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p53-Independent Elevation of p21 Expression by PMA Results from PKC-Mediated mRNA Stabilization

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The p21 (cip1/waf1) protein induces cell cycle arrest through inhibition of the activity of cdk (cyclin dependent kinase)/cyclin complexes. Expression of p21 is induced in a p53-dependent manner by DNA damage. p21 can also be induced independently of p53 by phorbol ester or okadaic acid. In this study, we have addressed the role of the PKC (protein kinase C) signaling pathway in the induction of p21 in response to PMA (phorbol myristate acetate) and okadaic acid. Levels of p21 (protein and mRNA) rapidly increased (within ~4 h) in U937 cells treated with PMA. The PKC-specific inhibitors RO 31-8220 and GF109203X down-regulated PMA or okadaic acid-induced p21 expression. Following persistent PKC activation, p21 mRNA levels remained elevated, indicating an enhanced stability of the mRNA. Using actinomycin D to measure mRNA stability and p21 promoter luciferase assays to measure activity, we provide evidence to support a role for the PKC signaling pathway in p21 mRNA stability. Thus, PKC regulates the amount of p21 in U937 cells at the level of mRNA accumulation and translation.

Cyclin/cdk complexes facilitate progression through the cell cycle and are activated at specific points during the cell cycle (1–3). The cdk inhibitor p21 plays a critical role in regulating cell cycle progression. p21 has been implicated in mediating growth arrest in response to a variety of conditions associated with DNA damage, cell differentiation, or growth factor deficiency (4–6). p21 gene expression is regulated by transcriptional and post-transcriptional mechanisms. Transcriptional regulation of p21 is characterized by activation of p53-dependent pathways after DNA damage. However, p53-independent expression of p21 is associated with serum stimulation and with cellular senescence and differentiation (4–9).

PMA and okadaic acid activate transcription of the p21 promoter through the Sp1 transcription factor but p21 can be induced by several transcription factors including p53, E2F, STAT, C/EBPα, etc. (4, 5, 10–13). Liu *et al.* reported that differentiation of U937 cells treated with vitamin D3 is facilitated by the transcriptional induction of the p21 gene by the vitamin D3 receptor (14). Other studies have demonstrated that p21 expression is regulated by post-transcriptional controls (15, 16). The Elav-like proteins (HuC, Hel-N1, HuD and HuR) are specific mRNA-binding proteins that regulate mRNA stability (17). p21 mRNA contains a conserved element in its 3′-untranslated region that is bound by the Elav-like mRNA-stabilizing proteins (17). Wang *et al.* have shown that post-transcriptional regulation of p21 by UV light induces elevation of HuR in the cytoplasm which enhances p21 mRNA stability (18). However, the underlying mechanisms controlling p21mRNA stability are poorly understood.

In this study, we show that activation of PKC regulates p21 expression at the posttranscriptional level by stabilizing p21 mRNA in U937 cells. We focused our studies on the characterization of the effect of PKC inhibitors on p21 mRNA destabilization.

MATERIALS AND METHODS

Cell culture and reagents. The human leukemia U937 cells were obtained from ATCC (Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle medium containing 10% FCS, 20 mM Hepes, 100 μg/ml gentamicin (complete medium). Anti-cdk2, anti-hsp70, anti-p21 and anti-p27 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). RO 31-8220, SB 203580, PD 098059, Rottlerin, GF109203X and LY 294002 were purchased from Biomol (Biomol Research Laboratories, Inc., PA). Other chemicals were purchased from Sigma.

Western blot analysis. Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM

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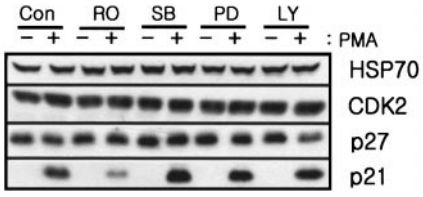

FIG. 1. Inhibition of PMA-induced p21 expression by various protein kinase inhibitors. U937 cells (2×10^6 cells) were pretreated with RO 31-8220 (2 μM), SB 203580 (10 μM), PD 098059 (50 μM) and LY 294002 (25 μM) for 2 h and then washed. Cells were incubated with or without 20 nM PMA for 24 h and then harvested in lysis buffer. Equal amounts of soluble lysates (50 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against p21, p27, cdk2, and Hsp70.

EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM Mops, 2 μg/ml proteinase inhibitor E64, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. Fifty micrograms of cell lysate were electrophoresed on 10% SDS–polyacrylamide gels. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Detection of the specific proteins was carried out with an ECL kit following the manufacturer's instructions.

Northern blot hybridization. Total RNA was isolated according to Chomczymski and Sacchi (19). Ten micrograms of total RNA were separated on a 1.2% agarose gel containing 6% formaldehyde in 20 mM Mops, pH 7.0, 8 mM sodium acetate and 1 mM EDTA, transferred to Hybond N^+ nylon membrane (Amersham Pharmacia Biotech) by the capillary methods, and cross-linked by UV irradiation. The membrane was then incubated overnight at 42°C in Northern-Max hybridization solution (Ambio, Inc.) with [α-^{32}P] dCTP-labeled probes of the 2.0 kb long *Xho*I fragment purified from the pBS-CIP1 plasmid. The membranes were washed under high stringency conditions: once with 2× SSC/0.1% SDS for 20 min at room temperature, once with 2× SSC/0.1% SDS at 42°C for 30 min, and once with 0.5× SSC/0.1% SDS for 30 min at 65°C before exposure to film for 1–2 days. The intensity of the band was measured using a phosphorimager.

DNA transfection and luciferase assay. The 2.4 kb promoter region of p21 gene was subcloned into the *Hin*dIII-digested pGL-2 basic vector (Promega, Madison, WI), and then p21 promoter fused luciferase gene was resubcloned into the pcDNA3.1 vector. 400 μl of U937 cells in RPMI 1640 (20×10^6 cells/ml) were transfected by preincubating with 15 μg of p21 promoter plasmid for 10 min at room temperature and then electroporating at 500 V, 700 μF. The sample was immediately put on ice for 10 min and then 10 ml of complete medium was added and then the cells incubated at 37°C for 24 h. The cells were selected in a medium containing 0.7 mg/ml of geneticin (G418) for 4 weeks. To asses the stable expression of p21 promoter luciferase, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions.

RESULTS

Induction of p21 Protein by PMA in U937 Cells

To examine the role of the cell signaling pathways in the induction of p21 by PMA, we measured expression of p21 in p53 negative U937 cells by Western blotting using a p21 monoclonal antibody (Fig. 1). U937 cells were exposed to RO 31-8220 (PKC inhibitor), SB 203580 (p38 MAPK inhibitor), PD 098059 (MEK inhibitor), LY 294002 (PI3 kinase inhibitor) prior to the addition of the 20 nM PMA. Preincubation with RO 31-8220 in the culture medium before the addition of PMA significantly decreased p21 expression level, whereas SB 203580, PD 098059 and LY 294002 did not induce p21 down-regulation. In contrast, the levels of p27, cyclin A and cdk2, which were readily detectable in exponentially growing cells, remained unaffected by the 6-h incubation with PMA. These results suggest that the PMA-induced increase in p21 was mediated via activation of a protein kinase C.

Effect of PKC Inhibitor, RO 31-8220, on Down-Regulation PMA-Mediated p21 Expression

To ascertain whether inhibition of PKC influenced PMA-induced increase of p21 level, U937 cells were treated 20 nM PMA for 4 h. PMA was removed before treatment with or without 2 μM RO 31-8220. As shown in Fig. 2A, p21 expression levels significantly decreased after 3 h of RO 31-8220 treatment. In contrast,

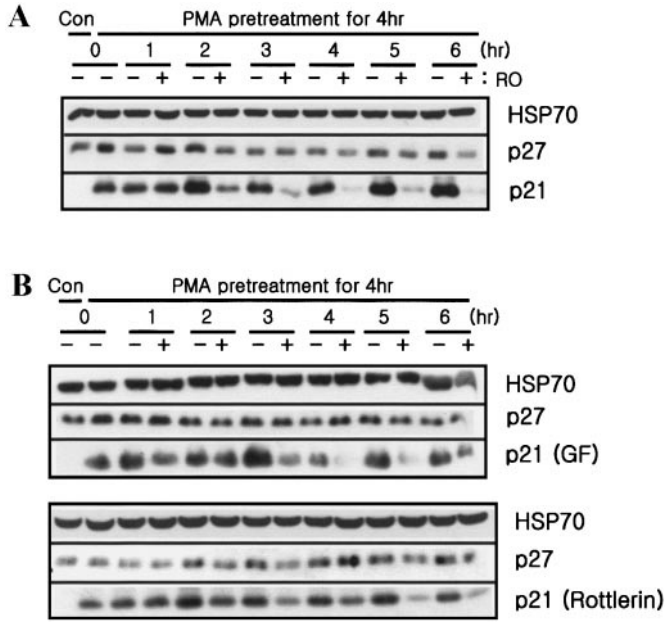

FIG. 2. Effects of PKC inhibitors on PMA-induced p21 expression. (A) U937 cells (2×10^6 cells) were pretreated with 20 nM PMA for 4 h and then washed. Cells were incubated with or without PKC inhibitor, RO 31-8220 (2 μM), for the indicated hour. Equal amounts of soluble lysates (50 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against p21, p27, and Hsp70. (B) U937 cells (2×10^6 cells) were pretreated with 20 nM PMA for 4 h and then washed. Cells were incubated with or without PKC inhibitors, GF 109203X (2 μM) and Rottlerin (20 μM), for the indicated hour. Equal amounts of soluble lysates (50 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against p21, p27, and Hsp70.

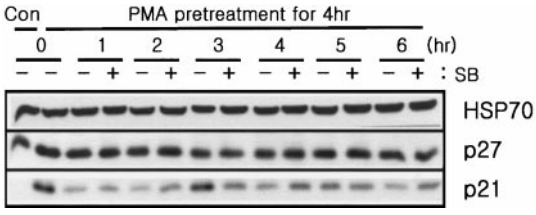

FIG. 3. Effects of p38 MAPK inhibitors, SB 203580, on PMA-induced p21 expression. U937 cells (2×10^6 cells) were pretreated with 20 nM PMA for 4 h and then washed. Cells were incubated with or without p38 MAPK inhibitor, SB 203580 (10 μM), for the indicated hour. Equal amounts of soluble lysates (50 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against p21, p27, and Hsp70.

the levels of p27 remained unaffected by treatment of RO 31-8820.

To confirm the effect of PKC inhibitors, we measured down-regulation of p21 expression using other PKC inhibitors. We employed Rottlerin and GF 109203X, which are PKC specific inhibitors. As shown in Fig. 2B, PMA mediated-p21 expression was down regulated by Rottlerin or GF 109203X, but Rottlerin had less effect than that of RO 31-8220. Taken together, these results suggest that down-regulation of PMA induced p21 protein levels was associated with the PKC signaling pathway.

Effect of p38 MAPK Inhibitor, SB 203580, on Down-Regulation PMA-Mediated p21 Expression

To investigate whether the effect of SB 203580 on down-regulation of the PMA-mediated p21 expression, U937 cells were first stimulated with PMA for 4 h to allow accumulation of p21 and then PMA was removed. The cells were treated with or without SB 203580 for an additional hour. As shown in Fig. 3, PMA mediated p21 expression was not changed in cells treated with SB 31-8220. These results suggest that down-regulation of p21 is indeed the specific effect of the PKC inhibitor.

Effect of PKC Inhibitor, RO 31-8220, on Down-Regulation of Okadaic Acid-Mediated p21 Expression

Because RO 31-8220 potentiated the inhibition of PKC activity in PMA treated U937 cells, we wanted to see whether RO 31-8220 could also down regulate p21 expression induced by other. p21 expression in U937 cells was induced by okadaic acid, an inhibitor of phosphatases 1 and 2A, which was removed before treatment with or without 2 μM RO 31-8220. As shown in Fig. 4, the p21 expression pattern was very similar to that of PMA-mediated p21 expression. Therefore, these results strongly indicate that PKC signal pathway is involved in p21 expression in U937 cells.

Effect of PKC Inhibitor, RO 31-8220, on the Level of p21 mRNA

We examined whether down-regulation p21 mRNA was induced in PMA-pretreated U937 cell following incubation with or without PKC inhibitor, RO 31-8220. The effect of RO 31-8220 on p21 mRNA accumulation in U937 cells was examined by Northern blot analysis. As shown in Fig. 5A, higher amounts of p21 mRNA were expressed when the cells were treated with PMA for 4 h. However, p21 mRNA expression drastically decreased in response to treatment with the PKC inhibitor. We therefore investigated the effect of the PKC inhibitor upon the stability of p21 mRNA. U937 cells were first stimulated with PMA for 4 hr to allow accumulation of p21 mRNA. Following this, actinomycin D was added to the cells to block any further transcription, with or without 2 μM RO 31-8220. In the absence of the RO 31-8220 the mRNA levels remained high for 2 h, but then rapidly decayed (Fig. 5B). However, the presence of RO 31-8220 strongly enhanced the decay (about 90% by 1 h).

To further strengthen this conclusion, we tested p21 promoter activity assay by luciferase reporter. To decrease variations in transfection efficiency, we established U937 cells which permanently expressed p21 promoter region. Transfectant cells were treated with PMA for 4 h and then PMA was removed. The cells were treated with or without RO 31-8220 for an additional hour. As shown in Fig. 5C, PMA mediated p21 promoter activity did not show a significant decrease after a 6 h treatment with RO 31-8220. The results of the reporter gene assay and Northern blot suggested that the down-regulation of p21 expression caused by RO 31-8220 was correlated with p21 mRNA destabilization.

DISCUSSION

Previous studies have demonstrated that induction of p21 is an important element in regulating cell cycle progression. It has been implicated in mediating

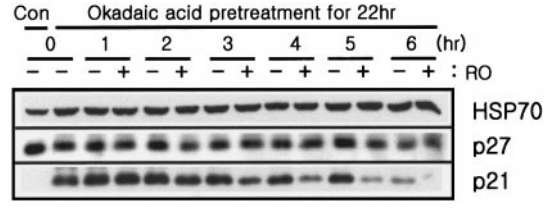

FIG. 4. Effects of PKC inhibitors on okadaic acid-induced p21 expression. U937 cells (2×10^6 cells) were pretreated with 60 nM okadaic acid for 22 h and then washed. Cells were incubated with or without PKC inhibitor, RO 31-8220 (2 μM), for the indicated hour. Equal amounts of soluble lysates (50 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against p21, p27, and Hsp70.

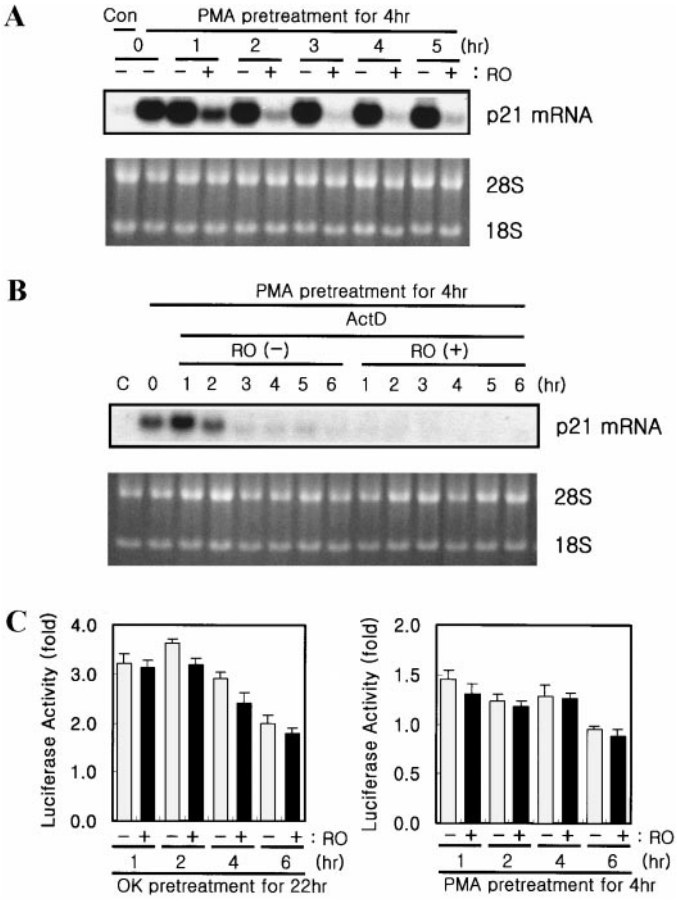

FIG. 5. Effects of PKC inhibitors on expression and stability of p21 mRNA by PMA in U937 cells. (A) U937 cells (2×10^6 cells) were pretreated with 20 nM PMA for 4 h and then washed. Cells were incubated with or without PKC inhibitor, RO 31-8220 (2 μM), for the indicated hour. Total cellular RNA was purified, and 10 μg of total RNA was subjected to Northern blotting, as described under Materials and Methods, using ^{32}P-labeled p21 cDNA. The levels of 28S and 18S RNA are shown in an ethidium bromide-stained formaldehyde gel, before Northern blotting. (B) U937 cells (2×10^6 cells) were pretreated with 20 nM PMA for 4 h and then washed. Cells were incubated with actinomycin D (5μg/ml) in the absence or presence of RO 31-8220 (2 μM) for the indicated hour. Total cellular RNA was purified, and 10 μg of total RNA was subjected to Northern blotting. The levels of 28S and 18S RNA are shown in an ethidium bromide-stained formaldehyde gel, before Northern blotting. (C) Stable p21 promoter expressed U937 cells were pretreated with PMA (20 nM) or okadaic acid (60 nM) and then washed. Cells were incubated with or without PKC inhibitor, RO 31-8220 (2 μM), for the indicated hour. Cells were harvested and assayed for luciferase. Data are mean values obtained from three independent experiments and bars represent standard deviations.

growth arrest in response to a variety of conditions, including DNA damage and terminal differentiation. The regulation of p21 gene transcription has been extensively studied and several transcription factors that bind to the p21 promoter regions have been identified (11–13). Posttranscriptional regulation has been implicated in the control of p21 gene expression by epidermal growth factor, UV light, okadaic acid and retinoid acid (14–16, 20–22). However, the mechanisms underlying such posttranscriptional regulation remain unknown. In this study, we show that p21 expression is induced by activation of PKC through a pathway that does not require p53. This up-regulated p21 expression by PMA and okadaic acid was inhibited by PKC inhibitors. The decreased expression of p21 by exposure to PKC inhibitors occurs mainly through stabilization of p21 mRNA.

To investigate the mechanisms by which the PKC inhibitors modulated p21 expression, we first performed several PKC specific inhibitors effect on p21 down-regulation. Rottlerin was less effective than RO 31-8220 and GF109203X for p21 down-regulation. Other studies have addressed the selectivity of staurosporine and its analogs to inhibit soluble PKC and membrane-associated PKC (23, 24). Rottlerin inhibited PKCδ 5-fold more potently than other PKC isoforms (25). Whereas, RO 31-8220 and GF109203X are an effective inhibitor of basal PKC activity composed of α and β activity (23–25). Additionally, SB 203580, p38 MAPK inhibitor, did not affect p21 protein expression levels. This probably accounts for the finding that down-regulation of PMA induced p21 protein levels was PKC signal pathway dependent, but not dependent on p38 MAPK.

The major effect of RO 31-8220 was to rapidly destabilize p21 mRNA. The transcriptional activity of PMA mediated-p21 did not regulated by RO 31-8220. These results of the reporter gene assay suggested that down-regulation of p21 by treatment of RO 31-8220 may be associated with posttranscriptional regulation. The fact that the inhibitor was effective in the presence of actinomycin D excluded any possible contribution of RO 31-8220 to transcription and showed that the destabilization occurs through modulation of existing factors.

The steady-state level of mRNA in the cells is dependent on both the rates of transcription and decay (26). Stabilization is one of the important mechanisms for accumulation of mRNA. Many labile mRNAs coding for oncogenes, including c-Myc and cytokines, have AUUUA motifs in the 3′ un-translated region (UTR) (27–29). The p21 mRNA has three repeats of the AUUUA motif (4). The 3′-UTR has been implicated in the regulation of p21 mRNA stability, but it remains to be seen whether PKC regulates p21 expression through this mechanism, and by which downstream targets.

In summary, we demonstrate that the PKC signaling pathway regulates expression of p21 at the posttranscriptional level by stabilizing p21 transcripts. PKC specific inhibitors including RO 31-8220 and GF109203X rapidly destabilized p21 mRNA. Our findings suggest a novel function of PKC for the regulation of p21 mRNA stability. It will be interesting to see if

other signals that increase p21 expression through PKC are controlled through a similar mechanism.

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