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Mechanisms of Cell Cycle Arrest in Response to TGF-β in Progestin-Dependent and -Independent Growth of Mammary Tumors

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TGF-β1 modulation of cell cycle components was assessed in an experimental model in which the synthetic progestin medroxyprogesterone acetate (MPA) induced mammary tumors in Balb/c mice. TGF-β1 inhibited both MPA-induced proliferation of progestin-dependent C4HD epithelial cells and proliferation of the progestin-independent variant cell type C4HI, arresting cells in G_1 phase of the cell cycle. Progestin-independent 60 epithelial cells evidenced reduced response to TGF-β1 antiproliferative effects. TGF-β1 inhibition of cyclins D1 and A expression and up-regulation of $p21^{CIP1}$ levels were the common findings in all three cell types. In addition, a significant content reduction of cyclin D1/cdk4 and cyclin A/cdk2 complexes was found after TGF-β1 inhibition of MPA-dependent and -independent proliferation. TGF-β1 inhibited cyclin D2 expression and up-regulated $p27^{KIP1}$ levels only when acting as inhibitor of MPA-induced proliferation of C4HD cells. Regulation of these two cell cycle components resulted in decreased cyclin D2/cdk2 complex and in increased $p27^{KIP1}$ association with cdk2 in C4HD cells treated with TGF-β1. These two molecular mechanisms, unobserved in progestin-independent growth of C4HI or 60 cells, were associated with a significantly higher degree of inhibition of cdk2 kinase activity in C4HD cells compared to that found in TGF-β-treated C4HI or 60 cells. Reduced sensitivity of 60 cells to the growth-inhibitory effects of TGF-β1 correlated with significantly lower levels of $p15^{INK4B}$, $p21^{CIP1}$, and $p27^{KIP1}$ expressed in these cells, compared to the levels present in C4HD or C4HI cells, and correlated as well with lack of expression of $p16^{INK4}$. Thus, common targets were found to exist in TGF-β1 inhibitory action on breast cancer cells, but regulation of specific targets was found when TGF-β1-inhibited proliferation driven by the progesterone receptor.

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INTRODUCTION

Transforming growth factor-β (TGF-β) comprises a family of multifunctional polypeptides involved in many cellular processes, including growth and differentiation [1]. One of the best-characterized and most important functions of TGF-β is its ability to arrest a wide variety of cell types in the G_1 phase of the cell cycle [reviewed in Ref. 2]. Though cytoplasmic events that propagate the TGF-β growth-arrest signal to the nucleus are not yet well understood, it is currently known that TGF-β binds to cell-surface receptors, which are transmembrane serine/threonine kinases [2, 3]. TGF-β binds to type II receptor (TGF-βRII) which then associates with and phosphorylates type I receptor. Upon phosphorylation, type I receptor becomes active and then phosphorylates a number of downstream effectors, including the Smad protein family [4]. After phosphorylation by TGF-βRII Smad2 and Smad3 heterodimerize with Smad4 and translocate to the nucleus in order to transactivate gene expression [4]. Although Smads are required for TGF-β-mediated growth arrest, the mechanism underlying their action is still unknown. In contrast, the nuclear signals by which TGF-β halts the cell cycle have been better characterized and appear to involve TGF-β links to the retinoblastoma protein (Rb) phosphorylation state. Thus, TGF-β-mediated growth arrest was found to correlate with an accumulation of hypophosphorylated Rb [5]. Control of the cell cycle-specific phosphorylation state of Rb is provided through the kinase activity of the enzyme complex formed by cyclins and cyclin-dependent kinases (cdks). In addition to cyclin association, kinase activity of the cdks can be regulated in three other ways. First, cyclins can be regulated through transcription. Second, activating and deactivating phosphorylations can regulate cdk activity. Third, a group of low-molecular-weight molecules

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known as cyclin-dependent kinase inhibitors (cdkIs) has been shown to associate with, and concomitantly block, cdk activity [reviewed in Ref. 6]. TGF-β induction of cell cycle arrest in G_1 has been found to involve several levels of control of the cell cycle components which appear to be cell type specific [reviewed in Ref. 7].

Regarding the role of TGF-β in breast cancer, it is well known that TGF-βs inhibit *in vitro* proliferation of normal mammary epithelial and breast cancer cell lines [8–11]. However, the net role of TGF-βs in the maintenance and progression of breast carcinoma is less clear. Several lines of evidence support the existence of a positive association between TGF-β and breast cancer cell tumorigenesis. This positive association involves both a reduced sensitivity of tumor cells to TGF-β autocrine growth inhibitory functions [11–13] and TGF-β-mediated effects on the host that are advantageous for tumor growth, such as modulation of extracellular matrix and stimulation of pericellular protease secretion [14].

We had already studied the expression and growth effects of TGF-β1 in the well-characterized model of hormonal carcinogenesis in which the synthetic progestin medroxyprogesterone acetate (MPA) induced mammary adenocarcinomas in virgin female Balb/c mice [15–17]. Most of the MPA-induced tumors are of ductal origin, express high levels of estrogen and progesterone receptors (ER and PR), and are maintained by syngeneic transplants in MPA-treated mice, evidencing a progestin-dependent (HD) pattern of growth [18, 19]. Ductal tumor variants with an MPA-independent (HI) pattern of growth have been generated by transplantation into untreated mice. These HI tumors express the same ER and PR content as their parental HD counterparts [19]. Finally, MPA induces a small number of lobular mammary carcinomas, which lack ER and PR expression and evidence an HI behavior [18, 19]. We had also already found that TGF-β1 inhibited both MPA-induced proliferation of primary cultures of C4HD epithelial cells derived from a ductal, progestin-dependent tumor line and proliferation of C4HI cells derived from the hormone-independent variant C4HI tumor line [17]. In addition, we found that 60 epithelial cells, which derive from a lobular MPA-independent tumor line, showed a reduced response to TGF-β antiproliferative effect [17]. Therefore, the present study was aimed at comparing TGF-β1 modulation of cell cycle components in these three different phenotypes of breast tumor cells. The availability of progestin-dependent tumor lines, and of their progestin-independent counterparts, provides us a model to investigate whether TGF-β1 regulation of the cell cycle machinery is different when TGF-β1 inhibits growth driven by the progesterone receptor from that when TGF-β1 inhibits growth of the variant tumor type that has become independent of progestins to proliferate. Finally, we sought to investigate whether the reduced response of 60 cells to TGF-β1 could be associated with defects or loss of expression of components of the cell cycle machinery required by TGF-β1 to induce growth arrest.

MATERIALS AND METHODS

Animals and tumors. Experiments were carried out in virgin female Balb/c mice raised at the National Academy of Medicine of Buenos Aires. All animal studies were conducted in accordance with the highest standards of animal care as outlined in the NIH guide for the Care and Use of Laboratory Animals. Hormone-dependent ductal tumors, C4HD, originated in mice treated with 40 mg MPA every 3 months for 1 year and have been maintained by serial transplantation in animals treated with 40 mg sc MPA deposited on the flank opposite to tumor inoculum [18, 19]. Their hormone-independent counterparts were C4HI tumors developed from HD tumors growing in mice that were not treated with MPA approximately 1 year after inoculum [18, 19]. The C4HI tumor line has been maintained by serial transplantation in untreated female mice. C4HD tumors express PR and ER [18, 19]. Although the C4HI tumor line has developed the capacity to grow in absence of progestin treatment, it has nevertheless retained PR and ER [18, 19]. The HI line 60 originated in MPA-treated mice and has been maintained by syngeneic transplants in untreated virgin female mice. The 60 line is of lobular origin and does not express PR or ER [19].

Primary cultures and cell proliferation assays. Primary cultures of epithelial cells from C4HD tumors growing in MPA-treated mice, from C4HI tumors, and from 60 tumors were carried out as described previously [20]. In brief, tumors were aseptically removed, minced, and washed with DMEM/F12 (Dulbecco's modified Eagle's medium/Ham's F12, 1:1, without phenol red, 100 U/ml penicillin and 100 μg/ml streptomycin). Tissue was suspended in 5 ml of enzymatic solution [trypsin 2.5 mg/ml, albumin 5 mg/ml, and collagenase type II (Gibco BRL, Gaithersburg, MD) 239 U/ml] in phosphate-buffered saline (PBS) and incubated at 37°C for 20 min, under continuous stirring. The liquid phase of the suspension was then removed and the undigested tissue was incubated with fresh enzymatic solution for 20 min. Enzyme action was stopped by adding DMEM/F12 + 5% heat-inactivated fetal calf serum (FCS) (Gen S.A., Buenos Aires). Epithelial and fibroblastic cells were separated as already described [20]. Briefly, the cell suspension was resuspended in 15 ml DMEM/F12 + 10% FCS and allowed to sediment for 20 min. The sedimented cells, corresponding to the epithelial-enriched fraction, were resuspended again in 15 ml DMEM/F12 + 5% FCS and allowed to sediment for another 20 min. The upper 15 ml was discarded and this procedure was repeated until no fibroblasts were observed in the supernatant. Cells were plated in culture flasks with DMEM/F12 + 5% steroid-stripped FCS (ChFCS) [20] and allowed to attach for 24–48 h. Purity of epithelial cultures was evaluated by cytokeratin staining. Cells were incubated in DMEM/F12 (without phenol red, 100 U/ml penicillin and 100 μg/ml streptomycin) with 2.5% ChFCS in the presence and in the absence of MPA or TGF-β1. After a 24-h incubation, 50% of the medium was replaced by fresh medium and cells were incubated for another 24 h in the presence of 0.8 μCi [^{3}H]thymidine (NEN, Dupont, Boston, MA; sp act 70–90 Ci/mmol). Cells were then trypsinized and harvested. Assays were performed in octuplicate. The differences between control and experimental groups were analyzed by ANOVA followed by Tukey *t* test between groups. In former experiments we had demonstrated that thymidine uptake correlates with the number of cells/well [20]. J. C. Calvo

(Instituto de Biología y Medicina Experimental, Buenos Aires) provided the normal murine mammary gland cells NMuMG.

Antisense studies. Cyclin D2 antisense ODNs (5′CCA CAT TGA TAC AGC TTT) correspond to the murine cyclin D2 mRNA translation start [21]. A scrambled sequence (5′TCC CCG AGC CAT TTC CTA) with the same overall oligodeoxynucleotide content as the antisense oligo was used as control. ODNs were purchased from NucleiCo (Buenos Aires, Argentina). The effect of ODNs was studied as follows: 0.2 ml/well of a cell suspension was seeded at a concentration of 10^5 cell/ml in a Falcon 96-well microtiter plate. After attachment (24–48 h), the cells were incubated for 48 h with medium (serum used in antisense studies was heated at 65°C for 30 min to inactivate exonucleases) containing the indicated concentrations of ODNs. The media were changed every 24 h adding fresh ODNs. After incubation, [^{3}H]thymidine was added and incorporation was determined as described above.

Flow cytometric analysis. Cells were harvested for flow cytometric analysis and fixed in 70% ethanol for 24 h at 4°C. They were then washed twice with PBS, followed by RNA digestion (RNase A 50 U/ml) and propidium iodide (20 μg/ml) staining for 30 min at room temperature in the dark before analysis, using a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA).

Cell cycle phases were determined with Modfit LT software.

Antibodies. Antibody to the retinoblastoma protein (pRb) was obtained from Pharmingen (San Diego, CA). Antibodies to cyclin D1 (72-13G), cyclin D3 (C-16), cyclin E (M-20), cyclin A (C-19), $p21^{CIP1}$ (C-19), $p16^{INK4}$ (M-156), $p27^{KIP1}$ (C-19), and cdk4 (C-22) were from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin E (Ab-1), cyclin D2 (clone DCS-5.2), cyclin D2 (clone DCS-3.1+DCS-5.2), cdk2 (clone 2B6+8D4), and $p15^{INK4B}$ antibodies were from Neomarkers (Freemont, CA).

Immunoblotting. Cells were lysed in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycolbis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 10% glycerol, 0.5% Nonidet P-40, 1 mM $ClMg_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 1 mM sodium orthovanadate, 5 mM NaF, 20 mM sodium molybdate, and 5mM sodium pyrophosphate. Lysates were centrifuged at 40,000*g* for 40 min at 4°C, and protein content in the supernatant was determined using a Bio-Rad kit (Richmond, CA). Proteins were solubilized in sample buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue) and subjected to SDS–PAGE. Proteins were electroblotted onto nitrocellulose. Membranes were blocked with PBS, 0.1% Tween 20 and immunoblotted with the indicated antibodies. After washing, the membranes were incubated with HRP-conjugated secondary antibody (Amersham International, UK). Enhanced chemiluminescence was performed according to the manufacturer's instructions (Amersham). For detection of abundance of the complexes cyclin D1/cdk4, cyclin A/cdk2, and cyclin E/cdk2, 1 mg protein from cell lysates was precleared with protein A–agarose or protein G–agarose (Santa Cruz Biotechnology), and 2 to 5 μg of cdk4, cdk2, or cyclin E antibodies was used. Immunoprecipitations were rocked 2 h at 4°C and then the immunocomplexes were captured by adding protein A–agarose or protein G–agarose and rocked for an additional 2 h. Beads were washed three times with lysis buffer, then boiled for 10 min in sample buffer, and subjected to SDS–PAGE. Proteins were electroblotted onto nitrocellulose. Membranes were blocked and filters were probed with anti cyclin D1, anti-cyclin A, or anti-cdk2 antibodies. To study association of cdk2 with $p21^{CIP1}$ or $p27^{KIP1}$, 1 mg protein precleared with protein G–agarose was incubated with anti-cdk2 antibody, and immunocomplexes were subjected to SDS–PAGE and electroblotted onto nitrocellulose. Filters were probed with $p21^{CIP1}$ and $p27^{KIP1}$ antibodies. For detection of association of cyclin D2 with cdk2 or cdk4, 1 mg protein extract was precleared with protein G–agarose and incubated with cyclin D2 antibody, complexes were resolved and blotted, and membranes were incubated with anti-cdk2 or -cdk4 antibodies. Protein extracts (100 μg total protein) were blotted in parallel with cdk2 or cdk4 antibodies. The intensities of the cdk2 and cdk4 bands in the lanes in which protein lysates were run and in the lanes in which cyclin D2 immunoprecipitates were run were quantitated densitometrically. The intensities of these bands were compared (correcting for the different quantities of protein loaded) to calculate the percentages of the total cdk2 and cdk4 pools that were associated with cyclin D2. In all the experiments in which associations between proteins were studied, protein-depleted lysate samples were incubated with the antibody used to immunoprecipitate so that absence of the protein could confirm that the amount of antibody used was sufficient to immunoprecipitate all of the protein of interest. To study the effects of antisense ODNs (ASODNs) to cyclin D2 on protein expression, lysates were prepared as described above from cells both treated and untreated with ODNs. Protein extracts were subject to Western blot analysis with anti-cyclin D2 antibodies. To quantitate either the intensities of cyclins, cdks, or cdkIs or the amount of the complexes of cyclins/cdks and cdks/cdkIs, band intensities were quantitated by densitometry of multiple autoradiograms of various exposures.

Cdk2 kinase assay. Cells were lysed in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM $ClMg_2$, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 1 mM sodium orthovanadate, 5 mM NaF, 20 mM sodium molybdate, and 5 mM sodium pyrophosphate (IP kinase buffer). Lysates were centrifuged at 40,000*g* for 40 min at 4°C and protein content in the supernatant was determined by using a Bio-Rad kit. Protein extracts were immunoprecipitated with an anti-cdk2 antibody. The precipitates were washed four times with IP kinase buffer and were then incubated with a kinase buffer containing 10 μg histone H1 protein (Calbiochem, La Jolla, CA), 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 1 mM sodium orthovanadate, 5 mM NaF, 20 mM sodium molybdate, 5 mM sodium pyrophosphate, 10 μCi [γ-^{32}P]ATP, 20 μM ATP. Samples were mixed gently to resuspend beads and incubated at 30°C for 30 min with gentle mixing every 10 min. The reaction was terminated by addition of 3× sample buffer (final concentration: 60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue) and subjected to SDS–PAGE on 12% gels. Band intensities were quantitated by densitometry of multiple autoradiograms of various exposures. In addition, identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with anti-cdk2 antibody to verify that nearly equal amounts of immunoprecipitated proteins were loaded.

RESULTS

TGF-β1 Inhibits MPA-Induced Proliferation of Progestin-Dependent Mammary Tumor Cells as Well as Proliferation of Progestin-Independent Cells

The present study was designed to compare TGF-β1 modulation of cell cycle components when acting either as inhibitor of progestin-dependent growth or as inhibitor of progestin-independent proliferation of mammary tumor cells. We used the well-characterized model of hormonal carcinogenesis in which the synthetic progestin MPA induced mammary adenocarcinomas in Balb/c mice [18, 19]. The availability of progestin-dependent tumor lines, and of their progestin-

independent counterparts, provides us a model to investigate whether TGF-β1 regulation of the cell cycle machinery is different when TGF-β1 inhibits growth driven by the progesterone receptor from when TGF-β1 inhibits growth of the variant tumor type that has become independent of progestins to proliferate. We first examined primary cultures of C4HD epithelial cells derived from a ductal, progestin-dependent tumor line. C4HD cells require MPA administration to proliferate and express high levels of ER and PR [20]. TGF-β1 inhibited MPA-induced proliferation of these cells, in a dose-dependent fashion, with an ED50 of 0.26 ± 0.14 ng/ml (Fig. 1A). We then studied C4HI cells, derived from the tumor hormone-independent variant C4HI [18, 19]. C4HI cells are able to proliferate in 2.5% ChFCS in the absence of MPA administration. However, they retain ER and PR expression [20]. TGF-β1 elicited a dose-dependent inhibition of serum-induced proliferation of C4HI cells with an ED50 of 0.27 ± 0.12 ng/ml, similar to that found in C4HD cells (Fig. 1A). Finally, we had in previous studies found that 60 epithelial cells evidenced a reduced response to TGF-β1 antiproliferative effect [17] and that they required significantly higher TGF-β1 concentrations than C4 cells to show an inhibitory response (ED50 7.85 ± 0.25 ng/ml) (Fig. 1A). 60 cells derive from a lobular MPA-independent tumor line, lack ER or PR expression, and are able to proliferate in the absence of MPA administration [18, 19].

Flow cytometric analysis showed that TGF-β1 treatment of asynchronous C4HD, C4HI, and 60 cells, for 48 h, led to an accumulation of cells in the G1 phase (Fig. 1B).

Anti-pRb immunoblot analysis of protein extracts showed that TGF-β1 treatment of the three cell types resulted in a down-regulation of total Rb protein, consistent with G_1 cell cycle arrest in many systems, including breast cancer cells [22], as well as in a decrease of the expression of inactive, slower migrating hyperphosphorylated Rb species (Fig. 1C).

TGF-β1 Down-regulates Cyclin D1 and A Expression at the Protein Level in All Three Cell Types: Cyclin D2 Protein Is Reduced Only When TGF-β1 Inhibits MPA-Induced Proliferation

We investigated TGF-β1 effects on the expression of cyclins, cdks, and cdk inhibitors under conditions in which TGF-β1 inhibited the strong proliferation induced by MPA in C4HD cells or in which TGF-β1 inhibited the high proliferative rate of C4HI and 60 cells in 2.5% ChFCS. For this purpose, TGF-β1 was added together with MPA on C4HD cells or was added to C4HI and 60 cells growing in 2.5% ChFCS. TGF-β1 concentrations corresponding to the maximal inhibitory effect on cell proliferation were used. Therefore, 10 ng/ml TGF-β1 was added to C4HD and C4HI cells and 70 ng/ml to 60 cells. Cyclin, cdk, and cdk inhibitor expression was examined after 48 h of TGF-β1 treatment. Figure 2A shows that protein levels of cyclin D1 were significantly inhibited when TGF-β1 inhibited MPA-proliferative effects on C4HD cells and when TGF-β1 inhibited proliferation of C4HI or 60 cells. Densitometric analysis of Western blots showed that TGF-β1 induced 78–90% reduction in cyclin D1 protein in all three cell types, compared with the levels found in cells proliferating either in MPA 10 nM or in 2.5% ChFCS. Cyclin D2 protein levels were reduced by 62–80% when TGF-β1 was added to C4HD cells growing in MPA 10 nM (Fig. 2A). Contrariwise, no significant effect of TGF-β1 on the levels of cyclin D2 were observed when TGF-β1 inhibited proliferation of C4HI or 60 cells (Fig. 2A). Cyclin D3 protein expression was not affected by TGF-β1 treatment of C4HD, C4HI, or 60 cells (Fig. 2A). Three cyclin E isoforms, ranging in size from 35 to 50 kDa, were detected in all three cell types using two different cyclin E antibodies (Fig. 2A). The same antibodies detected low levels of expression of one cyclin E isoform of approximately 50 kDa in normal mouse mammary tissue used as control for cyclin E expression (Fig. 2A). These findings are in accordance with previous results showing that cyclin E is abnormally expressed quantitatively and qualitatively in breast tumor tissues and breast cancer cell lines [23–25]. Thus, three overexpressed cyclin E isoforms, ranging in size from 35 to 50 kDa, were found in breast tumor tissues and breast cancer cell lines, whereas in normal breast tissue one major cyclin E protein of 50 kDa was expressed at low levels [23–25]. Levels of all three cyclin E isoforms remained unchanged after TGF-β1 treatment of C4HD, C4HI, or 60 cells (Fig. 2A). On the contrary, TGF-β1 dramatically reduced protein levels of cyclin A when acting either as inhibitor of MPA-induced growth of C4HD cells or as an inhibitor of C4HI and 60 cell proliferation (Fig. 2A).

Since the data presented above indicated that TGF-β1 down-regulated cyclin D1 when it inhibited MPA stimulatory effects, we examined the converse situation, whether MPA-induced proliferation of C4HD cells affected cyclin D1 levels. As can be seen in Fig. 2B, a three- to fourfold increase in cyclin D1 protein expression was induced by MPA treatment of these cells. This result is in agreement with what has been reported by other authors [6] and goes to show that induction of cyclin D1 expression is a critical component of the mitogenic response to progestins.

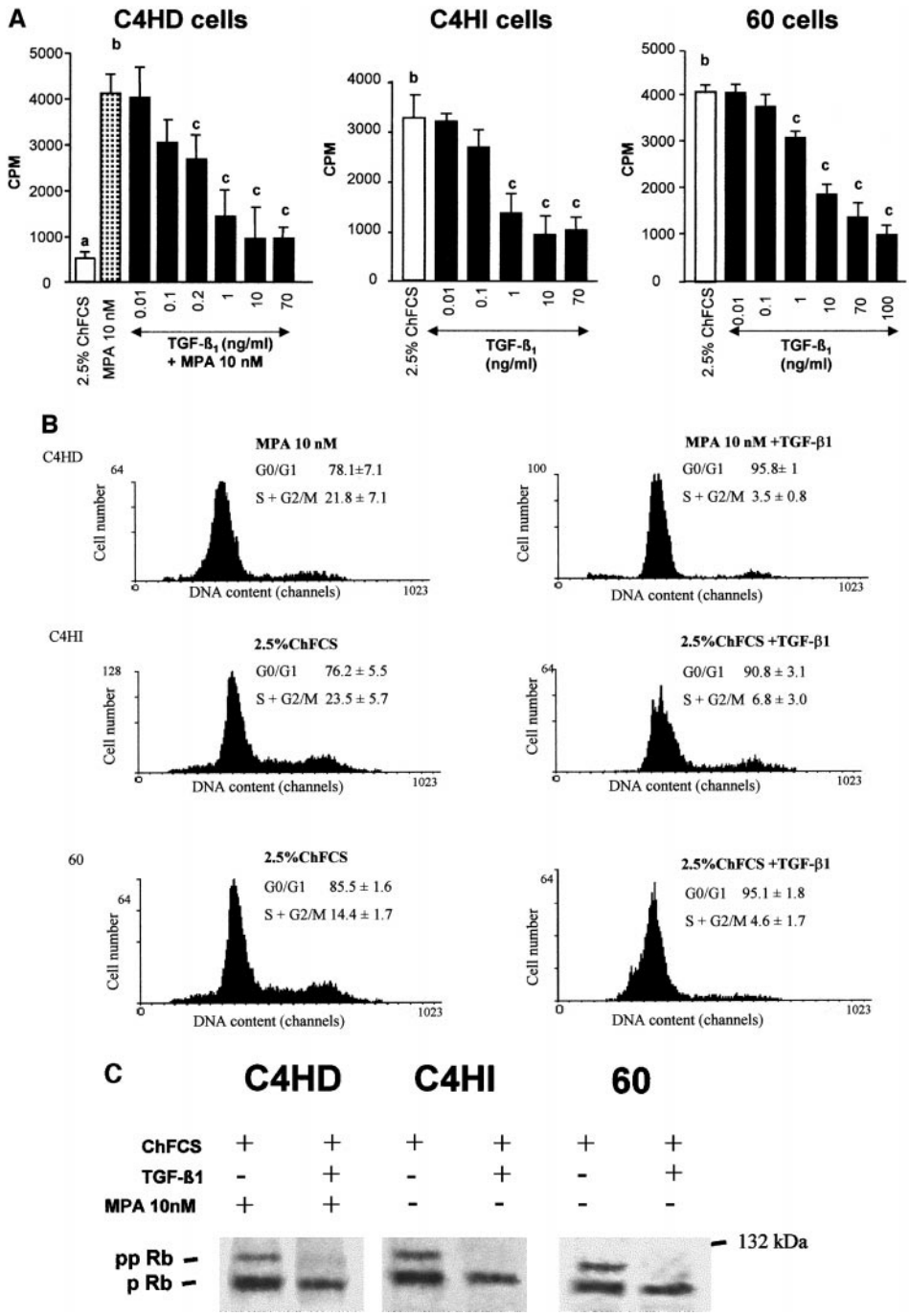


FIG. 1. Effect of TGF-β1 on the proliferation of HD and HI mammary tumor cells and on retinoblastoma protein. (A) [^{3}H]Thymidine incorporation assay. Primary cultures of epithelial cells from C4HD, C4HI, and 60 tumors were incubated in medium with 2.5% ChFCS with and without the addition of MPA, TGF-βs, or MPA + TGF-βs as indicated. Data are presented as means ± SD. b vs a and c vs b, $P < 0.001$. The experiments shown are representative of a total of four for each cell type. (B) G_1 arrest following TGF-β1 treatment. After being cultured in the absence (C4HD cells, 2.5% ChFCS + MPA; C4HI and 60 cells, 2.5% ChFCS) and in the presence of TGF-β1 (10 ng/ml for C4HD and C4HI cells and 70 ng/ml for 60 cells) for 48 h, cells were treated with propidium iodide and analyzed for cell cycle distribution by flow cytometry, as described under Materials and Methods. The DNA histograms are representative of a total of five for each cell type. The percentages of total cells in the cell cycle phases are indicated. (C) Phosphorylation of the Rb protein. Cell lysates from cells treated and untreated with TGF-β1 were resolved by 12% SDS–PAGE and immunoblotted with an anti-Rb antibody. pp Rb, hyperphosphorylated Rb; p Rb, hypophosphorylated Rb.

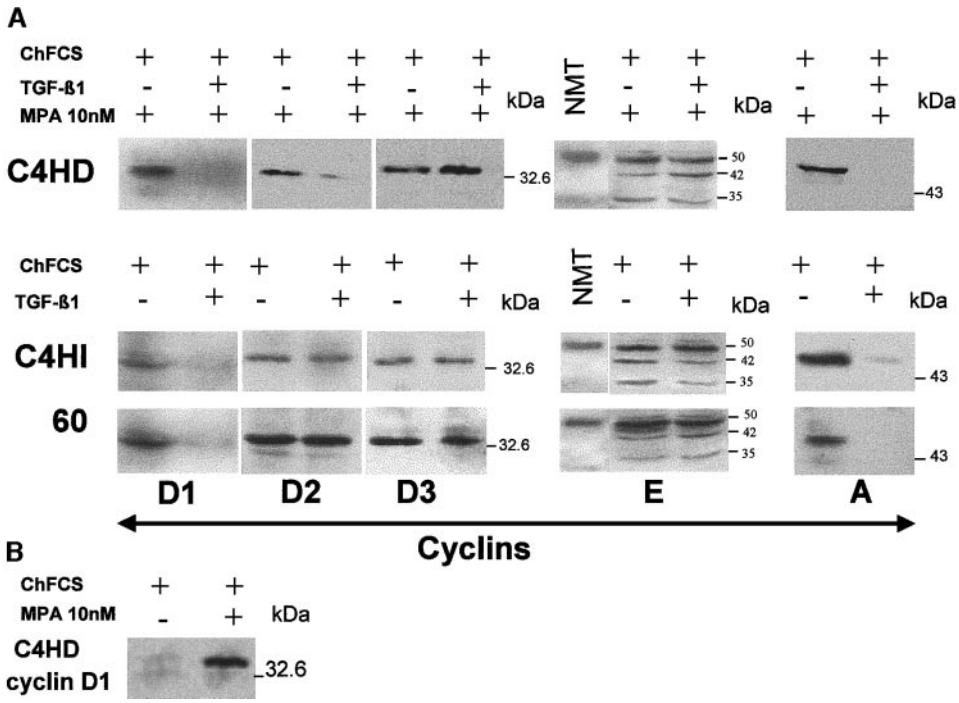


FIG. 2. Effect of TGF-β1 treatment on cyclin expression and MPA induction of cyclin D1 expression. Primary cultures of C4HD epithelial cells were incubated for 48 h in 2.5% ChFCS + MPA 10 nM and primary cultures of C4HI and 60 cells were incubated 48 h in 2.5% ChFCS, with and without the addition of TGF-β1 (C4HD cells, 10 ng/ml; C4HI and 60 cells, 70 ng/ml) as indicated. One hundred micrograms of protein from cell lysates was electrophoresed and immunoblotted for cyclins D1, D2, D3, E, and A. Cyclin E expression was studied using the two different antibodies detailed under Materials and Methods. Shown are the results obtained with the cyclin E antibody from Santa Cruz. NMT, normal mammary tissue, used as control for cyclin E expression. (B) Levels of cyclin D1 in primary cultures of C4HD cells growing for 48 h in 2.5% ChFCS or in 2.5% ChFCS supplemented with MPA 10 nM. One hundred micrograms of protein from cell lysates was electrophoresed and immunoblotted for cyclin D1. Band intensities were quantitated by densitometry of multiple autoradiograms of various exposures.

Effect of TGF-β1 on Cdk Protein Expression and Cyclin/Cdk Complex Abundance

Treatment with TGF-β1 did not affect cdk2 or cdk4 protein levels in any of the cell types (Fig. 3). Since we found inhibition of cyclin D1 expression by TGF-β1, we then investigated whether abundance of the complex cyclin D1/cdk4 was affected. Protein extracts from cells treated and untreated with TGF-β1 were immunoprecipitated with anti-cdk4 antibody and Western blotting was performed with an anti-cyclin D1 antibody. As expected, a significant reduction (80–90%) in cyclin D1/cdk4 content was found when TGF-β1 inhibited MPA–induced proliferation of C4HD cells and when TGF-β1 abolished C4HI and 60 cell growth (Fig. 4). Similarly, when cell extracts were immunoprecipitated with an anti-cdk2 antibody and blots were incubated with an anti-cyclin A antibody, the presence of cyclin A/cdk2 complex was barely detectable in TGF-β1-treated C4HD, C4HI, or 60 cells (Fig. 4). This might be explained by the dramatic reduction in cyclin A protein expression elicited by TGF-β1. Our results demonstrated that the levels of cyclin E and its major catalytic partner cdk2 were unaffected by TGF-β1 treatment in C4HD, C4HI, or 60 cells (Figs. 2 and 3). Therefore, we investigated the effect of TGF-β1 on the assembly of cyclin E/cdk2 complex. Protein extracts were immunoprecipitated with an anti-cyclin E antibody and Western blotting was performed with an anti-cdk2 antibody. As shown in Fig. 4, TGF-β1 treatment did not affect the abundance of the cyclin E/cdk2 complex in any cell type.

TGF-β1 Up-regulates $p21^{CIP1}$ in All Three Cell Types: $p27^{KIP1}$ Is Up-regulated Only When TGF-β1 Inhibits MPA-Induced Proliferation

TGF-β1 significantly up-regulated protein expression of $p21^{CIP1}$ when inhibiting MPA-induced proliferation of C4HD cells and when inhibiting proliferation of C4HI cells (Fig. 5A). Protein levels of $p27^{KIP1}$ were increased only when TGF-β1 antagonized MPA-stimulated growth of C4HD cells (Fig. 5A). Abundance of $p15^{INK4B}$ and of $p16^{INK4}$ remained unaffected by TGF-β1 treatment of these two cell types (Fig. 5A). No expression of any of these inhibitors was detected by Western

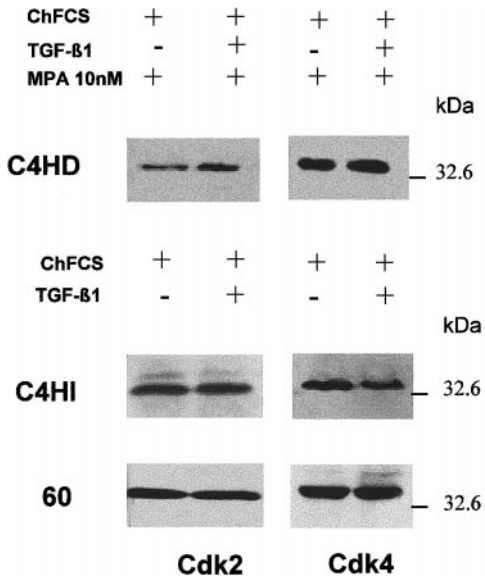


FIG. 3. Effect of TGF-β1 treatment on cdk expression. Protein extracts (100 μg) from cells growing as described for Fig. 2 were electrophoresed and immunoblotted for cdk2 and cdk4. The levels of the cdks were determined densitometrically.

blot in 60 cells, even after TGF-β1 treatment (data not shown). In an attempt to elucidate whether this was due to low levels of protein expression of these cdk inhibitors, we immunoprecipitated protein extracts with antibodies to each inhibitor and then performed Western blotting. Figure 5B shows that under these conditions $p21^{CIP1}$, $p27^{KIP1}$, and $p15^{INK4B}$ were readily detected in 60 cells. This result shows that 60 cells express significantly lower levels of these cdk inhibitors compared with C4HD and C4HI cells. We did not detect $p16^{INK4}$ expression in 60 cells even after immunoprecipitation (Fig. 5B). TGF-β1 treatment of 60 cells resulted in significant up-regulation of $p21^{CIP1}$ expression but did not affect $p27^{KIP1}$ or $p15^{INK4B}$ levels (Fig. 5B).

Cyclin D2 Role in MPA-Dependent and -Independent Proliferation

Since cyclin D2 protein levels significantly decreased when TGF-β1 inhibited MPA-induced growth of C4HD cells, but not when TGF-β1 abolished MPA-independent growth of C4HI or 60 cells, we investigated whether cyclin D2 had different roles in the proliferation of progestin-dependent and -independent cells. We first determined that MPA treatment of C4HD cells resulted in a two- to threefold increase in cyclin D2 protein expression (Fig. 6A), which indicates the involvement of cyclin D2 in MPA-induced growth. To further assess whether cyclin D2 was a critical component in progestin-dependent and -independent growth we used a strategy designed to block cyclin D2 expression by using antisense ODNs to cyclin D2 mRNA. The target was the translation start region, including the initiation codon of murine cyclin D2 mRNA [21]. Incubation of C4HD cells with antisense oligodeoxynucleotides to cyclin D2 resulted in a dose-dependent inhibition of MPA-mediated cell proliferation (Fig. 6B). Scrambled ODNs (SODNs) had no effect on cell growth (Fig. 6B). Similar results were obtained with C4HI cells in which cyclin D2 ASODNs inhibited proliferation in a dose-dependent manner and scrambled oligos had no effect on serum-induced growth of C4HI cells (Fig. 6B). The effect of cyclin D2 ODNs on protein expression was assessed by immunoblotting C4HD and C4HI cell lysates. Densitometric evaluation demonstrated that cyclin D2 levels were reduced by 63 $\pm$ 11% after 4 μM ASODN treatment of C4HD cells and by 71 $\pm$ 10% in C4HI cells. SODNs did not reduce cyclin D2 levels (Fig. 6B). ASODNs to cyclin D2 mRNA re-

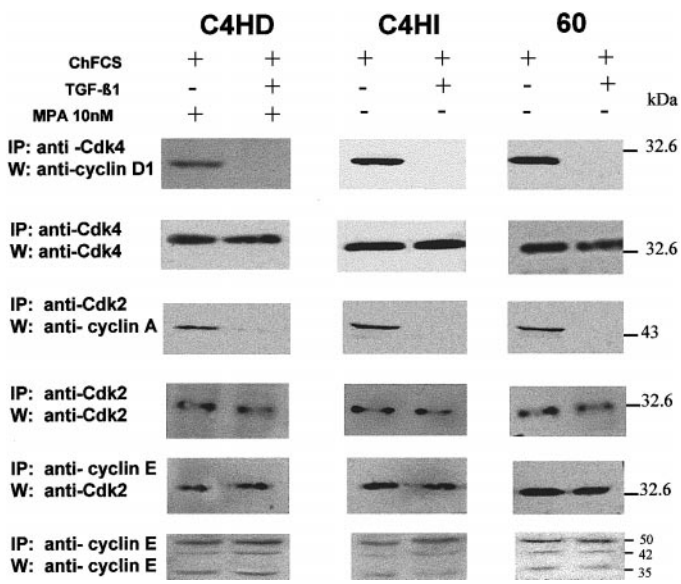


FIG. 4. Effect of TGF-β1 treatment on the abundance of cyclin/cdk complexes. Protein extracts (1000 μg) from cells treated and untreated with TGF-β1, as described, were immunoprecipitated with anti-cdk4 antibody and Western blotting was performed with an anti-cyclin D1 antibody (top row). Identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with the anti-cdk4 antibody to verify that nearly equal amount of immunoprecipitated proteins were loaded (second row). One hundred micrograms of protein was immunoprecipitated with an anti-cdk2 antibody and immunoblotted with an anti-cyclin A antibody (third row). As loading control, identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with the anti-cdk2 antibody (fourth row). Protein extracts (1000 μg) were immunoprecipitated with anti-cyclin E antibody and blotted with anti-cdk2 antibody (fifth row). As loading control, identical aliquots of the immunoprecipitates were blotted with anti-cyclin E antibody (sixth row). The levels of complexes were determined densitometrically.

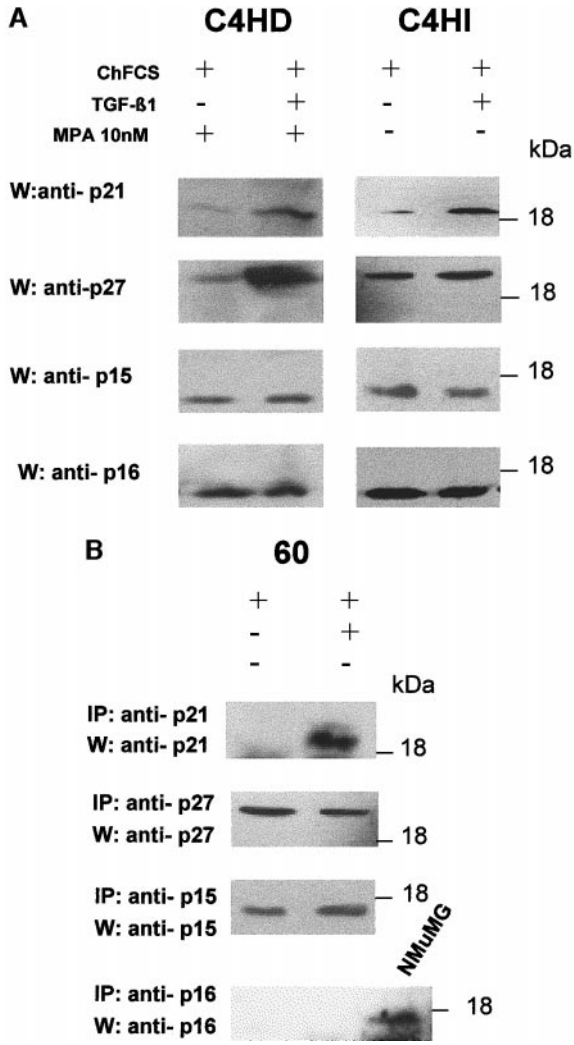


FIG. 5. TGF-β1 effects on the levels of cdk inhibitors. (A) Protein extracts (100 μg) from C4HD and C4HI cells treated with TGF-β as described were electrophoresed and subjected to Western blot analysis with anti-$p21^{CIP1}$, $p27^{KIP1}$, $p15^{INK4B}$, and $p16^{INK4}$ antibodies. (B) One milligram of protein from 60 cell extracts was immunoprecipitated with anti-$p21^{CIP1}$, $p27^{KIP1}$, $p15^{INK4B}$, and $p16^{INK4}$ antibodies and immunocomplexes were subjected to SDS–PAGE and analyzed by Western blotting with the same antibody used for immunoprecipitation. Normal murine mammary gland (NMuMG) cells were used as control for $p16^{INK4}$ expression.

duced protein expression by 68% but did not have any significant effect on the growth of 60 cells (Fig. 6B).

As cyclin D2 has been reported to bind and activate cdk2 in preference to cdk4 in breast cancer cells [26], we herein examined the relative abundance of the complexes cyclin D2/cdk4 and cyclin D2/cdk2 in proliferating cells. Protein extracts from C4HD cells growing in 10 nM MPA or from C4HI and 60 cells growing in 2.5% ChFCS were immunoprecipitated with anti-cyclin D2 antibody, and Western blots were performed with anti-cdk4 or anti-cdk2 antibodies. Protein extracts from each cell type (lanes marked lysate) were blotted in parallel with the specific cdk antibodies. As shown in Fig. 7, cyclin D2 binds both cdk4 and cdk2 in the three types of proliferating cells. No differences were detected in the relative abundance of cdk4 and cdk2 in the cyclin D2 complexes, since similar percentages of the total cdk4 and cdk2 pools were immunoprecipitated with cyclin D2 (table in Fig. 7).

Suppression of Cdk2 Activity by TGF-β1

Involvement of cdk2 in the phosphorylation of the progesterone receptor has been recently reported [27]. Thus, we sought to determine whether TGF-β1 regulated cdk2 activity differently when it inhibited growth driven by the progesterone receptor (C4HD cells) from when it inhibited MPA-independent proliferation (C4HI and 60 cells). First, we studied all the probable mechanisms leading to cdk2 inactivation in all three cell types. Since we found that TGF-β1 treatment of the three cell types induced an up-regulation of $p21^{CIP1}$, we studied whether this resulted in an increase in the association of $p21^{CIP1}$ with cdk2. Thus, cell lysates were immunoprecipitated with an anti-cdk2 antibody and Western was performed with an anti-$p21^{CIP1}$ antibody. As shown in Fig. 8, TGF-β1 induced an increase in the quantity of the complex $p21^{CIP1}$/cdk2 in C4HD, C4HI, and 60 cells. In addition, a number of reports have provided evidence suggesting that the ultimate function of $p27^{KIP1}$ in TGF-β-mediated growth arrest appears to be inactivation of cdk2. Therefore, we sought to determine whether TGF-β1 up-regulation of $p27^{KIP1}$ protein in C4HD cells resulted in an increase of the levels of $p27^{KIP1}$ complexed to cdk2. Cell lysates were immunoprecipitated with anti-cdk2 antibody and Western blot was performed with an anti-$p27^{KIP1}$ antibody. As shown in Fig. 8 the level of $p27^{KIP1}$ complexed with cdk2 substantially increased after TGF-β1 treatment of C4HD cells. As expected, since $p27^{KIP1}$ levels were nor regulated by TGF-β1 in C4HI cells, abundance of the complex $p27^{KIP1}$/cdk2 remained unaffected by TGF-β1 (Fig. 8). Therefore, the increase in $p27^{KIP1}$ observed in our model system when TGF-β1 abolished the MPA-proliferative effect could be a mechanism to inactivate cdk2. Since TGF-β1 down-regulation of cyclin D2 was observed only in C4HD cells, we also assessed whether abundance of cyclin D2/cdk2 complex was affected by TGF-β1 treatment of these cells. Figure 9 shows that there was in fact a decrease in the levels of cyclin D2/cdk2 complex after TGF-β1 treatment of C4HD cells, while, on the other hand, quantity of cyclin D2/cdk2 complex remained unaffected by TGF-β1 treatment of C4HI and 60 cells. Taking into account that these two mechanisms leading to cdk2 inactivation were present only when TGF-β1 inhibited

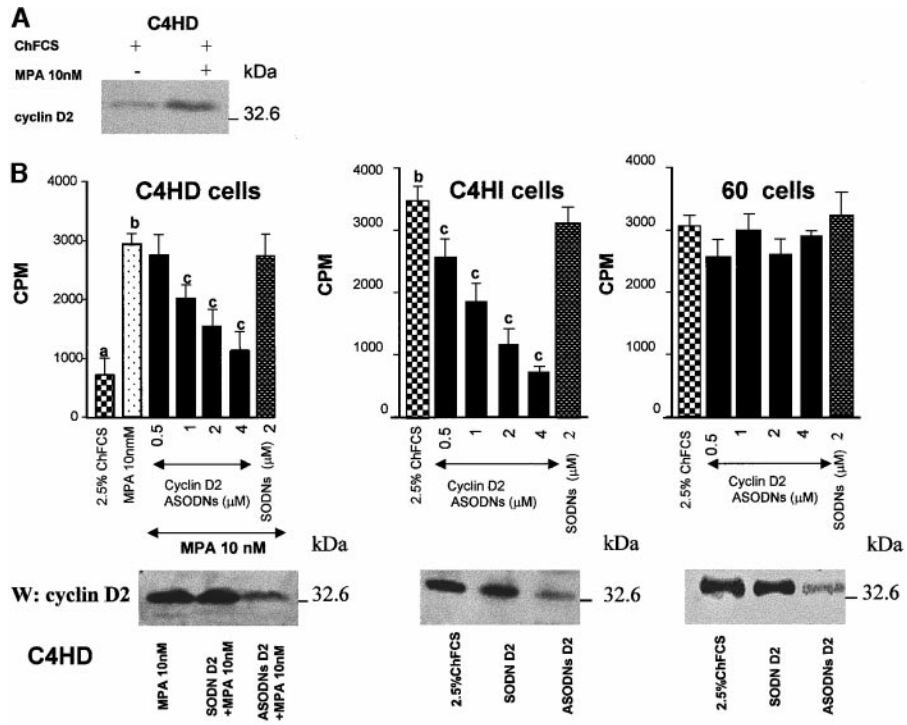


FIG. 6. Effect of MPA on cyclin D2 expression and growth effects of cyclin D2 ASODNs. (A) Protein extracts (100 μg) from C4HD treated and untreated with MPA 10 nM were electrophoresed and immunoblotted for cyclin D2. (B) Primary cultures of C4HD cells were incubated for 48 h in medium with 2.5% ChFCS supplemented with MPA 10 nM and MPA + ASODNs or SODNs to cyclin D2 mRNA. C4HI and 60 cells were incubated in medium with 2.5% ChFCS and ASODNs or SODNs to these ASODNs. Data are presented as means ± SD. b vs a and c vs b, $P < 0.001$. The experiments shown are representative of a total of four for each cell type. Bottom, 100 μg protein from C4HD, C4HI, and 60 cell lysates obtained from cells treated with 4 μM cyclin D2 ODNs was electrophoresed and immunoblotted for cyclin D2. Densitometric analysis of the cyclin D2 band expressed as a percentage of the control value (i.e., C4HD cells growing in 10 nM MPA or C4HI and 60 cells growing in 2.5% ChFCS) is 37% for C4HD, 29% for C4HI, and 32% for 60 cells treated with 4 μM ASODN. No significant differences were found in densitometric values of cyclin D2 bands between control cells and cells treated with 4 μM SODN. Shown are autoradiograms from representative experiments of a total of four for each cell type in which SE was within 10%.

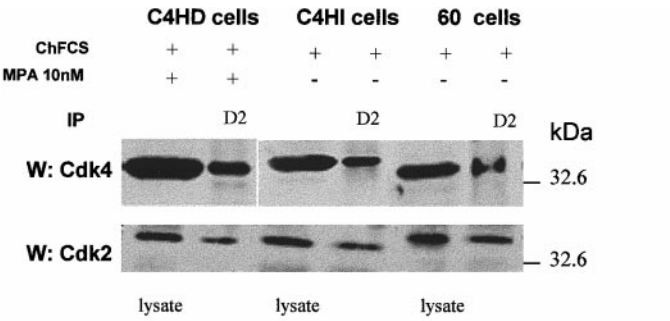
MPA-induced proliferation, we then investigated whether cdk2 activity was regulated in C4HD cells differently from in C4HI or 60 cells. Cell lysates were immunoprecipitated with anti-cdk2 antibody and the kinase activities of the immunoprecipitates were determined using histone H1 as substrate. Figure 10 shows that cdk2 activity markedly decreased after TGF-β1 treatment of all three cell types. However, a significant difference in the degree of TGF-β1 inhibition of cdk2 activity between them was observed. TGF-β1 caused reduction of cdk2 activity by 56% in C4HD cells, by 28% in C4HI cells, and by 15% in 60 cells.

DISCUSSION

Cyclins D1 and A and p21^{CIP1} Are Targets by Which TGF-β1 Causes Inhibition of MPA-Dependent and -Independent Growth of Mammary Tumor Cells

Cyclin D1 expression at the protein level was significantly inhibited when TGF-β1 inhibited MPA-induced growth (C4HD cells), when TGF-β1 abolished MPA-independent proliferation of ER$^+$, PR$^+$ C4HI cells, which are exquisitely sensitive to TGF-β1-mediated growth inhibition, and when TGF-β1 inhibited MPA-independent growth of ER$^-$, PR$^-$ 60 cells, which have a reduced sensitivity to TGF-β1-induced growth arrest. Therefore, cyclin D1 inhibition appears to be a general mechanism of TGF-β1 cell cycle arrest in the MPA-induced tumor model. Similarly, TGF-β1 inhibits cyclin D1 expression at the protein level when arresting rat intestinal epithelial cells RIE-1 and IEC-6 [28], Mv1Lu mink lung epithelial cells, and the human melanoma cell line WM35 [29, 30]. Unlike our findings, other authors found that TGF-β1 arrest of MCF-7 breast cancer cells or HMEC human mammary epithelial cells did not affect cyclin D1 mRNA or protein levels [13, 31].

Our results showing that TGF-β1 inhibits cyclin D1 expression when it abolishes MPA-induced growth of C4HD cells are in line with a series of findings, both *in*



	C4HD cells	C4HI cells	60 cells
D2/Cdk4	5.7 ± 0.9 %	6.3 ± 1.0 %	5.2 ± 0.9 %
D2/Cdk2	4.7 ± 1.1 %	4.8 ± 1.0 %	4.4 ± 0.9 %

FIG. 7. Cyclin D2 complex formation. One milligram of protein extracts from C4HD growing in 2.5% ChFCS + 10 nM MPA and from C4HI and 60 cells growing in 2.5% ChFCS were immunoprecipitated with an anti-cyclin D2 antibody. The immunocomplexes were separated by SDS–PAGE and blotted with anti-cdk4 or -cdk2 antibodies. The lanes marked "lysate" show C4HD, C4HI, and 60 cell lysates blotted in parallel with anti-cdk4 or -cdk2 antibodies. The intensity of the cdk4 or cdk2 bands in the lanes marked lysate and in the lanes in which protein extracts have been immunoprecipitated with the cyclin D2 antibody were densitometrically quantitated and the percentages of the total cdk4 (D2/cdk4) or the total cdk2 (D2/cdk2) pools immunoprecipitated with cyclin D2 were calculated as described under Materials and Methods and are shown in the table.

vivo and *in vitro*, that strongly support the conclusion that induction of cyclin D1 abundance is a critical component of the mitogenic response to progestins [reviewed in Ref. 6]. In agreement with these findings, we have demonstrated that MPA-induced proliferation of C4HD cells was also associated with an up-regulation of cyclin D1 expression.

Levels of cdk4 protein expression remained unaffected by TGF-β1 arrest of C4HD, C4HI, or 60 cells. However, in all three cell types, TGF-β1 growth inhibition was associated with a decrease in the abundance of cyclin D1/cdk4 complexes. Since TGF-β1 inhibited cyclin D1 protein expression, our results indicate that cyclin D1 was the limiting subunit of the holoenzyme. Therefore, a mechanism leading to cdk4 inhibition in our model system could be the decrease in the abundance of cyclin D1/cdk4 complex. Inhibition of cdk4 activity could explain the increase in the expression of underphosphorylated pRb found after TGF-β treatment of C4HD, C4HI, and 60 cells, since a number of studies have shown that cyclin D-associated kinases, cdk4 and cdk6, are the major contributors to pRb phosphorylation, both *in vivo* and *in vitro* [32–34]. Interestingly, in all epithelial cells studied to date, TGF-β1 induces G1 arrest by inhibiting cdk4 activity and preventing pRb phosphorylation, although different cell types appear to manifest different primary responses [35, 39].

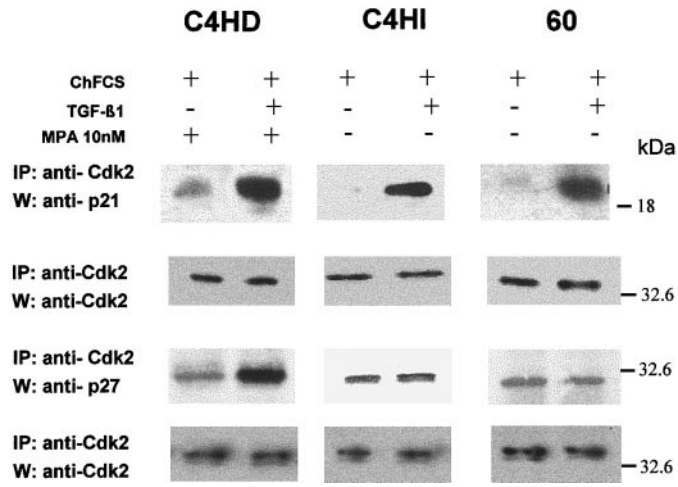


FIG. 8. Association of $p21^{CIP1}$ and $p27^{KIP1}$ with cdk2 in TGF-β1-treated cells. One milligram of protein from cells treated and untreated with TGF-β1 as described was immunoprecipitated with an anti-cdk2 antibody, electrophoresed, and subjected to Western blot analysis with an anti-$p21^{CIP1}$ (top row) antibody. As loading control, identical aliquots of the immunoprecipitates were blotted with anti-cdk2 antibody (second row). One milligram of protein from cells treated and untreated with TGF-β1 was immunoprecipitated with an anti-cdk2 antibody, electrophoresed, and subjected to Western blot analysis with an anti-$p27^{KIP1}$ antibody (third row). Fourth row, loading control performed as in the second row.

Cyclin A expression at the protein level was significantly inhibited when TGF-β1 inhibited MPA-induced growth of C4HD cells and when TGF-β1 inhibited the MPA-independent proliferation of C4HI and 60 cells. Similarly, TGF-β1 was reported to inhibit cyclin A at

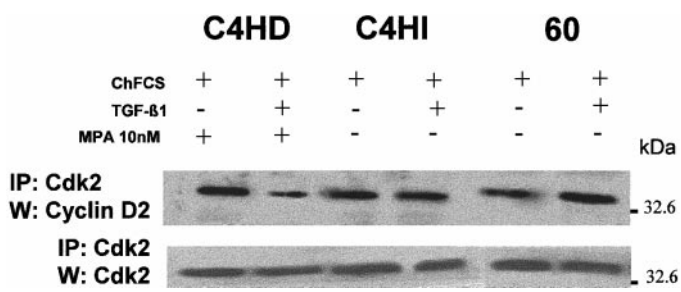


FIG. 9. TGF-β effect on the abundance of cyclin D2/cdk2 complex. Protein extracts (1 mg) obtained from cells treated and untreated with TGF-β1 were immunoprecipitated with an anti-cdk2 antibody, electrophoresed, and immunoblotted with an anti-cyclin D2 antibody. As loading control, identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with the anti-cdk2 antibody (bottom).

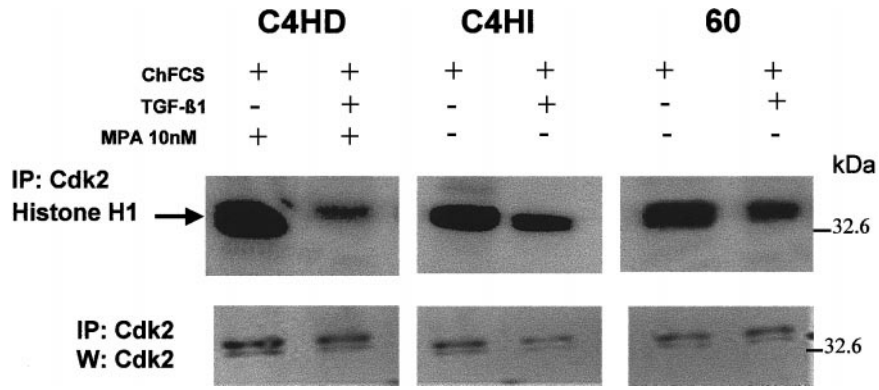


FIG. 10. Cdk2 kinase activity. Cell lysates (1 mg protein) from cells treated and untreated with TGF-β1 were immunoprecipitated with anti-cdk2 antibody and the kinase activities of the immunoprecipitates were determined using histone H1 as substrate. Bottom, as loading control, identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with the anti-cdk2 antibody. Band intensities were quantitated by densitometry of multiple autoradiograms of various exposures.

the mRNA and protein levels as well as its associated kinase activity during its growth arrest of Balb/MK mouse keratinocytes [29], of the human keratinocyte cell line HaCaT [40], and of Mv1Lu cells [41, 42]. Whether inhibition of cyclin A expression in TGF-β1-treated cells is a direct effect of TGF-β1 or an indirect consequence of the late G_1 cell cycle arrest still remains to be elucidated. Several studies support the idea that cyclin A inhibition may be mechanistically involved in the TGF-β1-induced late G_1 arrest [29]. Particularly, TGF-β1 was demonstrated to inhibit cyclin A promoter activity in Mv1Lu cells by a mechanism that appears to involve a decrease in the phosphorylation and activity of the activating transcription factor (ATF)-binding proteins ATF-1 and CREB [43].

We have demonstrated that $p21^{CIP1}$ protein levels increased when TGF-β1 acted as inhibitor of MPA-induced growth or as inhibitor of MPA-independent proliferation. Up-regulation of $p21^{CIP1}$ by TGF-β has been found in a variety of cell types [38, 44–50]. Up-regulation of $p21^{CIP1}$ in response to TGF-β1 resulted in an increase of $p21^{CIP1}$ bound to cdk2 in C4HD, C4HI, and 60 cells. Similarly, increased $p21^{CIP1}$ expression after TGF-β1 treatment was reported to correlate with a corresponding increase in cdk2-associated $p21^{CIP1}$, resulting in cdk2 inhibition, in Mv1Lu epithelial cells and human keratinocytes [38], in ovarian cancer cells [46], and in human prostatic epithelial cells [49]. $p21^{CIP1}$ was found to associate with cdk2 in MCF-7 cells treated and untreated with TGF-β1, and no variations in the levels of $p21^{CIP1}$ in this complex were found [13]. However, in TGF-β1-treated MCF-7 cells, a time-dependent increase in cells positive for $p21^{CIP1}$ nuclear localization was observed [13].

Reduction of Cyclin D2 Protein and Up-regulation of $p27^{KIP1}$ Expression as Molecular Mechanisms Involved in TGF-β1 Arrest of MPA-Induced Proliferation

We found that cyclin D2 protein levels decreased only when TGF-β1 inhibited MPA-induced growth in C4HD cells. This differential regulation of cyclin D2 suggests that cyclin D2 could be a target for MPA-induced growth. In fact, we have herein demonstrated that MPA stimulation of C4HD cells resulted in a significant induction of cyclin D2 expression. This is, to our knowledge, the first report that states that progestin-induced growth of mammary tumor cells involves cyclin D2 up-regulation. In addition, decrease of cyclin D2 synthesis has so far never been reported as a mechanism for TGF-β1 inhibition of breast cancer cells. However, in Mv1Lu mink lung epithelial cells TGF-β1 does inhibit cyclin D2 at the protein level [29, 51]. Though the role of cyclin D2 in breast cancer still remains to be ascertained, in human mammary epithelial cells expressing cyclin D2, microinjection of anti-cyclin D2 antibodies inhibited progress into S phase, despite the presence of significant amounts of cyclin D1, thus demonstrating that at least in some breast epithelial cells, cyclin D2 performs an essential role during G_1 progression [52]. Several of the findings we have herein reported may help to reveal the role of cyclin D2 in progestin-dependent and -independent growth of mammary tumor cells. First, we found that cyclin D2 binds cdk2 and cdk4 in proliferating cells of both progestin-dependent and -independent phenotypes. We found no preferential binding of D2 to any of these cdks, since similar percentages of the total cdk4 and cdk2 pools were immunoprecipitated with cyclin D2. This finding contrasts with a previous report show-

ing that cyclin D2 binds and activates cdk2 in preference to cdk4 in breast cancer cells [26]. Second, by using an antisense strategy we found that cyclin D2 is an essential component in the MPA-induced proliferation of the C4HD cells and in the proliferation of the progestin-independent variant C4HI cells. Contrariwise, abolishing cyclin D2 expression in the progestin-independent ER^-, PR^- 60 cells had no effect on cell growth. These results raise the possibility that in C4HD and C4HI cells, cyclin D1 and D2 functions are not redundant. Sutherland and co-workers [53] have reported that different nuclear substrates turned out to be phosphorylated by different complexes of cyclins and cdks in the T47D breast cancer cell line. Thus, a likely explanation for our findings could be that cyclin D2, bound to either cdk2 or cdk4, targets substrates whose phosphorylation is essential for the transit through G_1 and S phases of the cell cycle in C4HD and C4HI cells. 60 cell growth, which is not affected by cyclin D2 ASODNs, could require phosphorylation of a different set of substrates targeted in turn by other cyclin/cdk complexes. Ultimately, whether a key substrate in C4HD cell growth, that is, the progesterone receptor, is a target for cyclin D2/cdk phosphorylation will have to be ascertained. It has been demonstrated that the majority of the phosphorylation sites in the PR contain Ser-Pro sequences, suggesting that the proline-directed kinases such as the cdks play roles in PR phosphorylation [reviewed in Ref. 54]. In fact, cyclin A/cdk2 complexes have recently been reported to phosphorylate human progesterone receptor *in vitro* [27]. An interesting question worth raising is why cyclin D2 is essential to the proliferation of C4HI cells. We do not wish to rule out the possibility that cyclin D2/cdk complexes may be involved in the phosphorylation of the PR in C4HI cells. We have arguments to uphold this hypothesis. Thus, Montecchia *et al.* [55] have recently demonstrated that the growth of C4HI tumors *in vivo* is completely abolished by the anti-progestins onapristone and mifepristone and that ASODNs to the PR are able to block *in vitro* serum-induced growth of these cells [55], showing that the PR pathway is essential to the growth of C4HI cells. Since PR was found to be activated in the absence of its cognate ligands [56], it is not surprising that in C4HI cells PR may be activated either by serum factors or by several growth factors that we have already found are synthesized in an autocrine fashion by C4HI cells [57, 58]. Thus, cyclin D2 could be essential to directly phosphorylate PR or to phosphorylate PR coregulators or other protein factors interacting with the PR in C4HI cells.

Expression of $p27^{KIP1}$ was up-regulated only by TGF-β1 when it acted as inhibitor of MPA-induced proliferation of C4HD cells. Similarly, TGF-β growth inhibitory effect on Mv1Lu cells [41, 59] and in the 184 human mammary epithelial cells strain [37] was accompanied by an increase in the synthesis of $p27^{KIP1}$, which in turn blocked cyclin E/cdk2 activity. Results similar to ours have been shown in the WM35 early stage human melanoma cell line, in which TGF-β upregulates $p21^{CIP1}$ and $p27^{KIP1}$ proteins that act cooperatively to contribute to growth arrest by TGF-β [30]. Other studies in Mv1Lu cells, human mammary epithelial cells, and human keratinocytes HaCaT [31, 38] reported that TGF-β did not cause a rise in $p27^{KIP1}$ levels, but elevated expression of $p15^{INK4B}$, which in turn induced the release of $p27^{KIP1}$ from cdk4 and cdk6. $p27^{KIP1}$ then bound to and inactivated cdk2 [31, 38]. Thus, the ultimate function of $p27^{KIP1}$ in TGF-β-mediated growth arrest appears to be inactivation of cdk2. In line with this hypothesis, we found that TGF-β1 treatment of C4HD cells resulted in up-regulation of $p27^{KIP1}$ protein, as well as in an increase of the levels of $p27^{KIP1}$ complexed to cdk2, and in an inhibition of cdk2 activity significantly higher than that found in C4HI or 60 cells. A probable explanation for the higher cdk2 activity inhibition by TGF-β in C4HD cells could be provided, as discussed above, by the reported involvement of cdk2 in the phosphorylation of PR *in vitro.* Thus, suppression of cdk2 activity by TGF-β1 could be a means to antagonize MPA-proliferative effects through changes in the phosphorylation state of PR, which in turn affect the transcriptional activity of this receptor.

Reduced Sensitivity of 60 Cells to TGF-β1 Inhibitory Effects Correlated with Low Levels of $p21^{CIP1}$, $p27^{KIP1}$, and $p15^{INK4B}$ and with Lack of Expression of $p16^{INK4}$

Reduced sensitivity of 60 cells to the growth-inhibitory effects of TGF-β1 could be explained by the significantly lower levels of the cdk inhibitors $p21^{CIP1}$, $p27^{KIP1}$, and $p15^{INK4B}$ found in these cells, compared with the levels of expression found in C4HD or C4HI cells, and by the lack of expression of $p16^{INK4}$. Deregulation of TGF-β signaling cascades represents an important mechanism underlying TGF-β resistance, in addition to lack or reduced expression of TGFβ receptors. Our results are in agreement with those reported in cell lines derived from advanced primary melanomas and metastases which show that reduced or absent response to TGF-β growth inhibition correlates with low levels of $p21^{CIP1}$ expression [30]. In addition, our findings suggest that reduced expression of $p27^{KIP1}$ and of a member of the INK family, $p15^{INK4B}$, could also account for TGF-β resistance. Participation of $p15^{INK4B}$ in TGF-β-mediated growth arrest was also found since $p15^{INKB}$ is induced by TGF-β in sensitive cells [39, 49, 60]. $p16^{INKB}$ has been shown to be a tumor suppressor

and is functionally inactivated, most notably by deletion, in a high percentage of tumor cells, including breast cancer cell lines [61]. Therefore the lack of $p16^{INKB}$ protein in 60 cells suggests that the $p16^{INKB}$ gene could be deleted in these cells and provides a link between TGF-β resistance and absence of a tumor-suppressor gene. Finally, the percentage of inhibition of cdk2 in TGF-β-treated cells is significantly lower than in C4HD or C4HI cells, in agreement with previous findings in prostate carcinoma cells in which insensitivity to growth inhibition by TGF-β correlates with a lack of inhibition of the cdk2 activity [48].

In conclusion, our results demonstrated the existence of common targets in TGF-β1 inhibitory action on breast cancer cells. However, regulation of specific targets was found when TGF-β1 inhibited proliferation driven by the progesterone receptor. Moreover, our findings proved that cyclin D2 is an essential component in the growth of mammary tumor cells in which PR is expressed and involved in proliferation.

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