Inhibition of G_1 Cyclin-Dependent Kinase Activity during Growth Arrest of Human Breast Carcinoma Cells by Prostaglandin A_2

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Prostaglandin A_2 (PGA$_2$) potently inhibits cell proliferation and suppresses tumor growth in vivo, but little is known regarding the molecular mechanisms mediating these effects. Here we demonstrate that treatment of breast carcinoma MCF-7 cells with PGA$_2$ leads to G_1 arrest associated with a dramatic decrease in the levels of cyclin D1 and cyclin-dependent kinase 4 (cdk4) and accompanied by an increase in the expression of p21. We further show that these effects occur independent of cellular p53 status. The decline in cyclin D and cdk4 protein levels is correlated with loss in cdk4 kinase activity. cdk2 activity is also significantly inhibited in PGA$_2$-treated cells, an effect closely associated with the upregulation of p21. Immunoprecipitation experiments verified that p21 was indeed complexed with cdk2 in PGA$_2$-treated cells. Additional experiments with synchronized MCF-7 cultures stimulated with serum revealed that treatment with PGA$_2$ prevents the progression of cells from G_1 to S. Accordingly, the kinase activity associated with cdk4, cyclin E, and cdk2 immunocomplexes, which normally increases following serum addition, was unchanged in PGA$_2$-treated cells. Furthermore, the retinoblastoma protein (Rb), a substrate of cdk4 and cdk2 whose phosphorylation is necessary for cell cycle progression, remains underphosphorylated in PGA$_2$-treated serum-stimulated cells. These findings indicate that PGA$_2$ exerts its growth-inhibitory effects through modulation of the expression and/or activity of several key G_1 regulatory proteins. Our results highlight the chemotherapeutic potential of PGA$_2$, particularly for suppressing growth of tumors lacking p53 function.

The cyclopentenone prostaglandins A_1 (PGA$_1$) and A_2 (PGA$_2$) are potent inhibitors of growth in cultured cells (49, 50) and exhibit antitumor activity in vivo (10, 21, 22, 39, 50). Growth arrest following PGA$_2$ treatment occurs primarily through a block in cell cycle progression at the G_1-to-S transition, but the mechanisms responsible for eliciting this effect are still largely unknown (19). A number of genes (1, 18, 34) show altered expression following PGA$_2$ treatment, but the relationship between such changes in gene expression and the initiation and/or maintenance of the growth arrested state is unclear.

The eukaryotic cell cycle is regulated through the sequential activation and inactivation of cyclin-dependent kinases (cdks) that drive cell cycle progression through phosphorylation of key regulatory proteins (36). In normal cells, cdks exist predominantly in quaternary complexes consisting of a cdk, a cyclin, proliferating cell nuclear antigen, and a small protein of 21 kDa (p21) (55, 56). Among the proteins that are phosphorylated via these kinase complexes is the retinoblastoma tumor suppressor protein (Rb) (53), which plays an essential role in the progression of cells from G_1 to S through its regulation of the transcription factor E2F (for reviews, see references 26 and 37). In vitro, Rb has been shown to be phosphorylated by several cdk-cyclin complexes, including complexes containing the cyclins D1, D2, and D3 (7), in association with their main catalytic partners cdk4 and cdk6, and also by cdk2-cyclin E complexes (24). Temporally, as the cell progresses through G_1, activation of cyclin D-cdk4 complexes precedes that of cyclin E-cdk2 complexes (25). In most mammalian tissues, cells exist in a nonproliferating or resting state, known as G_0, which can be induced or mimicked in cultured cells by growth factor withdrawal. Current evidence favors a role for D-type cyclins in the emergence of the cells from quiescence (G_0-to-G_1 transition) and during progression through mid G_1, while cyclin E-cdk2 complexes are thought to participate in controlling passage through late G_1 into S (25, 40, 41, 45–47). However, the actual roles of these cyclin D- and cyclin E-cdk complexes in vivo and the precise nature of their collaboration in promoting cell cycle progression remain to be elucidated.

The cell cycle is subject to numerous surveillance mechanisms that are responsible for arresting cells in the event of unfavorable conditions and/or if damage is incurred. These mechanisms operate primarily at major points of restriction termed checkpoints, which occur in the G_1 and G_2 phases of the cell cycle, and include at least four important regulatory systems that modulate cdk activity: (i) alteration of cdk levels; (ii) changes in the expression of the cyclins with which they interact; (iii) regulation of the cdk kinase activity through reversible phosphorylation; and (iv) the presence of small inhibitory proteins (cdk inhibitors [CKIs]) which interact reversibly with cdks to regulate their activity. The growing number of CKIs includes p16 (Ink4/Mts1) (31), p14/p15 (Ink4B/Mts2) (11, 13), p18 (11), p27 (Kip1, p28^{Ick1}) (16, 44, 52), p19 (56), p57 (Kip2) (28, 34), and the above-mentioned p21 (Waf1/Cip1/ Sdi1).

p21, the first of the CKIs to be described, has been shown to be capable of inhibiting virtually every cdk-cyclin complex tested in vitro (54). However, the majority of in vivo studies suggest that the inhibitory effect of p21 is largely exerted during the G_1 phase of the cell cycle, with preferential binding to cdk4- and cdk2-containing complexes. By different approaches, p21 was identified as a protein upregulated in senescent cells (Sdi1) (38), as a protein that associates and inhibits cyclin-cdk complexes (Cip1) (15), and as a gene (*WAF1*) regulated by the tumor suppressor gene p53 (6). Indeed, p21 has been impli-

cated in the growth arrest that follows treatment with DNA-damaging agents, and this effect is largely dependent on the presence of functional p53 (5). However, recent studies indicate that p21 is regulated by p53-independent mechanisms in other situations, such as during development and terminal differentiation (12, 20, 32, 35, 43).

In this study, we demonstrate that treatment of breast carcinoma MCF-7 cells with PGA$_2$ results in growth arrest that is independent of p53. To further investigate the molecular mechanisms associated with the effects of PGA$_2$, we examined the influence of this prostaglandin on the factors involved in regulating G$_1$ progression. We demonstrate that PGA$_2$ treatment results in the rapid downregulation of cdk4 and cyclin D1 gene expression, thereby reducing cdk4 kinase activity. This is accompanied by an increase in the expression of the CKI p21 and downregulation of cdk2 activity. Thus, PGA$_2$ appears to exert its antiproliferative effects via modulation of several G$_1$-regulatory proteins. As a chemotherapeutic agent, it may be of particular value for suppressing tumor growth in p53-deficient malignancies.

MATERIALS AND METHODS

Cell culture and treatments. The human breast carcinoma MCF-7 cell line, cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, Md.), and mouse embryonal fibroblasts (MEFs) derived from a p53-knockout mouse (30), cultured in Dulbecco's modified essential medium (Gibco BRL), were maintained in a humidified atmosphere containing 5% CO$_2$ in air and supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) and 50 μg of gentamicin (Gibco BRL) per ml. PGA$_1$ and PGA$_2$ (Sigma, St. Louis, Mo.), prepared in ethanol, were added directly into the medium to a final concentration of 36 μM. For the starvation-synchronization experiments, MCF-7 cells were grown to 50% confluence, rinsed twice with phosphate-buffered saline (PBS) and placed in serum-free medium for 3 days. Cells were then released from growth arrest by the addition of fetal bovine serum to a final concentration of 10%.

Northern (RNA) blot analysis. Total RNA was isolated by the STAT-60 method (Tel-Test "B", Friendswood, Tex.) as specified by the manufacturer. Twenty-microgram RNA samples were denatured, size fractionated by electrophoresis in 1.2% agarose-formaldehyde gels, and transferred onto GeneScreen Plus nylon membranes (DuPont/NEN, Boston, Mass.). For the detection of cdk4 and p21 mRNAs in MCF-7 cells, corresponding cDNAs excised from pRSVcdk4 (46) and pCEP-WAF1 (6), respectively, were labeled with [α-^{32}P]dCTP with a random primer labeling kit (Boehringer Mannheim, Indianapolis, Ind.). For the detection of p16 and cyclin D1 in MCF-7 cells and of p21 in MEFs, oligonucleotides complementary to p16 mRNA (5′-GACCCCGGGCCGCGGCCGAGGC CAGCCAGTCAGCCGAAGGCTCCATGCTGCTCCCCGCCG-3′), human cyclin D1 mRNA (5′-GAGGTTGGCATCGGGGTACGCGCGGCGGAT GGTTTCCACTTCGCAGCACAGGAGCTGGTG-3′), the mouse p21 mRNA (5′-ACGGTATCTGATCGTCTTCGAACC-3′), and 18S rRNA (5′-ACGGTAT CTGATCGTCTTCGAACC-3′) (Integrated DNA Technologies, Coralville, Iowa) were 3′ end labeled with [α-^{32}P]dATP by terminal deoxynucleotidyltransferase (Life Technology Laboratories, Gaithersburg, Md.). Hybridization and washes were performed by the method of Church and Gilbert (3), and the hybridization signal was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Signals obtained with the cyclin D1, cdk4, and p21 probes were normalized to 18S signals obtained on the same blot to control for variations in loading and transfer among samples.

Cell counts and flow cytometric cell cycle analysis. Cell counts were performed with a hemocytometer. Cell cycle distribution was analyzed by flow cytometry as described previously (23). Briefly, 2 × 10^6 to 5 × 10^6 cells were trypsinized, washed once with PBS, and fixed in 70% ethanol for 30 min. Fixed cells were washed with PBS, incubated with 1 μg of RNase A per ml for 30 min at 37°C, and stained with propidium iodide (Boehringer Mannheim). The stained cells were analyzed on a FACscan flow cytometer for relative DNA content based on red fluorescence levels. Doublets were excluded from the DNA histograms by using a double-discrimination method. The percentages of the cells in the various cell cycle compartments were determined by using the MULTICYCLE software program (Phoenix Flow Systems, San Diego, Calif.).

Immunoprecipitation and kinase assays. For immunoprecipitation of cdk4, cells were washed twice with ice-cold PBS, harvested in cdk4 lysis buffer containing 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.5), 10% glycerol, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, and 0.1% Tween 20, supplemented with the phosphatase and protease inhibitors 5 mM NaF, 0.1 mM sodium orthovanadate, 5 μg of leupeptin per ml, 10 μg of aprotinin per ml, 50 μg of phenylmethylsulfonyl fluoride per ml, and 5 μg of pepstatin A per ml, and lysed by repeated passages through a 25-gauge needle. Cellular debris was removed from soluble extracts by centrif-

ugation at 16,000 × g for 10 min at 4°C. Following normalization of protein content, lysates were precleared by incubation with protein A-Sepharose (Sigma) and preimmune rabbit serum for 30 min at 4°C. Endogenous cdk4-containing complexes were immunoprecipitated for 3 h at 4°C, using a rabbit polyclonal anti-human cdk4 antiserum (Pharmingen, San Diego, Calif.). Immunoprecipitates were washed twice with cdk4 lysis buffer and four times with glutathione S-transferase–(GST)-Rb kinase buffer (50 mM HEPES [pH 7.5], 10 mM MgCl$_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM orthovanadate, 1 mM NaF) and then resuspended in 50 μl of GST-Rb kinase buffer. For immunoprecipitation of cyclin E and cdk2, cells were processed as described above except that they were lysed in cdk2 lysis buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 0.1% Triton X-100, supplemented with the inhibitors specified above, and immunoprecipitated for 3 h at 4°C with a rabbit polyclonal anti-human cyclin E antibody (Upstate Biotechnology Inc., Lake Placid, N.Y.) and rabbit polyclonal anti-human cdk2 antiserum (Pharmingen), respectively. Immunoprecipitates were washed twice with cdk2 lysis buffer and four times with H1 kinase buffer (50 mM Tris-HCl [pH 7.4], 10 mM MgCl$_2$, 1 mM dithiothreitol) and then resuspended in 50 μl of H1 kinase buffer.

The kinase activity associated with anti-cdk4 immunocomplexes was assayed in 50 μl of GST-Rb kinase buffer containing 10 μg of GST-Rb substrate, prepared as described previously (17), and that associated with anti-cyclin E and anti-cdk2 immunoprecipitates was assayed in 50 μl of H1 kinase buffer containing 10 μg of histone H1 (Ambion, Austin, Tex.), in each case supplemented with 2 mM EGTA and 10 μCi of [γ-^{32}P]ATP. Reactions were carried out for 30 min at 30°C; cold ATP (final concentration, 30 μM) was then added to each reaction mixture to reduce background signal. Reactions were stopped by addition of Laemmli sample buffer, and the reaction products were electrophoresed in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels, whereupon the gels were dried, visualized by autoradiography, and quantitated with a PhosphorImager (Molecular Dynamics).

For the analysis of p21-associated proteins, cells were metabolically labeled for 12 h in the presence of [^{35}S]methionine and [^{35}S]cysteine (Expre^{35}S^{35}S; Du Pont, NEN) in RPMI medium without methionine or cysteine, supplemented with 10% dialyzed fetal bovine serum. Cells were harvested in cdk2 lysis buffer, and the lysates were subjected to immunoprecipitation of p21-containing complexes as described above, using a monoclonal antibody against p21 (Ab-3; Oncogene Science, Uniondale, N.Y.). The labeled proteins present in the immunocoprecipitate were resolved on SDS–15% polyacrylamide gels and visualized by autoradiography.

Western blot (immunoblot) analysis. Fifty-microgram samples of total cell lysates or aliquots from ^{35}S-labeled immunoprecipitates were size fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes, using standard techniques (14). p27, p21, cdk2, cdk4, cyclin D1, cyclin E, and Rb proteins were detected with the ECL (enhanced chemiluminescence) system (Amersham, Arlington Heights, Ill.) following incubation with the appropriate antibodies: polyclonal rabbit anti-human cdk4 antiserum, polyclonal rabbit anti-human p27, monoclonal mouse anti-human cyclin D1, monoclonal mouse anti-human cdk2, monoclonal mouse anti-human cyclin E, and monoclonal mouse anti-human Rb antibodies from Pharmingen; monoclonal mouse anti-human p21 from Oncogene Science and Transduction Laboratories (Lexington, Ky.); polyclonal rabbit anti human pan-cyclin D1 from Upstate Biotechnology Inc.; and monoclonal mouse anti-human cdk4 antibody from Transduction Laboratories. Histidine-tagged mouse p27^{KIP1} was from Pharmingen.

RESULTS

PGA$_2$ treatment causes growth inhibition in MCF-7 cells associated with G$_1$ arrest. Exponentially growing MCF-7 cultures exposed to 36 μM PGA$_2$ rapidly underwent growth inhibition, as evidenced by the lack of cell number increase over time (Fig. 1A). A similar inhibition of cellular proliferation was observed with PGA$_1$ (not shown). Trypan blue dye exclusion indicated little toxicity associated with this dose of PGA$_2$ (>90% viability after 48 h). Fluorescence-activated cell sorter (FACS) analysis revealed that a 24-h treatment of PGA$_2$ (Fig. 1B) resulted in a higher proportion of cells in G$_1$ (86%) relative to that seen in untreated cells (50%), consistent with a G$_1$ arrest. Figure 2 shows the effect of PGA$_2$ on the cell cycle distribution of MCF-7 cultures that were serum starved (i.e., quasi-synchronized) and then restimulated to proliferate by the addition of fresh serum. In these experiments, PGA$_2$ was added to the cultures during the last 12 h of a 72-h serum starvation period. As shown in Fig. 2, in the absence of PGA$_2$, serum-stimulated cells readily proceeded to enter the S and G$_2$/M phases. In contrast, PGA$_2$-treated cells failed to proceed

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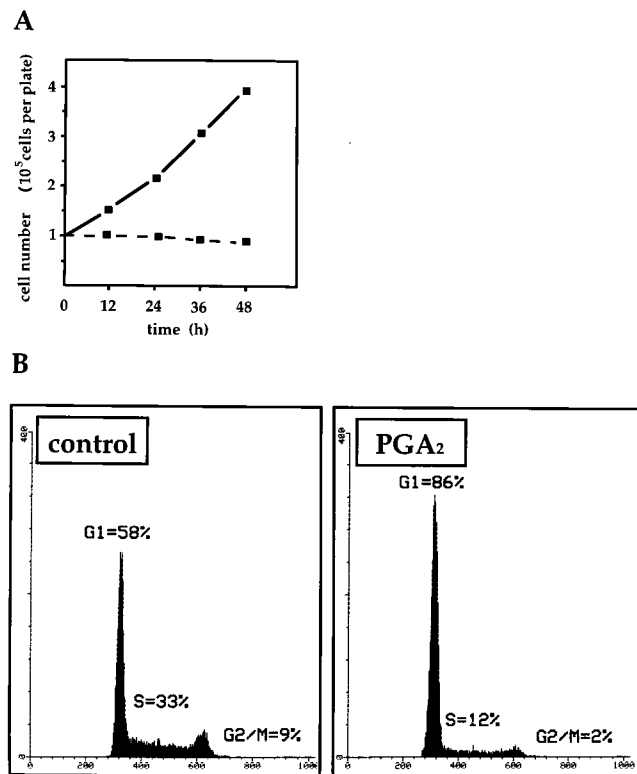

FIG. 1. Effect of PGA$_2$ on growth of MCF-7 cells. (A) MCF-7 cells were either left untreated or treated with 36 μM PGA$_2$, and cell number was determined every 12 h thereafter, using a hemocytometer. Solid lines, untreated control cells; dashed lines, PGA$_2$-treated cells. (B) FACScan analysis of untreated (control) or PGA$_2$ (36 μM)-treated MCF-7 cultures. Cells were treated for 24 h prior to fixation and FACS analysis as described in Materials and Methods. Numbers indicate the percentages of cells in the different phases of the cell cycle.

into S and G$_2$/M following stimulation but remained arrested in the G$_1$ phase.

Expression of cdk4 and cyclin D1 in MCF-7 cells following PGA$_2$ treatment. Western analysis was used to examine the effect of PGA$_2$ treatment on the expression of proteins to participate in regulating G$_1$ progression (Fig. 3). We first analyzed the expression of cdk4 and cyclin D1, components of the cdk-cyclin complex that is thought to be active in early G$_1$. Our analysis of cyclin D family expression was limited to cyclin D1, as preliminary experiments using polyclonal antibodies that recognize all D-type cyclins failed to detect cyclins D2 and D3 in MCF-7 cells (unpublished observations). Interestingly, cyclin D1 and cdk4 protein levels decreased dramatically (>90% decline by 16 h of treatment) in PGA$_2$-treated cells (Fig. 3A). Northern analysis examining changes in the expression of cyclin D1 and cdk4 mRNAs is shown in Fig. 3B. Consistent with the decrease in protein levels following PGA$_2$ treatment, mRNA expression for cyclin D1 and cdk4 similarly declined. However, the kinetics of the response differed for the two genes, with loss in D1 expression being the most rapid effect. It is important to note that in further experiments (discussed in greater detail below), in which earlier time points following PGA$_2$-treatment were examined for cdk4 and cyclin D1 protein expression (see Fig. 9), marked changes in protein levels precede those seen for mRNA expression, indicating that there is a strong translational component involved in controlling the levels of these proteins.

The expression of cdk4 and cyclin D1 mRNA was further monitored in synchronized MCF-7 cells (Fig. 4A). Cells were growth arrested by serum starvation and then were either pretreated with PGA$_2$ for 12 h or left untreated prior to serum stimulation. Both cyclin D1 and cdk4 transcripts were abundant in control cells, with serum addition leading to a transient elevation in the levels of cyclin D1. In contrast, cyclin D1 and cdk4 mRNAs were found to be extremely low or undetectable in PGA$_2$-treated cells throughout the entire time course of the experiment (Fig. 4A).

The effect of PGA$_2$ on cdk4 kinase activity was also examined in these serum-stimulated MCF-7 cultures. cdk4 was immunoprecipitated with anti-cdk4 antibodies at various times following the addition of serum to synchronized cultures, and the kinase activity associated with the immunocomplexes was assayed by using GST-Rb as a substrate for in vitro phosphorylation (Fig. 4B). In the absence of PGA$_2$, the GST-Rb-kinase activity associated with cdk4 immunocomplexes increased in a time-dependent manner and with maximal incorporation seen at ~15 h following release from serum starvation. However, cdk4 kinase activity remained extremely low in PGA$_2$-treated cells throughout the entire time course of the experiment.

Regulation of cdk2 kinase activity in MCF-7 cells following PGA$_2$ treatment. In contrast to what was observed for cdk4 and cyclin D1, cyclin E and cdk2 protein levels were found to be unaltered by PGA$_2$ treatment (Fig. 5A). However, to determine if changes in cdk2 kinase activity might be contributing to the G$_1$ arrest, cdk2-containing complexes were immunoprecipitated from PGA$_2$-treated cultures and assayed with histone H1 as a substrate. Indeed, cdk2 kinase activity was high in untreated cells but decreased markedly with exposure to PGA$_2$ (Fig. 5B). Among the known regulators of cdk activity is a group of CKIs that reversibly interact with cdks, resulting in their inactivation. The expression of several of these inhibitors known to act in G$_1$ was monitored in PGA$_2$-treated cultures (Fig. 5C). No change in p27 expression was observed in PGA$_2$-treated cells, and no expression of p16, a cdk4/cdk6 inhibitor, was detected in MCF-7 cells, in agreement with the findings of others that the gene is deleted in MCF-7 cells (31). However, p21 protein levels were greatly elevated following PGA$_2$ treatment (>15-fold elevation with a 16-h treatment). Northern analysis revealed that p21 mRNA likewise increased following PGA$_2$ treatment, with the highest levels of expression seen by 16 h of treatment (Fig. 5D).

As p21 has been shown to act as an inhibitor of cdks in vitro, we sought to determine if p21 was associated with cdk2 and/or other cdks in PGA$_2$-arrested cells. To this end, asynchronously growing control and PGA$_2$-treated cells (16-h treatment) were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 12 h prior to harvesting of the cells. Equivalent quantities of ^{35}S-labeled lysate were immunoprecipitated with an anti-p21 monoclonal antibody, and the immunoprecipitate was analyzed by SDS-PAGE (Fig. 6A). Two distinct bands corresponding to proteins of 21 and 33 kDa were apparent with higher intensity in lysates from PGA$_2$-treated cells than in the control population. As expected, the 21-kDa band was confirmed to be p21 by a second immunoprecipitation using different antibodies (not shown). Western blot analysis identified the 33-kDa band as cdk2 (Fig. 6B). The increased expression of p21 mRNA in PGA$_2$-treated cells correlated temporally with the decline in cdk2 kinase activity (Fig. 5B and D). Although p21 has been shown to be associated with cdk4, no enrichment of cdk4 was seen in these experiments (Fig. 6B). This finding was predicted from the observed decline in cdk4 protein levels following 16 h of PGA$_2$ treatment (Fig. 3A).

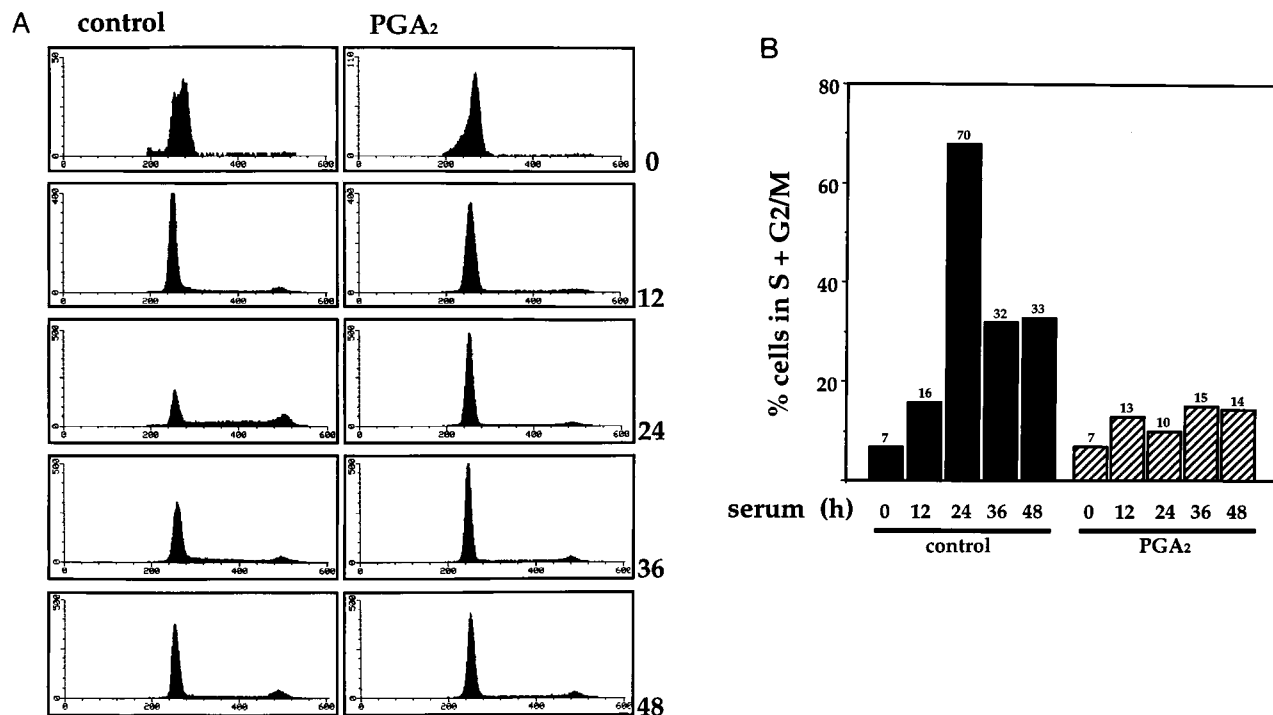The expression of p21 mRNA in synchronized, serum-stim-

FIG. 2. Cell cycle profiles of MCF-7 cultures in the presence or absence of PGA₂. (A) Synchronized MCF-7 cells were left untreated (control) or treated with PGA₂ 12 h prior to the addition of serum for the indicated times. Fixed cells were incubated with RNase A, stained with propidium iodide, and subjected to FACScan analysis to determine the distribution of cells throughout the G_1, S, and G_2/M phases. (B) Percentages of cells in the S and G_2/M phases following serum addition in the control (solid bars) and PGA₂-treated (hatched bars) cultures.

ulated cells is shown in Fig. 7. Prior to the addition of serum, p21 mRNA levels were low both in the control cells and in those receiving PGA₂ for 12 h. Nevertheless, although low, the levels of p21 mRNA in PGA₂-treated cells were still approximately fivefold higher than those seen in the absence of PGA₂ treatment (Fig. 7A, 0-h time point). Serum addition led to a rapid elevation in p21 mRNA expression in the PGA₂-treated cells. In fact, the kinetics of p21 mRNA induction in these cells synchronized by serum starvation was much faster than that seen following the addition of PGA₂ to asynchronous cells maintained in high serum concentrations (compare Fig. 7A with Fig. 5D). The slight increase in p21 mRNA seen in the control cells 12 h after serum addition is consistent with previous reports showing that p21 expression is transiently increased following growth factor stimulation (29).

The effect of PGA₂ on cdk2 kinase activity in synchronized, serum-stimulated MCF-7 cultures is shown in Fig. 7B. Cells were either left untreated or treated with PGA₂ for 12 h prior to serum stimulation. Lysates were immunoprecipitated in parallel at various times following the addition of serum with anti-cyclin E (the main cyclin associated with cdk2 during G_1) and anti-cdk2 antibodies, and the kinase activities associated with the various immunocomplexes were assayed. In the absence of PGA₂, the histone H1-kinase activities associated with the cyclin E and cdk2 immunocomplexes increased in a time-dependent manner. Although in both cases cdk kinase activity was detected by 8 h of serum addition (not shown) and substantially elevated by 14 h of stimulation (Fig. 7B), maximum kinase activity associated with cyclin E and cdk2 immunocomplexes was seen at ~18 and ~24 h, respectively. Together with our results for cdk4 activity, these findings are consistent with the current model for G_1-to-S progression whereby activation of cdk4 (in association with cyclin D) precedes that of cdk2 (in

association with cyclin E). Cyclin E-associated kinase activity peaks earlier (18 h) than that associated with cdk2 immuno-complexes (24 h), as cdk2 activity extends into the S phase in association with cyclin A. As was found for cdk4 kinase activity, cyclin E- and cdk2-associated kinase activities were extremely low in PGA₂-treated cells throughout the entire time course of the experiment.

PGA₂-mediated inhibition of cdk4 and cdk2 kinase activities correlates with hypophosphorylated Rb in serum-stimulated cultures. Since the existing evidence strongly supports a role for the G_1 cyclins and their associated kinases in the phosphorylation of endogenous Rb, and given the important role of Rb in the regulation of cell cycle progression, we examined the phosphorylation states of Rb in synchronized PGA₂-treated and control MCF-7 populations following serum stimulation. Hypophosphorylated forms of Rb migrate faster than hyper-phosphorylated forms and are thus easily discerned by Western analysis. Rb readily became hyperphosphorylated in control cells following serum addition (Fig. 8); however, in PGA₂-treated cells, Rb remained hypophosphorylated, even after 44 h of serum stimulation. This finding is consistent with the lack of cdk activity in PGA₂-treated cells and the failure of these cells to progress from G_1 into S.

PGA₂ effects occur independent of p53 function. p53-dependent as well as p53-independent mechanisms have been shown to contribute to the regulation of p21 expression. p53 has also recently been shown to be required for the downregulation of cdk4 expression that occurs in transforming growth factor β (TGF-β)-treated Mv1Lu cells (8). To address whether the growth-inhibitory response to PGA₂ is dependent on the p53 status of the cell, MCF-7 cultures transfected with the viral oncoprotein E6, and thereby rendered p53 deficient, were used (9). Expression of cyclin D1, cdk4, and p21 was examined following PGA₂
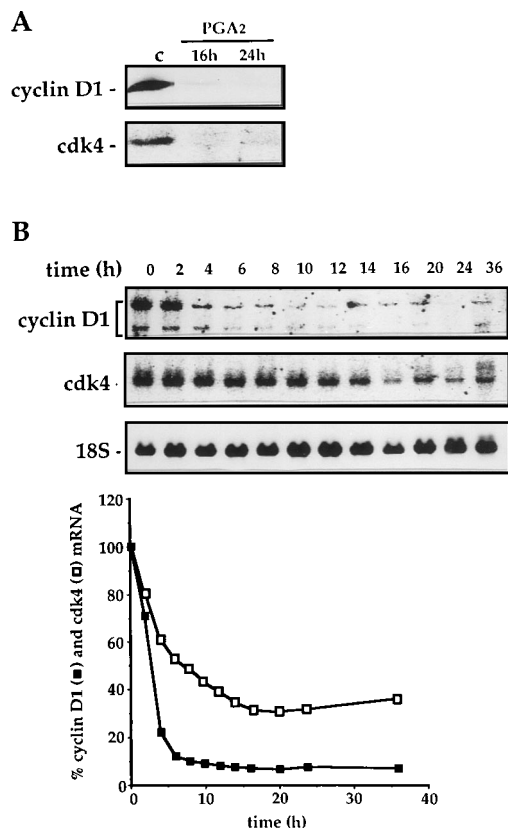
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FIG. 3. Effect of PGA$_2$ on the expression of cdk4 and cyclin D1. (A) Cells that were left untreated (lane c) or treated with 36 μM PGA$_2$ for 16 h and 24 h were analyzed for the expression of cyclin D1 (35 kDa) and cdk4 (33 kDa) by Western blot analysis as described in Materials and Methods. (B) Time course of changes in cyclin D1 and cdk4 mRNAs in PGA$_2$-treated cells. Cells were treated with 36 μM PGA$_2$ for the times indicated, and the expression of cyclin D1 and cdk4 mRNAs was analyzed on Northern blots as described in Materials and Methods. The 18S rRNA signal was used to normalize for differences in loading and transfer of the RNA samples. Hybridization signals were quantitated and normalized (graph) with a PhosphorImager (Molecular Dynamics).
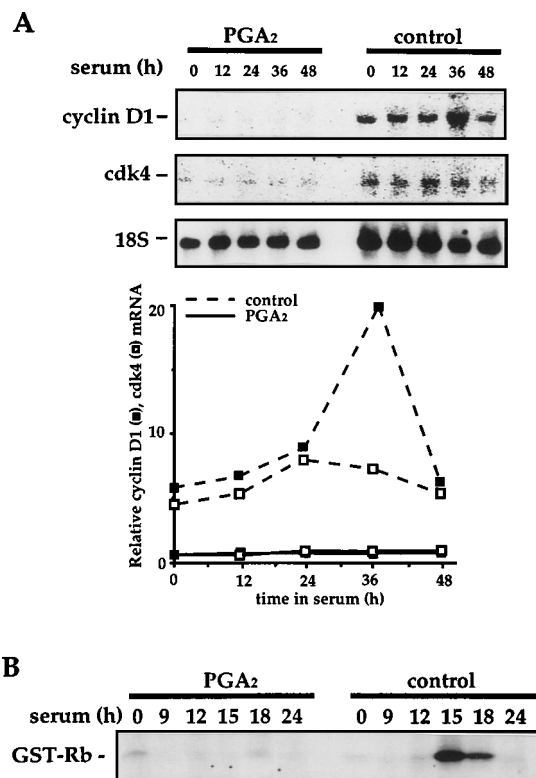
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FIG. 4. Expression of cyclin D1 and cdk4 mRNA and cdk4 kinase activity in synchronized MCF-7 cultures serum stimulated in the absence or presence of PGA$_2$. (A) Northern blot analysis of RNA extracted from synchronized MCF-7 cultures serum stimulated in the presence (PGA$_2$) or absence (control) of 36 μM PGA$_2$. Cyclin D1, cdk4, and 18S signals were quantitated with a PhosphorImager, and the mean values from three independent experiments are shown. (B) Kinase activities associated with anti-cdk4 immunocomplexes in PGA$_2$-treated and untreated MCF-7 cells. Synchronous MCF-7 cultures, either untreated or treated with 36 μM PGA$_2$, were serum stimulated for the indicated times. By using 1 mg of protein lysates for immunoprecipitations, the kinase activity associated with the cdk4 immunocomplexes was assayed with histone GST-Rb as a substrate. ^{32}P-labeled GST-Rb is shown.

treatment of two clones displaying high E6 expression (E6.1 and E6.9) and compared to that seen in parental MCF-7 cells similarly transfected with vector DNA only (*neo* control). As shown in Fig. 9A, the p53-deficient and parental MCF-7 cells contained similar levels of cdk4 and cyclin D1 prior to treatment, and both showed similarly low of these proteins in response to PGA$_2$ (results shown for E6.9 cells only). It is also worth noting that the decrease occurred rapidly following treatment with the prostaglandin. This finding is particularly striking for cyclin D1, wherein greater than 90% of the protein disappeared within 2 h of treatment.

The magnitude of p21 induction by PGA$_2$ (and PGA$_1$) also did not differ in the parental and p53-deficient cells (Fig. 9B and C). However, induction of p21 in response to methyl methanesulfonate, a DNA-alkylating agent, was significantly reduced in the p53-deficient cells, consistent with the role of p53 in mediating the cellular response to DNA damage and verifying the p53 deficiency of the clones. Finally, in accordance with the effects of PGA$_2$ on the expression of these cell cycle-regulatory proteins in p53-deficient cells, treatment of the E6.9 cells with PGA$_2$ also resulted in growth arrest that was indistinguishable from that of the parental cell lines (compare Fig. 9D with Fig. 1A). On the basis of these findings, we

conclude that functional p53 neither is required for nor contributes to the response of MCF-7 cells to PGA$_2$.

To further establish that p53 function is not required for either growth arrest or p21 induction by PGA$_2$, the response to PGA$_2$ was studied in MEFs derived from p53-knockout mice (30). As shown in Fig. 10A, PGA$_2$ treatment resulted in complete growth arrest of MEFs, while untreated cultures continued dividing. Accordingly, the expression of p21 mRNA (Fig. 10B) was substantially elevated following treatment of MEFs with either PGA$_1$ or PGA$_2$. These findings demonstrated that p53 is not required for growth arrest or p21 induction by PGA$_2$.

DISCUSSION

In this report, we have demonstrated that PGA$_2$ induces G$_1$ growth arrest in MCF-7 cells associated with an inhibition in the activities of both cdk2 and cdk4. However, the cellular mechanisms leading to the loss of these two kinase activities differ. In the case of cdk4, the lack of cdk4 activity appears to be due to the rapid loss in cyclin D and cdk4 protein expression following PGA$_2$ treatment. cdk2 protein levels, on the other hand, are unaffected by PGA$_2$ treatment; rather, cdk2 inactivation occurs concomitantly with the enhanced expression of
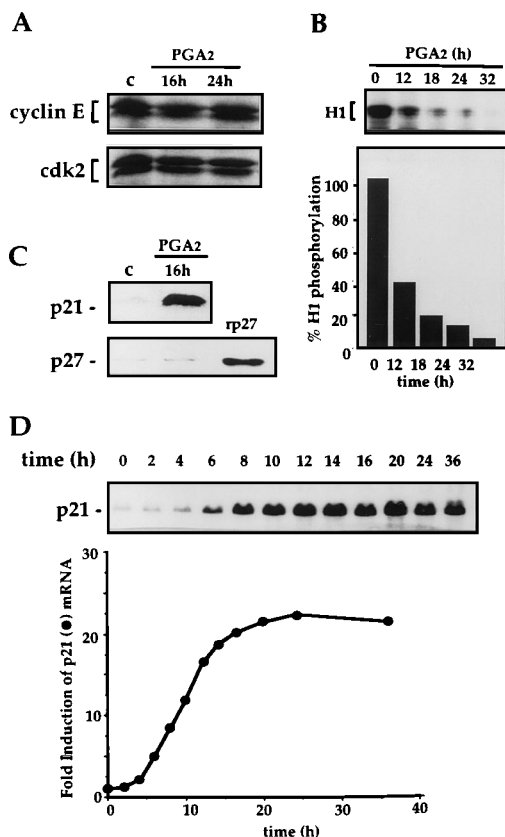
FIG. 5. Effect of PGA₂ on the expression of cyclin E and cdk2. (A) Cells that were left untreated (lane c) or treated with 36 μM PGA₂ for 16 h and 24 h were analyzed for the expression of cyclin E (50 kDa) and cdk2 (33 kDa) on Western blots. (B) Effect of PGA₂ treatment on cdk2 kinase activity. Asynchronously growing MCF-7 cultures were treated with 36 μM PGA₂ for the times indicated, and 1 mg of protein lysate was subjected to immunoprecipitation with an anti-cdk2 antiserum. The kinase activity associated with the resulting immunocomplexes was measured with histone H1 as a substrate as described in Materials and Methods. The incorporation of ^{32}P into histone H1 is shown. The bar graph represents a summary of the values obtained by quantitation of the radioactivity associated with histone H1, using a PhosphorImager. (C) Western blot analysis for p21 (21 kDa) and p27 (27 kDa) protein expression from cells that were either left untreated (lane c) or treated with 36 μM PGA₂ for 16 h. Ten nanograms of recombinant histidine-tagged mouse p27 protein (lane rp27) was included as a positive control. (D) Time course of changes in p21 mRNA in PGA₂-treated cells. Cells were treated with 36 μM PGA₂ for the times indicated, and the expression of p21 mRNA was analyzed on Northern blots. Values were normalized to the 18S rRNA level and quantitated (graph) with a PhosphorImager (Molecular Dynamics) as described in Materials and Methods.

FIG. 6. Analysis of p21-associated proteins in PGA₂-treated cells. (A) ^{35}S-labeled MCF-7 cells were either left untreated (lane c) or treated with 36 μM PGA₂ for 16 h, and the lysates were immunoprecipitated with a monoclonal anti-p21 antibody. The 21-kDa peptide (●) was confirmed to be p21 by subsequent immunoprecipitation using independent antibodies against p21 (not shown). (B) The 33-kDa protein (○) was identified as p33^{cdk2} by performing Western blot analysis on the ^{35}S-radiolabeled lysates. Very low levels of cdk4 were detected in these lysates with monoclonal antibodies against p34^{cdk4}.

FIG. 7. Expression of p21 mRNA and kinase activity in cdk2 and cyclin E immunoprecipitates in synchronized MCF-7 cultures serum stimulated in the absence or presence of PGA₂. (A) Northern blot analysis of RNA extracted from synchronized MCF-7 cultures serum stimulated in the presence (PGA₂) or absence (control) of 36 μM PGA₂. p21 mRNA and 18S signals were quantitated with a PhosphorImager, and the mean values from three independent experiments are shown. (B) Kinase activities associated with anti-cyclin E and anti-cdk2 immunocomplexes in PGA₂-treated and untreated MCF-7 cells. Synchronous MCF-7 cultures, either untreated or treated with 36 μM PGA₂, were serum stimulated for the indicated times. By using 1 mg of protein lysates for immunoprecipitations, the kinase activity associated with cyclin E and cdk2 immunocomplexes was assayed with histone H1 as a substrate. ^{32}P-labeled histone H1 is shown.

the cdk inhibitor p21. The pattern of cdk activity closely correlated with the phosphorylation profile of Rb: in PGA₂-treated cells it remained hypophosphorylated, and in control cells it was substantially phosphorylated by 8 h poststimulation (not shown), submaximally phosphorylated by 14 to 15 h of serum addition (Fig. 7B), and extensively phosphorylated thereafter. These findings are consistent with the current view that G₁ cyclin-cdk complexes play a major role in the phosphorylation of Rb, rendering it inactive and thereby allowing for the release of the transcription factor E2F from Rb and the enhanced expression of E2F-regulated genes (26, 37). Inhibition of cdk activity by a combination of cdk4 and cyclin D1 downregulation and p21 upregulation would therefore be expected to prevent changes in E2F-regulated gene expression. Indeed, it has previously been shown that the expression of one such E2F-regulated gene, *c-myc*, decreases in response to
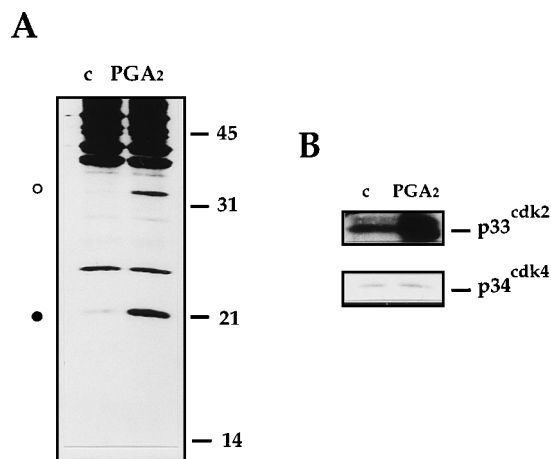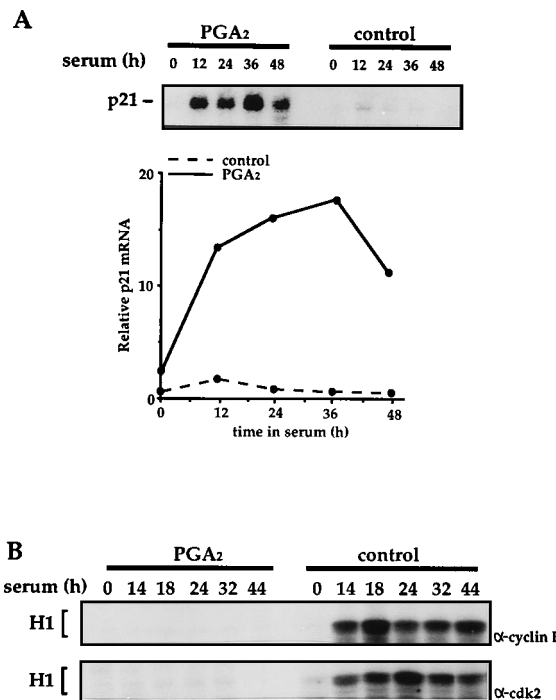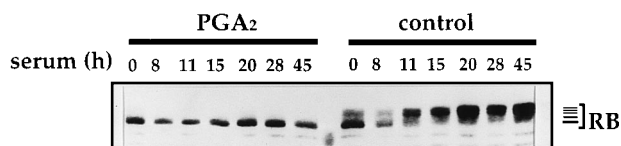
FIG. 8. Rb phosphorylation patterns in PGA$_2$-treated and untreated MCF-7 cells. Synchronous MCF-7 cultures were either left untreated (control) or treated with 36 μM PGA$_2$ prior to serum stimulation for the indicated times. The pattern of phosphorylation was detected by Western blot analysis using 50 μg of whole cell lysate. Faster-migrating (lower) band, unphosphorylated Rb; more slowly migrating bands, phosphorylated Rb.

PGA$_2$ (33, 42), and here we have demonstrated that the expression of cyclin D1 mRNA, another E2F-regulated gene (48), decreases following treatment with the prostaglandin.

That PGA$_2$ treatment would result in the concomitant inactivation of cdk2 and cdk4 by different mechanisms is intriguing.
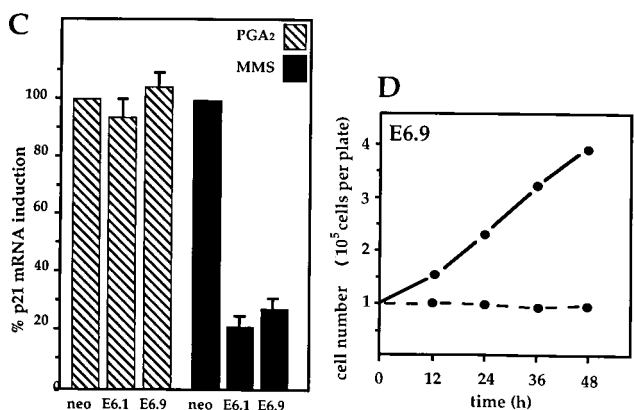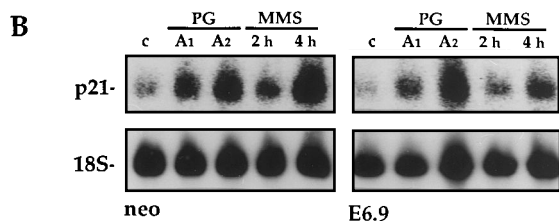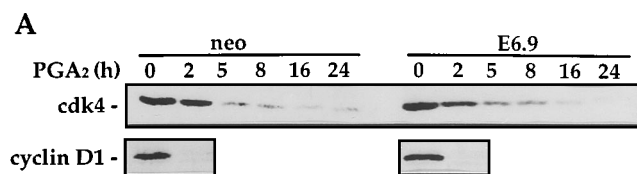

FIG. 9. Effect of p53 status on the growth-inhibitory response to PGA$_2$ in MCF-7 cells. (A) Western blot analysis of expression of cdk4 and cyclin D1 in p53-wild type (neo) and p53-deficient (E6.9) MCF-7 cells treated with PGA$_2$ (36 μM). (B) Induction of p21 mRNA in p53-wild type and p53-deficient MCF-7 cells. Parental (neo) and E6-transfected (E6.9) MCF-7 cells were treated with either PGA$_1$ (A$_1$) or PGA$_2$ (A$_2$) (36 μM) for 12 h or with 100 μg of methyl methanesulfonate (MMS) per ml for 2 or 4 h, after which the cells were harvested and analyzed for expression of p21 mRNA by Northern blot analysis. (C) Quantitation of p21 mRNA induction by PGA$_2$ or MMS in MCF-7 parental and E6.9 cells. The results, represented as percentages of fold induction of p21 mRNA relative to parental cells, are the means of four independent experiments. (D) Effect of PGA$_2$ on the growth of p53-deficient MCF-7 E6.9 cells. MCF-7 E6.9 cells were treated with 36 μM PGA$_2$ as described in the legend to Fig. 1A. Solid lines, untreated control cells; dashed lines, PGA$_2$-treated cells.
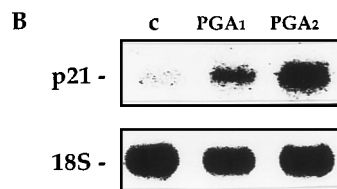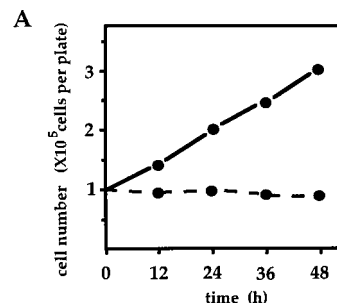

FIG. 10. Effect of PGA$_2$ treatment on p53$^{-/-}$ MEFs. (A) MEFs derived from a p53 knockout mouse were either treated with 36 μM PGA$_2$ or left untreated, and cell number was determined every 12 h thereafter with a hemocytometer. Solid lines, untreated control cells; dashed lines, PGA$_2$-treated cells. (B) Northern blot analysis of p21 expression in MEFs treated for 12 h with 36 μM PGA$_1$ or PGA$_2$ or left untreated (lane c). Values were normalized to the 18S rRNA level and quantitated with a PhosphorImager (Molecular Dynamics) as described in Materials and Methods.

Events associated with the inhibition of cdk4 kinase activity (downregulation of cdk4 and cyclin D1 expression) clearly preceded those involved in the inactivation of cdk2 kinase activity (i.e., induction of p21). However, whether p21 induction is dependent on the loss of cdk4 activity is presently unclear. Experiments aimed at further discerning the relative contribution and interdependence of alterations in cdk4 and cdk2 activities on the growth arrested state are under way. In this regard, the recently reported MEFs carrying targeted deletions of the gene encoding p21 (4) should be particularly useful in directly assessing the requirement of p21 in PGA$_2$-mediated growth arrest. Furthermore, our findings that growth arrest and p21 induction follow treatment of p53-deficient MEFs provide definitive evidence that p53 is not required for either one of these PGA$_2$-mediated effects (Fig. 10).

It remains to be determined how PGA$_2$ treatment decreases the expression of cdk4 and cyclin D1. Cyclin D1 mRNA levels are known to be regulated through changes in its rate of transcription as well as through alterations in its stability (27, 51). It is conceivable that PGA$_2$ modulates cyclin D1 levels by either or both of these mechanisms, as there are examples of PGA$_2$-induced changes in gene expression through modulation of transcription as well as through changes in mRNA stability (2). Importantly, however, PGA$_2$-stimulated changes in cyclin D1 protein levels preceded those of mRNA expression, indicating that translational effects are also clearly involved in regulating cyclin D1 expression by PGA$_2$.

With respect to the regulation of cdk4, Ewen et al. (8) recently demonstrated that TGF-β-induced growth arrest in Mv1Lu cells occurs concomitant with a reduction in cdk4 protein levels. This reduction in cdk4 protein was found to be largely mediated through repression of the translation of cdk4 mRNA and was shown to be dependent on the presence of wild-type p53. The p53 status of MCF-7 cells does not influence the response to PGA$_2$, as shown here. However, a translational mechanism similar to that occurring in TGF-β-treated

Mv1Lu cells is likely to contribute to the reduction in cdk4 protein expression seen in PGA$_2$-treated cells, as changes in cdk4 mRNA occur over a slower time course.

The mechanisms through which PGA$_2$ induces p21 expression also remain to be elucidated. Our previous observation that PGA$_2$ treatment results in a rapid (within 100 s) increase in intracellular calcium (2) suggests that it is possible that changes in intracellular calcium are important in initiating the response. Whether the induction involves transcriptional or posttranscriptional control is also unclear. However, it is worth noting that we have consistently failed to detect any activation of a p21 promoter-luciferase reporter construct following PGA$_2$ treatment of transiently transfected MCF-7 cells (unpublished observations).

We have shown that the growth-inhibitory response to PGA$_2$, including the reduction of cdk4 and cyclin D1 and the induction of p21, occurs independent of p53 activity. This is interesting given the role of p53 in mediating G$_1$ arrest in response to genotoxic stress (5, 32) as well as TGF-β treatment (8). Such information could be of practical significance for the treatment of cancers lacking functional p53. Moreover, increased knowledge of how PGA$_2$ acts to alter the expression of specific G$_1$-regulated proteins to arrest proliferation could likewise be beneficial in devising new treatment regimens utilizing combinations of different agents which act at different stages in the cell cycle.

ACKNOWLEDGMENTS

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