shown that activation of signal transducers and activators of transcription by factors including epidermal growth factor and interferon was blocked by inhibition of PKC [46–48]. Our transcriptional run-on and luciferase assays, using the natural promoter element of p21, however, showed that exposure to PMA increased p21^{WAF1}

transcription only 2-fold as compared with untreated SKOV-3 cells. Therefore our results suggest that increased levels of p21^{WAF1} mRNA in PMA-treated cells are not due entirely to transcriptional activation.

The steady-state level of mRNAs in the cell is dependent on both the rates of transcription and decay. Stabilization is one of the important mechanisms for accumulation of mRNAs, especially short-lived ones [49]. A recent study has shown that the cell-cycle-coupled accumulation of topoisomerase II α transcripts is regulated through a post-transcriptional mechanism [50]. Furthermore, studies have shown that another cell-cycle related protein, c-Myc, is modulated, in part, by changes in the stability of its mRNA [51]. Various extracellular stimuli such as protein synthesis inhibitors and stimulators of PKC are able to stabilize c-Myc RNA [52]. Indeed, proteins important in the cell cycle must be able to undergo rapid changes as the cell comes in contact with various positive and negative stimuli of cellular proliferation. Changes in the stability of specific mRNA afford an extremely rapid mechanism to change the levels of a critical cell-cycle-related protein. In the present study, the $t_{1/2}$ of p21^{WAF1} mRNA was less than 1 h in untreated SKOV-3 cells; PMA markedly stabilized p21^{WAF1} mRNA in these cells ($t_{1/2} > 4$ h). These results suggest that p21^{WAF1} mRNA levels in PMA-treated cells were mainly regulated by a post-transcriptional mechanism. Previous studies have reported sequence determinants affecting mRNA stability, including poly(A) and the 3'-untranslated region (3'UTR) [53]. However, no mammalian RNase which might be programmed to degrade specific mRNAs has yet been identified. Thus the molecular or enzymological mechanism underlying the stabilization of p21^{WAF1} mRNA is not fully understood, although the degradation of mRNA appears to involve endoribonucleases. A recent study on p21^{WAF1} protein reported PMA-induced expression of a novel form of p21^{WAF1} protein, which mediated G₂/M arrest through protein-modification and that this protein may be truncated at the C-terminus as a result of PKC-mediated proteolytic cleavage [54]. Many labile mRNAs coding for oncogenes, including c-Myc and cytokines, have AUUUA repeats in the 3'UTR [55,56]. Previously, we found that PMA could stabilize more effectively a reporter RNA containing three or more repeats of the AUUUA motif in the 3'UTR than those with two or less. The p21^{WAF1} mRNA has three repeats of the AUUUA motif [8]. Indeed, there are several observations which suggest increased levels of AU-binding protein interacting with AU-rich elements in PMA-treated cells [56,57]. Other studies have shown that a calcium ionophore, A23187, stabilized mRNAs coding for interleukin-3 and granulocyte/macrophage colony-stimulating factor through a pathway which requires AU-rich elements in the 3'UTR [58,59]. These studies suggest that the transient accumulation or redistribution of intracellular calcium may be also one of mechanisms for the stabilization of mRNAs by phorbol esters and PKC activation. Redox is also known to modulate the stability of mRNAs coding for cytokines and oncogenes by the AU-binding protein [60] and treatment of cells with PMA causes oxidative stress [61]. Taken together, the PMA may stabilize p21^{WAF1} mRNA through the AU-rich region in 3'UTR. Further studies are required to determine the mechanism resulting in stabilization of these transcripts.

In the present work, Northern- and Western-blot analyses clearly showed that PMA induced p21^{WAF1} expression at both the mRNA and protein level. However, these analyses revealed the discrepancy between the induced levels of p21^{WAF1} protein and mRNA in SKOV-3 cells. These results suggest that the increased levels of p21^{WAF1} following exposure to PMA may involve modification at protein level. A recent study has shown that

vitamin D₃ regulated p21^{WAF1} expression at the protein level; vitamin D₃ increased levels of the p21^{WAF1} protein without altering those of p21^{WAF1} mRNA [62]. Therefore, we examined the effect of PMA-treatment on the stability of p21^{WAF1}. Our results demonstrated that PMA markedly stabilized the p21^{WAF1} protein. Levels of regulatory proteins important for the cell cycle, including p53 and p21^{WAF1}, are usually low because of the short half-life. The turnover of these proteins is regulated by ubiquitin-mediated proteolysis [63]. The p53 protein is stabilized in response to various DNA-damaging agents such as irradiation, UV, actinomycin D, cisplatin or etoposide [63]. By contrast, a recent study has reported that γ - or UV-irradiation failed to stabilize p21^{WAF1}, whereas p53 was stabilized [64]. These results demonstrate that stabilization of the p21^{WAF1} protein is regulated through different mechanism(s) from the p53 protein, which may have repercussions on p53-independent induction of p21^{WAF1}.

The ability of p21^{WAF1} to inhibit kinase activity depends upon its stoichiometric ratio to the complex of Cdk, cyclin and proliferating-cell nuclear antigen. p21^{WAF1}-containing cyclin kinases exist in both active and inactive states [65] and it has been shown that p21^{WAF1} inhibits the Cdk complex when more than one molecule of p21^{WAF1} binds to the complex and changing the ratio of p21^{WAF1} to cyclin-Cdk results in conversion of active complexes into inactive ones [65,66]. In the present study, the magnitude of induced levels of p21^{WAF1} mRNA in SKOV-3 cells was less than that of the control cells, SK-HEP1, which have rearrangement of the p53 gene but normal size transcripts and levels of protein [67]. To determine whether a relatively small magnitude of p21^{WAF1} induction is physiologically relevant, we studied the cell-cycle profile in PMA-treated SKOV-3 cells. By flow cytometric analysis it was found that PMA delayed cell-cycle progression but failed to induce cell-cycle arrest in SKOV-3 cells. We also determined the effect of PKC-activating phorbol esters on the expression of p21^{WAF1} protein, by Western blotting, in WI38 human fibroblasts which carry wild-type p53. Treatment of these fibroblasts with phorbol esters induced p21^{WAF1} expression without affecting p53 levels. These results suggest that activation of PKC induces p21^{WAF1} expression through a p53-independent mechanism in WI38 human fibroblasts. However, the induced levels of p21^{WAF1} were relatively low and the results of cell-cycle analysis in PMA-treated fibroblasts were similar to those of SKOV-3 cells. The levels of p53-independent p21^{WAF1} expression have been reported to be greatly decreased when compared with p53-dependent expression [65]. Taken together, the p53-independence of p21^{WAF1} expression by PKC may occur at subsaturating levels, which may be unable to arrest the cell cycle.

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