Novel Form of p21WAF1/CIP1/SDI1 Protein in Phorbol Ester-induced G₂/M Arrest*

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Cell cycle progression requires activation of different cyclin-dependent kinases (CDKs) which are positively regulated by cyclins and negatively regulated by CDK inhibitors. Growth inhibition of the Calu-1 lung carcinoma cells induced with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent activator of protein kinase C, is associated with G₂/M arrest and induction of expression of a novel, faster-migrating form of p21WAF1/CIP1/SDI1 (p21) protein, an inhibitor of cyclin-dependent kinases. This faster-migrating p21 protein was also expressed in TPA-treated A549 lung carcinoma cells which also exhibited G₂/M arrest but not in TPA-treated U937 leukemia cells, which only expressed a slower-migrating form of p21 protein. However, reverse transcriptase-polymerase chain reaction and Southern analysis demonstrated no evidence of novel splice in TPA-treated Calu-1 cells. On the other hand, immunoblotting analysis demonstrated that the faster-migrating p21 protein could be detected only by peptide antibody directed against the N terminus but not the C terminus, suggestive of truncation of the latter or protein modification that results in the loss of the C-terminal epitope. Correlation of G₂/M arrest with expression of the faster-migrating p21 protein suggests that this novel form of p21 protein may be a mediator of G₂/M arrest and growth inhibition.

Transitions between different cell cycle states are regulated at checkpoints controlled by cyclin-dependent kinases (CDKs) which are activated by cyclins and inhibited by CDK inhibitors (reviewed in Refs. 1–4). Many checkpoints are deregulated in oncogenesis, and, hence, negative controls on cell cycle progression may play an important role in preventing tumorigenesis. By inhibiting cyclin-dependent kinase activities, CDK inhibitors can act as negative regulators of cell growth and therefore can potentially function as tumor suppressors. Two major classes of CDK inhibitors have been identified: first, p21WAF1/CIP1/SDI1 (p21), p27KIP1, and p57KIP2 belong to a family of related CDK inhibitors that inhibits a broad range of CDK-cyclin complexes, and second, p15INK4B/MTS2, p16INK4A/MTS1, p18, and p19 constitute a different family of specific inhibitors of Cdk4 and Cdk6. Hence, elucidating the regulation of CDK inhibitors and their mechanisms of braking the cell cycle may contribute to controlling cancer growth and development.

The gene encoding p21 (5) was also cloned as WAF1, a p53-inducible gene (6), CIP1, a potent inhibitor of cyclin-dependent kinases (7), and SDI1, senescent cell-derived inhibitor (8). Transient expression of p21 resulted in inhibition of growth and DNA synthesis (6–8), and expression of antisense p21 RNA in G₀-arrested cells resulted in induction of DNA synthesis and entry into mitosis (9). In addition, p21 may also play an important role in cellular transformation because it is a downstream effector of p53. p53-dependent p21 expression can be induced by ionizing radiation that triggers G₁ arrest or apoptosis in cells expressing wild-type p53 (10). On the other hand, p21 can also be induced through p53-independent mechanisms by growth factors and the phorbol ester TPA (11). Induction of p21 expression by TPA in p53-null leukemia cells is also coupled to G₁ arrest and differentiation (12, 13).

Protein kinase C (PKC) is the major receptor for TPA, which is widely used as a pharmacological tool to investigate PKC functions in cells (reviewed in Refs. 14–16). The PKC gene family consists of 12 different isotypes, namely, α, β₁, β₂, γ, δ, ε, ζ, η, θ, τ, λ, and μ, which have different tissue distribution, subcellular localization, cofactor dependence, and substrate specificities. Activation of PKC can have pleiotropic effects on growth and tumorigenicity, depending on the cellular background in which it is expressed. This is supported by our previous observations that overexpression of PKC-β₁ caused growth inhibition and tumor suppression in colon cancer cells (17) but resulted in enhanced cell growth and tumorigenicity in fibroblasts (18). On the other hand, overexpression of the same PKC-β₁ gene was incompatible with growth of T leukemia cells (19).

We are interested in investigating the p53-independent, negative growth-regulatory pathways for controlling proliferation of cancer cells because mutations in p53 are common in cancers. Hence, PKC-mediated pathways of p21 induction could represent alternative approaches for controlling aberrant growth of cancers containing p53 mutations. Although p21 has been commonly associated with the G₁ checkpoint, it has been shown that during the cell cycle of normal human fibroblasts, the levels of p21 mRNA peak twice, during G₁ and G₂/M, which is suggestive of a potential role of p21 in G₂/M (20). In support of the latter, addition of p21 to cycling Xenopus egg extracts resulted in a block to mitosis (21). In addition, there is also precedent for induction of G₂/M arrest by overexpression of p19ARF, a gene product translated from an alternative reading frame of the p16INK4a gene (22). In this report, we demonstrate that TPA-induced growth arrest in a p53-null lung cancer cell
line is associated with induction of G2/M arrest and expression of a novel, faster-migrating form of p21 protein which may play a role in G2/M arrest and growth inhibition.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—Calu-1, A549, and U937 cell lines were purchased from American Type Culture Collection and cultured according to their respective instructions. p21-specific monoclonal antibody 6B6 was purchased from Pharmingen, and polyclonal antibodies against N-terminal peptide (amino acids 2–21) and C-terminal peptide (amino acids 146–164), N20 and C19, respectively, were purchased from Santa Cruz Biotechnology. Monoclonal antibodies against Cdk2 and Cdc2 were purchased from Pharmingen.

Growth Inhibition and Cell Cycle Analysis—Calu-1 cells were seeded at 2 × 10^5 cells/well in 6-well dishes, and TPA (1 nM or 10 nM) was added the following day. At various times after TPA treatment, cells were trypsinized, and cell growth analysis was performed by quantitating the number of viable cells by trypan blue exclusion using a hemacytometer. For cell cycle analysis, ethanol-fixed cells were treated with RNase A, stained with propidium iodide (100 μg/ml), and analyzed by a Becton-Dickinson FACS analyzer. Cell cycle distribution was based on 2N and 4N DNA content.

Northern Analysis and Reverse Transcriptase-PCR—RNA was extracted from the guanidinium isothiocyanate-cesium chloride ultracentrifugation method. Twenty μg of total RNA was loaded and separated by 0.8% agarose-formaldehyde gel, and RNA was transferred onto Hybond-N membrane (Amersham). RNA was hybridized to gel-purified p21 cDNA excised from pC-WAF1 (a gift from Dr. B. Vogelstein, Johns Hopkins University), which was labeled with [32P]dCTP by random priming. RNA loading was evaluated by probing the same filter with similarly labeled β-actin cDNA.

Reverse transcriptase-PCR (RT-PCR) was performed using the SuperScript Preamplification System (Life Technologies, Inc.). Based on the reported cDNA sequence of p21 (6), the following primers were used in this study: PCR1, 5′-GGGCCCATGTCAAGACGCGGTGGGTGATG-3′ (70–97); PCR2, 5′-CATCCTGGTCTGGCCGTTTTCCTCGACC-3′ (516–489); PCR3, 5′-GCTCTTCTCTGGGTGTTACAGTGTCGCTC-3′ (586–558); SQ1, 5′-GGACCTGGAGACTCTCAGGGTCG-3′ (472–494).

The conditions for the PCR reaction were: denaturation at 94 °C for 1 min, annealing/extension at 72 °C for 2 min, for 35 cycles (23). Primers PCR1 and PCR2 were used for amplification of the exon 2 coding sequence while primers PCR1 and PCR3 were used to amplify the entire coding sequence. PCR amplification of cDNA using PCR1 and -2 or PCR1 and -3 generated fragments of 446 and 516 base pairs, respectively. Amplified products were separated on 1.2% agarose gel and transferred to Hybond-N membrane by the Southern transfer method. The filter was hybridized with end-labeled primer SQ1.

Immunoblotting Analysis—Protein extraction was performed by lysing cells for 30 min on ice in a buffer containing 1% Triton X-100, 250 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 25 μg/ml aprotinin, 1 mM benzamidine, and 10 μg/ml soybean trypsin inhibitor. Lysed cells were centrifuged for 10 min at 4 °C, and supernatants were frozen until analysis. Equal amounts of protein determined by the Bradford method (Bio-Rad) were loaded and electrophoresed through 15% SDS-polyacrylamide gels, transferred onto Immobilon-P membrane (Millipore). Antibodies were used according to instructions of their respective manufacturers, and antigen-antibody complexes were detected by the ECL system (Amersham).

RESULTS AND DISCUSSION

To study the mechanism of p53-independent, PKC-mediated growth arrest, the effects of TPA, a potent activator of PKC, on the growth of the Calu-1 lung carcinoma cells, which contain a homozygous deletion of the p53 gene (24), was studied. As shown in Fig. 1A, growth inhibition was observed when Calu-1 cells were treated with 10 nM TPA but not with a 10-fold lower dose of TPA (1 nM). We have previously shown that TPA-induced growth inhibition in PKC-β1 overexpressing colon cancer cells was also dose-dependent and was maximal at 10 nM (17). Because p53-independent induction of p21 by TPA in human leukemia cells has been correlated with growth arrest (12, 13), induction of p21 mRNA expression in TPA-treated Calu-1 cells was first examined by Northern blot analysis. Low levels of p21 mRNA were expressed in untreated cells (Fig. 1B).

In a dose-dependent manner, TPA (1 nM and 10 nM) up-regulated p21 mRNA expression as early as 4 h, and maximal induction occurred at 8 h. Treatment with the noninhibitory dose of TPA (1 nM) resulted in only a transient induction of p21 mRNA expression which declined rapidly by 24 h. In contrast, sustained, higher levels of p21 mRNA were induced with 10 nM TPA which lasted for 72 h. Since growth inhibition was observed only when Calu-1 cells were treated with 10 nM TPA, it is conceivable that prolonged, high levels of p21 expression may be necessary for growth inhibition.

Because induction of p21 expression has commonly been associated with G1 arrest, we examined the cell cycle distribution of TPA-treated Calu-1 cells, determined by propidium iodide staining and flow cytometry. Interestingly, despite the induction of p21 mRNA expression (Fig. 1B), no G1 arrest was observed in TPA-treated cells as compared with untreated cells, as indicated by the lack of an increase in the G1 (2N) cell population (Table I). However, treatment with 10 nM TPA, which resulted in growth inhibition, elicited a prominent, prolonged accumulation of cells with 4N DNA content in the G2/M phase beginning on day 1 and lasting for 3 days. On the other hand, consistent with the lack of growth inhibition in cells treated with the noninhibitory dose of TPA (1 nM), the accumulation of cells in the G2/M phase was not observed. Hence, G2/M arrest appears to be the underlying mechanism of TPA-induced growth inhibition of Calu-1 cells.

To investigate if the absence of G1 arrest was due to the lack of p21 protein expression, the latter was analyzed by immunoblot analysis. Consistent with p21 mRNA induction (Fig. 1B), a
higher level of p21 protein was expressed at 8 h in cells treated with 10 nM TPA than in cells treated with 1 nM TPA (Fig. 2B). At day 1, as the level of p21 mRNA decreased in cells treated with 1 nM TPA, the corresponding level of protein also decreased. Interestingly, as G2/M arrest was induced in cells treated with 10 nM TPA at day 1 (Fig. 2A), in addition to detecting the 21-kDa protein band, an additional band with a faster electrophoretic mobility of approximately 20 kDa was also detected (Fig. 2B). After 2 days of treatment with 10 nM TPA, the upper 21-kDa band could not be detected while the faster-migrating band was prominently expressed and its level decreased at day 3. More significantly, induction of expression of this faster-migrating form of p21 protein coincided temporally with induction of G2/M arrest (compare Fig. 2A with Fig. 2B). Treatment with 1 nM TPA which did not induce growth inhibition or expression of the faster-migrating form of p21 protein also failed to induce G2/M arrest, suggesting that G2/M arrest may be mediated by the faster-migrating form of p21 protein.

Because of the small differences in electrophoretic mobility between the two forms of p21 protein, it is conceivable that the faster-migrating protein may be the result of PKC-induced post-translational modifications or small protein truncation. Phosphorylation of serines 98 and 130 of the p21 protein has been reported to cause electrophoretic mobility shifts, and kinase-bound p21 displayed a slower electrophoretic mobility under conditions in which the cyclin A-Cdk2-p21 complex was active (25). In contrast to kinase-bound p21 which migrated slower, TPA treatment induced a modification that resulted in a faster electrophoretic mobility of the p21 protein. Hence, to explore the alternative possibility of protein truncation as the cause of the latter, we hypothesized that a small truncation of the p21 protein could occur at the N or C terminus. To this end, we performed immunoblotting analysis using polyclonal antibodies against p21 (6B6) and the N-terminal peptide (amino acids 2–21) N20, indicating that the N terminus was intact in both forms of p21 protein. Expression of the faster-migrating form of p21 protein detected with the N-terminal peptide antibody N20 corresponded exactly with that detected with the monoclonal antibody 6B6 (Fig. 2B). In contrast, the C-terminal peptide antibody C19 only detected the upper form but failed to detect the faster-migrating form of p21 protein (Fig. 2D). Hence, induction of G2/M arrest correlates with expression of a second form of p21 protein that lacks the C-terminal epitope.

Effects of TPA on cell cycle distribution of Calu-1 cells

| Time in culture | Cell cycle | Cell cycle distribution (%) |
|-----------------|------------|-----------------------------|
|                 | Untreated  | TPA (1 nM) | TPA (10 nM) |
| 8 h             | G1         | 42.0        | 36.9         | 36.3         |
|                 | S          | 40.6        | 38.1         | 38.1         |
|                 | G2/M       | 17.4        | 25.1         | 25.7         |
| Day 1           | G1         | 46.3        | 47.6         | 41.9         |
|                 | S          | 30.2        | 24.0         | 11.8         |
|                 | G2/M       | 23.5        | 28.4         | 46.3         |
| Day 2           | G1         | 55.5        | 52.1         | 42.3         |
|                 | S          | 27.3        | 29.4         | 16.1         |
|                 | G2/M       | 17.2        | 17.5         | 41.7         |
| Day 3           | G1         | 72.3        | 63.1         | 37.3         |
|                 | S          | 16.8        | 23.4         | 29.7         |
|                 | G2/M       | 11.0        | 13.5         | 33.0         |

TABLE I

![Fig. 2](image-url)

**Fig. 2. Induction of expression of a novel form of p21 protein in G2/M arrest.** A, cell cycle distribution of TPA-treated Calu-1 cells was analyzed by propidium iodide staining of DNA content and flow cytometry and correlated with induction of p21 expression by 1 nM TPA (T1) or 10 nM TPA (T10) by immunoblotting with a monoclonal antibody against p21 (6B6) (B). Induction of expression of a faster-migrating form of p21 protein correlates temporally with induction of G2/M arrest. The expression of p21 was also detected using polyclonal antibodies against the N-terminal peptide (amino acids 2–21) N20 (C) and the C-terminal peptide (amino acids 146–164) C19 (D). The levels of protein expression of Cdk2 (E) and Cdc2 (F) were also analyzed by immunoblotting with the respective monoclonal antibodies.

At the G2/M transition, mitosis is initiated by the mitosis-promoting factor consisting of Cdc2-cyclin B complex (reviewed in Ref. 26). The activity of Cdc2 is regulated positively by phosphorylation of Thr-161 and negatively by phosphorylation of Tyr-15 and Thr-14. At the onset of mitosis, the Cdc25 phosphatase activates mitosis-promoting factor by dephosphorylating Cdc2 on Tyr-15 and Thr-14. The addition of p21 to cycling Xenopus egg extracts which inhibited Cdk2 kinase activity resulted in blockade in mitosis and accumulation of inactive Cdc2-cyclin B. In fact, Cdk2 kinase has been shown to be required for entry into mitosis as a positive regulator of Cdc2-cyclin B kinase activity (21). Immunoblotting analysis demonstrated a modest decrease in the protein level of Cdk2 at day 2 with 10 nM TPA treatment (Fig. 2E). On the other hand, a significant decrease in the level of Cdc2 protein expression was observed at day 2 after treatment with 10 nM but not 1 nM TPA (Fig. 2F). Interestingly, the decrease in the protein levels of Cdk2 and Cdc2 at day 2 paralleled the high level of expression of the faster-migrating p21 protein. Whether down-regulation of the protein levels of Cdc2 and Cdk2 is the cause or consequence of G2/M growth arrest remains to be determined. In addition, the exact role of the faster-migrating form of p21 protein in regulating the levels and kinase activities of Cdc2 and Cdk2 will also be investigated.

To further characterize the faster-migrating p21 protein, expression of the latter in U937 leukemia cells was analyzed. Previous reports have demonstrated the induction of expression of only one form of p21 protein in the p53-null HL60 and...
U937 leukemia cells upon exposure to TPA (27, 28), which caused growth arrest in the G1 phase (12, 13). To determine which form of p21 protein was expressed in leukemia cells, expression of p21 protein in TPA-treated U937 cells was evaluated alongside that in TPA-induced Calu-1 cells. In contrast to TPA-treated Calu-1 cells, only the upper form of p21 protein was expressed in TPA-treated U937 cells as detected by the monoclonal antibody 6B6 and confirmed with the N-terminal peptide antibody N20 and the C-terminal peptide antibody C19 (Fig. 3A). Treatment with a higher dose of TPA (100 nM) also resulted in the induction of only one form of p21 protein expressed. Cell cycle analysis confirmed that G1 arrest was induced in TPA-treated U937 cells as evident by an increase in the G1 cell population and a concomitant decrease in the S cell population at 2–3 days post-treatment (Fig. 3B). Hence, the lack of expression of the novel form of p21 protein is consistent with the absence of G2/M arrest in TPA-treated U937 cells.

With respect to the origin of the novel form of p21 protein, the absence of a C-terminal epitope coupled with a faster electrophoretic mobility suggested that the novel p21 protein may lack the C terminus due to alternative splicing or proteolytic cleavage. To evaluate the former possibility, we performed RT-PCR using primers to amplify the exon 2 coding sequence (PCR1–2) or the entire p21 coding sequence (PCR1–3) (Fig. 4A). Southern analysis of the amplified products demonstrated the induction of only a single band of the expected size in TPA-treated U937 cells which comigrated with that in Calu-1 cells (Fig. 4B). Thus, these results argued against novel splice as the cause of the faster-migrating p21 protein and suggested that the latter may be truncated at the C terminus due to proteolytic cleavage. However, direct proof of truncation of the p21 protein will require protein sequencing of the C terminus. The C terminus has previously been reported to be involved in proliferating cell nuclear antigen binding but not to be required for DNA synthesis inhibitory activity (29). In addition, the C terminus also contains two putative bipartite nuclear localization signals (6, 7). However, Nakaneishi et al. (30) have shown that a C-terminal truncated protein which lacks nuclear localization signals can still inhibit DNA synthesis and translocate to the nucleus.

To further evaluate the cell-type specificity of expression of this faster-migrating p21 protein, expression of the latter was investigated in the A549 lung carcinoma cells. Treatment of A549 cells with TPA also resulted in growth inhibition and expression of the faster-migrating form of p21 protein, which comigrated with that in TPA-treated Calu-1 cells (Fig. 5A). In contrast to Calu-1 cells, there was a basal level of the upper form of p21 protein expressed in untreated A549 cells which was strongly induced at 8 h with TPA treatment. Similar to that in Calu-1 cells, expression of the novel, faster-migrating form of p21 protein in A549 cells was not detected until 1 day after treatment with TPA (Fig. 5B) and coincided temporally with induction of G2/M arrest in A549 cells (Fig. 5C). These results further support the role of the novel form of p21 protein in G2/M arrest.

Based on these results, we hypothesized that TPA activates a PKC-mediated, negative-growth regulatory pathway leading to the induction of expression of a novel form of p21 protein which mediates G2/M arrest. Since RT-PCR and Southern analysis of cDNA from TPA-treated Calu-1 demonstrated no alternative splicing, the novel p21 protein may be modified at the protein level. Because of the faster electrophoretic mobility coupled with the lack of a C-terminal epitope recognized by peptide antibody, this novel form of p21 protein may be truncated at the C terminus.

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