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Molecular cell cycle mechanisms of human prostate crcinoma insensitivity to TGF-β?

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MOLECULAR CELL CYCLE MECHANISMS OF HUMAN PROSTATE CARCINOMA INSENSITIVITY TO TGF-β

by

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DEDICATION

This thesis is dedicated to my mom, in loving memory.

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INTRODUCTION

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine known to be involved in many cellular processes including growth and differentiation (Massague, 1990). TGF-β is both a potent stimulator and inhibitor of cell growth depending on the cell type and growth conditions. TGF-β1, one of five known isoforms of TGF-β, is stimulatory for cells of mesenchymal origin. In contrast, it is also one of the most potent inhibitors for cells of epithelial, endothelial, hematopoietic, and neuroectodermal origin (Alexandrow and Moses, 1995; Massague, 1990). However, many malignancies of epithelial and hematopoietic origin are resistant to the growth inhibitory effects of TGF-β. This suggests that the development of resistance to the growth inhibitory effects of this cytokine may play an important role in tumorigenesis (Filmus and Kerbel, 1993; Kerbel, 1992).

TGF-β and Cancer

Loss of negative growth response is generally considered to be a late event of carcinogenesis and usually correlates with developing invasiveness as demonstrated in a mouse skin model (Haddow et al., 1991). Tumor cell responsiveness to growth inhibition by TGF-β has been shown to decrease with increasing stage in many human tumors including those of the lung, thyroid, and colon (Masui et al., 1986; Wyllie et al., 1991; Hoosein et al., 1989; Hsu et al., 1994). In addition, TGF-β1 has been shown to inhibit the formation of benign skin tumors, but enhance progression to invasive spindle cell carcinomas in transgenic mice (Wei et al., 1996). TGF-β has also been shown to stimulate the proliferation of some advanced stage tumors (Jennings et al., 1991;

Mulder, et al., 1988). Therefore, this late stage loss of negative regulation in tumor cells may be due to a defect(s) in TGF-β signaling.

The response pattern of tumor cells to TGF-β covers a complete spectrum from cells which maintain responsivity to those which are no longer inhibited (Roberts and Sporn, 1993). Reports on the effects of growth inhibition by TGF-β in mammary tumor derived cell lines have been controversial. Analysis of the growth effect of TGF-β on eleven breast carcinoma cell lines revealed that no absolute distinction can be made. However, it seems that ER^- (estrogen receptor negative) cell lines may be somewhat more responsive to growth inhibition by TGF-β than ER^+ (estrogen receptor positive) cell lines (Wakefield et al., 1991). Squamous carcinomas of the head and neck and some colorectal and thyroid papillary carcinomas retain sensitivity to the growth inhibitory effects of TGF-β (Hebert and Birnbaum, 1989; Suardet et al., 1992; Blaydes et al., 1995).

Conversely, TGF-β is known to act as a growth stimulator in several human and mouse mesothelioma cell lines. Administration of antisense TGF-β2 oligonucleotides delivered locally to malignant mesotheliomas in mice proved to reduce tumor growth (Marzo et al., 1997).

Most mammalian cells possess three types of TGF-β receptors at the cell surface. The type I and type II receptors are the most relevant to TGF-β signaling. These receptors interact with the ligand TGF-β to elicit TGF-β signal transduction and hence cellular responses. The type III receptor has been postulated to regulate access of TGF-β to its signaling receptors. To support this, de novo expression of the type III receptor in L6 cells, which express only the type I and II receptors, increased TGF-β1 binding to the

type II receptor by 2.5 fold (Wang et al., 1991).

Tumors can become resistant to TGF-β as a result of loss or decreased expression of the type I and or type II receptors. While loss of both of the TGF-β signaling receptors (type I and type II) occurs infrequently in tumors, this has been observed in retinoblastomas and certain colon carcinoma cell lines (Coffey et al., 1987; Kimchi et al., 1988). Decreased expression of either the type I or type II receptor has been frequently observed in certain tumor cell lines (Filmus et al., 1992). Additionally, TGF-β type II receptor mutations were shown to be associated with microsatellite instability in a subset of human colorectal carcinoma cell lines (Markowitz et al., 1995). Inactivating mutations of the TGF-β type II gene have been identified in a high proportion (71-90%) of colorectal cancers in patients with hereditary nonpolyposis colorectal cancer (Myeroff et al., 1995). Also, deletions of the type II receptor gene have been demonstrated in human gastric carcinoma cell lines (Park et al., 1994).

TGF-β and Prostate Cancer

While TGF-β1 is know to be a potent growth inhibitor of epithelial cells, several in vivo studies have shown significantly higher levels of expression of TGF-β1 and TGF-β receptor expression in many epithelial cancers including the prostate than that in their normal tissue counterparts (Anzano et al., 1985; Barrett et al., 1990; Steiner et al., 1990; Schmid et al., 1995). One in vivo study using Dunning R3327-MATLyLu prostate cancer epithelial cell sublines showed that TGF-β1 mRNA levels were dramatically increased in rat prostate adenocarcinomas compared to normal prostate (Steiner et al., 1990). Additionally, the same model showed a correlation between

increasing TGF-β1 mRNA levels and increasing degrees of anaplasia and growth rate. Another study showed that TGF-β1 overproducing MATLyLu tumor cells produced larger and less necrotic tumors and more extensive metastatic disease in vivo compared to control MATLyLu cells (Steiner and Barrack, 1992). These effects of TGF-β1 in vivo may be explained in three ways. First, TGF-β1 has been shown to stimulate angiogenesis (Massague, 1990; Yang and Moses, 1990). Second, TGF-β1 can suppress the immune system (Kehrl et al., 1986). Finally, TGF-β1 has been shown to stimulate invasion and metastatic potential of mammary adenocarcinoma cells (Welch et al., 1990). This suggests that overexpression of TGF-β may impose a survival advantage for tumor cells (Kim et al., 1996). In conclusion, these in vivo studies show that TGF-β1 and its receptors are overexpressed in epithelial tumor tissue versus normal tissue.

Relatively few studies have been done studying the effect of TGF-β1 on prostate carcinoma cells in vitro. In addition, the reported findings were inconsistent. One in vitro study of the effects of TGF-β on prostate carcinoma cell line PC3 showed no growth inhibitory effect even at very high concentrations (12 nM) (Franzen, et al., 1993). Another study showed that following treatment with TGF-β, growth was initially inhibited in a dose-dependent manner (maximally at 200 pM) in prostate carcinoma cell lines PC3 and DU145, but not in prostate carcinoma cell line LNCaP. However, the rate of growth of PC3 and DU145 eventually returned to control levels despite re-treatment with TGF-β (Wilding et al., 1989). Thus, continuous exposure to TGF-β1 might desensitize cells to any inhibitory effect. No studies to date have investigated the effect of TGF-β1 on normal prostate cell growth in vitro.

The type I and type II TGF-β receptors are wild-type in PC3 and DU145 cell lines

(Wilding et al., 1989). Thus, the lack of response to the growth inhibitory effects of TGF-β in these two cell lines is unlikely due to loss or mutation at the receptor level. In contrast, the LNCaP cell line has a defective type I TGF-β receptor which may explain its lack of response to growth inhibition (Kim et al., 1996).

While TGF-β1 is known to be a potent inhibitor of epithelial cell growth, in vitro and in vivo evidence indicates that, even in the presence of excess TGF-β1, prostate epithelial tumor cells remain unresponsive to growth inhibition. Therefore, it is likely that some defect(s) exist in the TGF-β1 signaling pathway which renders these cells unresponsive. The molecular mechanism of this unresponsiveness is unknown. One possibility lies in the role of cell cycle regulators.

TGF-β Signaling

There are many possible points along the TGF- signaling pathway at which aberrations can occur leading to lack of cell cycle arrest. For example, there can be a mutation in the TGF- ligand itself or a defect at the level of the receptor. Other possibilities include a defect in a signal transducer such as the Smads or in the transcription factors themselves. Lastly, deregulation of cell cycle proteins such as the Cdks, cyclins, and or CKIs may also lead to lack of cell cycle arrest (Fig. 1).

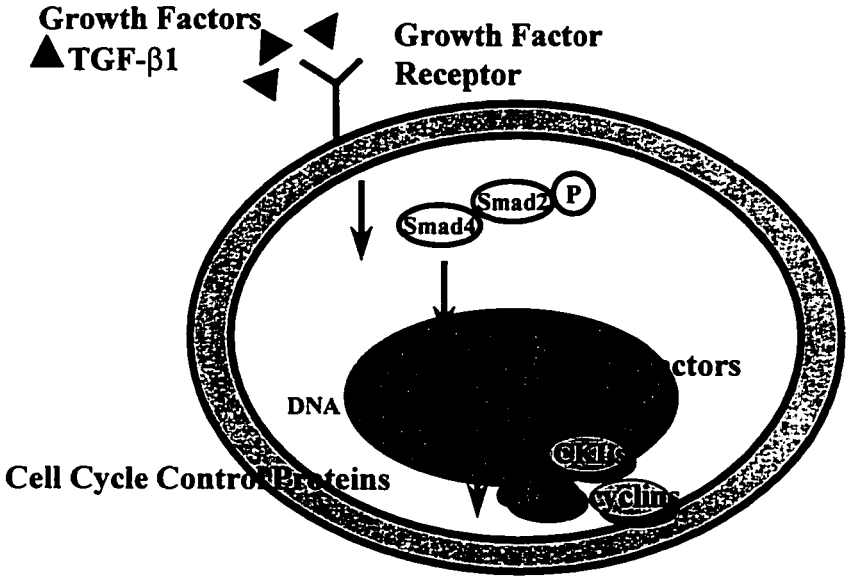


Figure 1. Control points in TGF- signaling.

TGF-β and Smads

Recently, a group of seven signal transduction proteins called Smad have been identified and shown to be components of cell signaling pathways. In particular, Smad2, Smad3 and Smad4 have been shown to be involved in TGF-β signaling (Fig. 1). The activated TGF-β receptor recognizes Smad2 or its close isoform Smad3 and phosphorylates them. Smad2 then binds to Smad4 and the entire complex is translocated to the nucleus where it either directly or in combination with other complexes directs transcription of specific genes (Wrana, J. and Pawson, T., 1997).

Smad2 and Smad4 when stimulated by TGF-β inhibit growth (Lagna et al., 1997). Lack of phosphorylation of Smad2 or 3 could be a possible mechanism of inactivation of Smads and therefore lack of growth inhibition. Studies in this area of

prostate cancer have not yet been explored. Alternatively, a mutation in one or more of these Smads may inactivate the TGF-β pathway. Indeed, loss of Smad4 expression has been identified in a variety of TGF-β resistant cancer cells including half of pancreatic carcinomas (Schutte, M, 1996). In addition transfection of Smad4 into these cells restored their responsiveness to TGF-β (Zhang et al., 1996). Smad4, the product of tumor suppressor gene DPC4 (deleted in pancreatic carcinoma-4), has been shown to be rarely if ever mutated during prostate tumorigenesis (MacGrogan, D., 1997). Studies of Smads 2 and 4 expression in prostate cancer have yet to be determined.

TGF-β and Cell Cycle Regulation

The fact that TGF-β plays an inhibitory role in epithelial cell growth implies that it regulates the cell cycle. The cell cycle consists of progression through a series of phases. The regulation at each phase is controlled by the presence and specificity of interaction of a host of proteins, among them are the cyclins, cyclin-dependent kinases (Cdks), and cyclin-dependent kinase inhibitors (CKIs) (Table 1).

		CKIs						
Cell cycle phase	cyclin-Cdk complex	p15	p16	p18	p19	p21	p27	p57
G1	cyc D-Cdk4/6	+	+	+	+	+	+/-	+/-
G1/S	cyc E-Cdk2	-	-	-	-	+	+	+
S	cyc A-Cdk2	-	-	-	-	+	-	+
G2/M	cyc B-Cdk1	-	-	-	-	+	-	-

Table 1. Cyclin/Cdk/CKI complex formation in different phases of the cell cycle

Cdks comprise a large family of proteins consisting of Cdks 1 through 7 which have now been identified. When bound together in specific combinations, cyclin-Cdk

complexes allow progression through the cell cycle. CKIs are proteins that can bind and inhibit Cdk activity preventing cell phase transition. Overexpression of cyclins and or CDKs or underexpression of their CDK inhibitors (CKIs) may deregulate the cell cycle leading to uncontrolled cell proliferation.

TGF-β is intricately linked to cell cycle arrest. TGF-β1 has been shown to inhibit the ability of cells to enter S phase when this cytokine is added to culture in early and late G_1. (Geng and Weinberg, 1993; Laiho et al., 1990; Pietenpol et al., 1990; Howe et al., 1991). After the G_1-S transition, the cells are mostly unresponsive to TGF-β. Cyclins, Cdks, and CKIs important for regulation at the G_1 to S checkpoint are likely mediators of TGF-β induced G_1 cell cycle arrest.

Mammalian CKIs, the inhibitors of cyclin-dependent kinases can be divided into two families based on structural and functional properties. One family includes $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$ highly related proteins with tandem ankyrin repeats and no sequence similarity to other known inhibitors. The other family include $p27^{KIP1}$, $p21^{CIP1/WAF1}$, and $p57^{KIP2}$. These three have a homologous region defining a domain that is responsible for binding and inhibiting Cdks (Polyak, 1996; Polyak et al., 1994; Luo et al., 1995; Nakanishi et al., 1995; Toyoshima and Hunter, 1994).

One proposed mechanism explaining the cells inability to progress to the S phase is that TGF-β1 induces accumulation of hypophosphorylated pRB (Laiho et al., 1990; Pietenpol et al., 1990; Koff et al., 1993; Ewen et al., 1993). Upon TGF-β treatment, a negative growth signal is initiated in the cell which increases the level of CKI(s). CKI(s) then bind to either a Cdk or cyclin-Cdk complex inhibiting its kinase activity. This then leads to accumulation of hypophosphorylated pRB. The hypophosphorylated pRB then

binds to the transcription factor E2F (Weinberg, 1995). This, in turn, suppresses cell division by preventing cells in G1 from entering into the S phase (Weinberg, 1992; Benedict et al., 1990).

There have been five molecular mechanisms proposed to explain the role of TGF-β in preventing pRB phosphorylation (Weinberg, 1995). The first involves CKI, p27. Several studies suggest that p27 mediates G1 arrest in epithelial cell lines induced by contact inhibition, TGF-β, or serum deprivation (Polyak et al, 1994). Specifically, TGF-β elevates the expression of p15 and promotes the release of p27 from Cdk4 and 6. p27 is then able to bind to the cyclin E-Cdk2 complex inhibiting its activity (Reynisdottir et al., 1995) leading to accumulation of hypophosphorylated pRB. While levels of p27 are not affected by treatment with TGF-β, Cdk2 in treated cells have been found to be associated with p27 (Ewen et al., 1993; Polyak et al., 1994).

A second proposed mechanism is based on the fact that CKI, p15, protein and mRNA levels were found to be induced 10-30 fold following treatment with TGF-β of exponentially growing HaCaT keratinocytes (Hannon and Beach, 1994) and Mv1Lu mink lung epithelial cells (Reynisdottir et al., 1995). p15 can inhibit Cdk4 and Cdk6 (Guan et al., 1994; Serrano et al., 1995).

Third, CKI p21 can inhibit multiple Cdks. p21 has been shown to be a transcriptional target of p53 (Xiong et al., 1993; Gu et al., 1993; El-Deiry et al., 1993; El-Deiry et al., 1994). However, transcriptional activation of p21 by TGF-β1 is through a p53-independent mechanism in many cell lines (Datto et al., 1995; Elbendary et al., 1994; Li et al., 1995; Jiang et al., 1994; Akagi et al., 1996). Increased p21 expression after TGF-β treatment correlates with a corresponding increase in Cdk2 associated p21

with Cdk2 inhibition (Reynisdottir et al., 1995).

The fourth proposed mechanism is that TGF-β acts to dramatically reduce levels of Cdk4 (Ewen et al., 1993). The TGF-β induced decrease in Cdk4 levels may result in liberation of substantial amounts of p27. This in turn may antagonize the functioning of other Cdks in the cell (Weinberg, 1995).

The final mechanism involves the Cdk tyrosine phosphatase Cdc25A. TGF-β has been shown to decrease expression of Cdc25A which results in tyrosine phosphorylation and inactivation of Cdk4 and Cdk6 in a human mammary epithelial cell line (Ivarone and Massague, 1997). The same study also showed that TGF-β induction of p15 and decreased expression of Cdc25A are independent events even though they both mediate inhibition of Cdk4 and Cdk6.

In contrast, TGF-β has been shown to have no effect on p16 induction in normal epithelial cells. Inactivation of $p16^{INK4A}$ has been shown to occur only rarely in prostate cancer (Chen, W. et al, 1996; Gaddipati, J.P. et al., 1997; Tamimi, Y. et al., 1996) and only with inactivation of p15 (Herman et al., 1996). Thus, it is unlikely that p16 plays a major part in the TGF-β signaling pathway (Polyak, K., 1996). Mutations in p18 and p19 are uncommon in many types of human cancer (Gemma, A., et al., 1996). While a p18 point mutation has been identified in BT-20 breast cancer cells, no p18 mutations have been reported in prostate tumor cells in vitro or in vivo (Lapointe, J. et al., 1996). Therefore, their role in human cancer progression is uncertain.

Purpose of This Study

TGF-β is known to inhibit normal epithelial cell growth. While TGF-β is highly

expressed in tumor tissue, prostate cancer cells seem to have lost their sensitivity to it. The molecular mechanism of such insensitivity to TGF-β is unknown. This lack of responsiveness to TGF-β in prostate tumor cells may be due to a defect(s) in the TGF-β signaling pathway and specifically in cell cycle regulation. The defect(s) may result in lack of induction of CKIs, or lack of inhibition of Cdk activity. Current methods of treatment for prostate cancer such as androgen deprivation ultimately fail to prevent tumor growth. Therefore, there is a need for development of nonhormonal treatment regimens. Elucidation of a mechanism linking cell cycle deregulation and loss of cell cycle arrest in prostate tumor cells may provide alternative means for treatment of prostate cancer.

Aim #1

Determine the sensitivity of prostate epithelial cells to growth inhibition by TGF-β1.

Aim #2

Determine the effect of TGF-β1 on induction and association of G_1 Cdks, cyclins, and CKIs, as well as, Cdk kinase activity in normal prostate and carcinoma cells.

Aim #3

Determine a possible mechanism that can explain prostate tumor cells loss of sensitivity to TGF-β1.

MATERIALS AND METHODS

Cell Culture

PC3, DU145, and LNCaP prostate carcinoma cell lines were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (Gibco/BRL Laboratories). These three cell lines were obtained from American Tissue Type Culture Collection. PN (non-immortalized normal prostate cells) were cultured in PrEBM medium supplemented with BPE, insulin, hydrocortisone, gentamycin-1000, retinoic acid, transferrin, T3, epinephrine, and hEGF (Clonetics). PN cells were obtained from three sources. Cells were obtained from Dr. Jer Tsong Hsieh (Johns Hopkins University), Dr. Dharam P. Chopra, and Dr. Clement A. Diglio (Wayne State University). Cells obtained at Wayne State were from patient samples at Harper Hospital. These cells were verified histologically by a pathologist to be normal using Hematoxylin and Eosin staining. Further, PN cells obtained from Dr. Chopra have been characterized (Chopra, D.P. et al., 1997). PN (1-8) normal prostate cell populations were derived from 8 patients and placed in culture at passage 1. PN cells at passage 3 were used for experiments. HaCaT, an immortal human keratinocyte cell line, was cultured in Dubecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. PC3, DU145, LNCaP, and HaCaT cells when grown to confluency were detached with 0.25% trypsin and split at a ratio of 1:3 or 1:4. PN cells when reaching confluency were washed with 1X $PBS^{=}$ buffer (1.47 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 0.146 M NaCl, pH 7.0), trypsinized with mild EDTA/trypsin buffer (1X $PBS^{=}$, 0.01% EDTA, 0.025% trypsin), neutralized with neutralization buffer (1X $PBS^{=}$, 0.5% fetal bovine serum) and split at a ratio of 1:2 or 1:3.

TGF-β1

TGF-β1 (human recombinant) protein (Gibco/BRL Laboratories) was reconstituted in sterile dH_2O at a concentration of 0.8 μM then filtered through a 0.22 μM filter.

Antibodies

Rabbit polyclonal anti-human $p15^{INK4B}$, Cdk2, Cdk4, Cdk6 antibodies and Cdk2 blocking peptide were purchased from Santa Cruz. Purified mouse monoclonal anti-human $p27^{KIP1}$ and cyclin D1 antibodies were purchased from Oncogene Research Products and anti-β-actin antibody from Sigma. Purified mouse monoclonal anti-human cyclin D2, cyclin D3, and cyclin E antibodies were purchased from Pharmingen. Monoclonal anti-human $p21^{CIP1/WAF1}$ antibody was purchased from Upstate Biotechnology.

Histone H1 Kinase Assay Substrate

Histone HI substrate was purchased from Boehringer Mannheim.

Expression and Purification of GST/RB Fusion Protein for Use as a Kinase Assay Substrate

GST/RB fusion protein was purified using the RediPack GST Purification Module (Pharmacia Biotech) with modifications.

Preparation of Competent Cells

BL21 (DE3) glycerol culture was used to inoculate 2 ml of LB medium and grown overnight with shaking at 37°C. Two hundred ml of LB medium was inoculated with the 2 ml overnight culture and grown with vigorous shaking at 37°C to an $O.D._{600}$ 0.8. Cells were then chilled on ice for 20 minutes and centrifuged at 3000 rpm, 15

minutes, 4°C. The pellet was resuspended in 200 ml of ice cold sterile 10% glycerol. Cells were then centrifuged under the same conditions and the pellet was resuspended in 100 ml of ice cold sterile 10% glycerol. Cells were centrifuged and the pellet resuspended in 4 ml of ice cold sterile 10% glycerol. Cells were centrifuged and resuspended in a final volume of 600 µl of 10% glycerol. Cells were aliquoted as 60 µl per tube, snap frozen in a slurry of dry ice with ethanol, and stored at -80°C.

Transformation and Determination of Optimum Induction Time of GST/RB Protein

BL21 (DE3) competent cells were transformed with pGEX-RB. Sixty µl of competent BL21 (DE3) cells were thawed on ice and then mixed with 10-40 ng of pGEX-RB and electroporated. Cells were inoculated in 1 ml of Circle Growth medium and grown at 37°C for 30 minutes with shaking. Transformed cells were plated at 10 and 100 µl on LB plus 50 µg/ml ampicillin plates and incubated overnight at 37°C. The following day, eight colonies were grown in 2 ml cultures of LB plus 50 µg/ml ampicillin with shaking overnight at 37°C. The following day, 0.5 ml of each culture was placed into 10 ml of LB broth plus 50 µg/ml ampicillin and grown with shaking at 30°C until $O.D._{600}$ reached 0.7 to 0.8. Two ml of each culture was aliquoted into 2 sets of conical tubes. One set served as the controls (no IPTG added). The other set was induced by adding 0.2 mM final IPTG. Both sets of cultures were grown with shaking at 33°C for 2 hours. After 2 hours, 1 ml of each culture was pelleted and resuspended in 1X Sample Buffer. The remaining 1 ml cultures were placed back into the shaker and grown at 33°C for another 2 hours (4 hours total) then pelleted and resuspended in 1X Sample Buffer. All samples were boiled, run on a 12% SDS-polyacrylamide gel, and stained with Commassie Blue stain.

Large Scale Induction of GST/RB Protein

The gel was used to choose the clone which was maximally induced by IPTG. Clone #5 was then used to inoculate 4 liters of LB medium plus 50 μg/ml ampicillin and shaken at 37°C for 3 hours. Once the culture reached $O.D._{600}$ 0.3 to 0.7, 0.2 mM final IPTG was added followed by continued shaking at 34°C for 4 hours. Cells were pelleted at 6000 rpm, 15 minutes, 4°C and resuspended in ice cold 1X $PBS^{=}$. One mg/ml lysozyme pH 8.0 was added and the resuspension was incubated on ice for 20 minutes. Lysate was snap frozen and thawed several times using liquid nitrogen. Twenty percent Triton X-100 was added at 1ml/100ml lysate and incubated on ice 30 minutes with intermittent mixing. Lysate was then pelleted at 6000 rpm, 10 minutes, 4°C. Using a QIAGEN syringe filter, the lysate was filtered to remove cell debris.

Column Purification of GST/RB Protein

Filtered lysate was applied to a pre-made 2 ml glutathione sepharose 4B column and allowed to bind to the column for 20 minutes. The column was washed with at least 10 bed volumes of cold 1X $PBS^{=}$. The fusion protein was eluted by adding 8 ml of glutathione elution buffer to the column and letting it stand at 4°C for 20 minutes. Fractions were collected and analyzed with a 12% SDS-polyacrylamide gel and stained with Commassie Blue.

Maximally induced fractions were pooled and charged with ammonium sulfate at a final saturation of 55%, incubated on ice for 30 minutes, and centrifuged at 13,200 rpm, 5 minutes, 4°C. The pellet was resuspended in 2 ml of 50 mM Tris-HCl pH 7.5. Protein resuspension was then dialyzed with 50 mM Tris-HCl pH 7.5 overnight at 4°C using tubing of 3500 kDa cut-off. Dialyzed protein was then applied to an FPLC Mono-S ion

exchange column and run through the column with a NaCl gradient of 80 to 200 mM. Fractions were collected and run on a 12% SDS-polyacrylamide gel followed by staining with Commassie Blue. Maximally induced fractions were pooled and run on a large-scale 12% SDS-polyacrylamide gel. The band protein at 41 kDa representing the GST/RB fusion was cut out of the gel, placed in dialysis tubing with a 3500 kDa cut-off point, and electro-eluted with 1X SDS-Page running buffer (24.0 mM Tris, 192 mM glycine, 3.5 mM SDS). Protein was collected from the tubing and concentrated in an Amicon concentrator #3. Protein concentration was determined using a BCA assay (Pierce).

Growth Curves

Dosage Experiments

In 24-well plates, a total of 2 X 10^3 HaCaT cells/well, 3 X 10^3 DU145, PC3, and PN cells/well, and 1 X 10^4 LNCaP cells/well were seeded and 24 hours later treated with either 0, 20, 40, or 80 pM TGF-β1. Cells were supplemented with fresh media with or without the appropriate dosage of TGF-β1 every other day. Cells were collected on day 5 and counted with a hemacytometer. All experiments were done in triplicate. Three independent experiments were performed.

Timing Experiments

In 24-well plates, a total of 2 X 10^3 HaCaT cells/well, 3 X 10^3 DU145, PC3, and PN cells/well, and 1 X 10^4 LNCaP cells/well were seeded and 24 hours later treated with either 0 or 80 pM TGF-β1. Cells were supplemented with fresh media with or without the appropriate dosage of TGF-β1 every other day. Cells were collected on days 1 through 5 and counted with a hemacytometer. All experiments were done in triplicate. Three

independent experiments were performed.

RNA Isolation

RNA was isolated from cells harvested in 100 mm plates using 1ml of TRIzol Reagent (Gibco/BRL Laboratories) per plate. Cells were incubated in TRIzol for 10 minutes at room temperature. Suspended samples were transferred to microfuge tubes, shaken vigorously for 15 seconds, and incubated for 2 to 3 minutes. Suspensions were centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase of the samples were transferred to fresh tubes and 0.5 ml of 2-propanol was added (per 1 ml of TRIzol previously) followed by incubation for 10 minutes at room temperature or -20°C overnight. Samples were centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatants were removed and the pellets washed 1 time (per 1 ml of TRIzol reagent) with 1 ml of ice cold 75% ethanol in DEPC H_2O. Samples were briefly vortexed and centrifuged at 7,500 rpm for 5 minutes at 4°C. RNA pellets were air-dried for 5 to 10 minutes and resuspended in TE/DEPC + ANA (10 mM Tris pH 6.8, 1 mM EDTA, 1 mM aurintricarboxylic acid) and incubated for 10 minutes at 55°C.

Northern Blotting

Ten μg of total RNA of each sample was electrophoresed on a 1% agarose gel containing formaldehyde, followed by transfer onto a nylon Hybond-N+ membrane (Amersham) using either a Possiblotter pressure system (Stratagene) or capillary transfer. $P15^{INK4B}$, $p21^{WAF1}$, and $p27^{KIP1}$ mRNA was detected using a DIG DNA labeling and detection kit (Boerhinger Mannheim) with modifications. $P15^{INK4B}$ and $p21^{WAF1}$ cDNA was labeled with digoxigenin by random priming. The membrane was prehybridized in 5 to 10 ml of hybridization buffer (250 mM Na_2HPO_4 pH 7.0, 1.0 mM EDTA, 20% SDS,

0.5% blocking reagent) for 1 hour, 68°C. Buffer was then removed and 5 to 10 ml of fresh hybridization buffer added containing 17 μg of labeled probe and hybridized overnight at 68°C with rotation. The membrane was washed 3 times 5 minutes with 100 ml each of wash buffer 1 (20 mM Na_2HPO_4 pH 7.0, 1.0 mM EDTA pH 8.0, 1.0% SDS) at room temperature. Afterward, the membrane was washed 3 times 20 minutes with 50 to 100 ml each of wash buffer 1 at 65°C with rotation followed by 1 wash for 5 minutes with 100 ml of wash buffer 2 (3 M NaCl, 100 mM maleic acid-1.2% Tween 20) at room temperature.

The RNA membrane was incubated in 10 ml of blocking buffer (100 mM maleic acid/0.3% Tween 20, 0.5% blocking powder, 8 M NaCl) for 1 hour at room temperature then 30 minutes in blocking buffer containing mouse anti-digoxigenin antibody conjugated with aikaline phosphatase (1:15,000 dilution). The membrane was washed 4 times 10 minutes in 50 to 100 ml each of wash buffer 2 at room temperature followed by 1 wash in 10 mM Tris pH 8.8. The membrane was then laid atop a Lumi-phos 530 chemiluminescent substrate sheet (Schleicher & Schuell) and sealed in a plastic bag. Signal was detected by exposing the sealed membrane to x-ray film for 30 minutes to 48 hours at 37°C. De-probing the membrane included 1 wash in dH_2O for 5 minutes, 1 wash in 5X SSC for 20 minutes, and 1 wash in 0.1% SDS for 2 minutes at 95°C. The membrane was then dried and re-probed with a β-actin DIG-labeled probe.

De-methylation/Northern Blotting

HaCaT, DU145, and PC3 cells were seeded in 100 mm plates. The following day, 6 plates for each cell line received fresh medium plus treatment as described below. Two plates were seeded per cell line per experimental condition. There were 6 conditions. For

the first condition, the 2 plates were treated with 1 μm final de-methylating agent 5-aza-2′-deoxycytidine (Sigma). The second condition was treatment with only 100 pM TGF-β1. The third was treatment with both 1 μm final 5-aza-2′-deoxycytidine and 100 pM TGF-β1. The fourth condition was no treatment serving as a negative control. The fifth was treatment with 1 μm final DMSO (Sigma). DMSO treated plates served as a negative control as DMSO was used to resuspend the de-methylating agent. The sixth condition was treatment with 50 mM NaAc. This served as another negative control as the TGF-β1 was resuspended in NaAc. Treatment with 5-aza-2′-deoxycytidine or DMSO was for 3 days. Treatment with TGF-β1 was for 6 hours prior to collection of the cells on day 3. RNA was isolated and Northern blotted as described previously.

Western Blotting

Forty μg of protein for each sample was separated on a 10% (cyclin D1, D2, D3, E) or 16% polyacrylamide gel (p15, p21, p27) and transferred onto a nitrocellulose membrane. The membrane was then blocked with 20 ml of 10% non-fat dry milk/PBS-Tween 20 for 1 hour at room temperature. The 20 ml was discarded and 3 ml added which contained 1.5 μg/ml of antibody and incubated with rotation, 1 hour, at room temperature. The membrane was washed 3 times 10 minutes with 25 ml each of 1X PBS-Tween 20 (146 mM NaCl, 0.1% Tween 20, 1.47 mM $NaH_2PO_4 \cdot H_2O$). The membrane was incubated in 3 ml of 10% non-fat dry milk/PBS-Tween 20 which contained 2 μl of horseradish peroxidase-conjugated goat anti-mouse (or anti-rabbit) IgG (2:3000 dilution) (BIORAD) for 1 hour with rotation at room temperature. The membrane was washed 4 times 10 minutes with 25 ml each 1XPBS-Tween 20 followed by detection using ECL Western detection kit (Pierce). Stripping the membrane included 2 times 10 minute

washes with 25 ml of 1X PBS-Tween 20, 1 wash in stripping solution (70 mM SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris pH 6.8) for 30 minutes at 70°C, and 2 times 10 minute washes with 25 ml of 1X PBS-Tween 20. The membrane was then dried and re-blotted with β-actin antibody.

Immunoprecipitation

Cells were washed 2 times 5 ml per 100 mm or 10 ml per 150 mm plate with cold $PBS^{=}$ followed by addition of 1 ml of lysis buffer with inhibitors (50 mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, 1.0% NP-40, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 1X protease inhibitor (Boehringer Mannheim)) per 100 mm plate or 2 ml per 150 mm plate. Plates were placed on ice for 20 minutes with occasional shaking. Lysate was collected into 1.5 ml microfuge tubes, incubated on ice for 30 minutes with intermittent vigorous vortexing and then centrifuged at 13,200 rpm, 10 minutes, 4°C. Protein concentration was determined using either a BCA assay or TCA precipitation assay recommended by the manufacturer (Pierce).

Immunoprecipitation/Western Blot Analysis

Cells were incubated in the absence or presence of 80 pM TGF-β1 at time points indicated and harvested for protein lysate. Cells were washed 2 times with PBS followed by addition of 1 ml of lysis buffer with inhibitors (50 mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, 1.0% NP-40, 1 mM Na_3VO_4, 1 mM DTT, 1X protease inhibitor (Boehringer Mannheim), 1 mM PMSF) per 100 mm plate or 2 ml per 150 mm plate. Plates were placed on ice for 20 minutes with occasional shaking. Lysate was collected, incubated on ice for 30 minutes with intermittent vigorous vortexing, and then centrifuged at 13,200 rpm, 10 minutes, 4°C. Protein concentration was determined using

either a BCA assay or TCA precipitation with BCA assay recommended by the manufacturer (Pierce). Equal amount of protein (200 μg) were added to 1 μg of polyclonal anti-Cdk2, anti-Cdk4, or anti-Cdk6 antibody (Santa Cruz) and rotated overnight at 4°C. Protein G-agarose beads (Boehringer Mannheim) were washed 1 time with 1 ml lysis buffer without inhibitors (50 mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, 1.0% NP-40), added to the protein sample (30 μl/tube) and incubated at 4°C, 2 h with rotation. The beads were washed 4 times with 1 ml lysis buffer without inhibitors with centrifugation at 6000 rpm for 1 to 2 minutes in between washes. Beads were resuspended in 4X sample buffer (240 mM Tris pH 6.8, 280 mM SDS, 40% glycerol, 60 mM bromophenol blue, 4.0 mM DTT), boiled for 10 minutes, then centrifuged at 6000 rpm for 2 minutes. Protein was loaded onto a 10% (cyclin D1, D2, D3, and E) or 16% (p15, p21, p27) polyacrylamide gel followed by Western blot analysis with monoclonal antibodies anti-cyclin D1, anti-p27 (Oncogene Research Products) anti-cyclin D2, anti-cyclin D3, anti-cyclin E (Pharmingen), anti-p21 (Upstate Biotechnology), or polyclonal anti-p15 (Santa Cruz) antibody as described previously.

Immunoprecipitation/Kinase Assay

Cells were incubated in the absence or presence of 80 pM TGF-β1 at time points indicated, harvested for protein lysate, and immunoprecipitated as described previously. Equal amounts of protein lysate (500 μg) were added to 1 μg of either anti-Cdk2, anti-Cdk4, or anti-Cdk6 antibodies (Santa Cruz) and rotated overnight at 4°C. Thirty μl of Protein G-agarose beads was added to each tube and samples were incubated for an additional 2 h at 4°C. The beads were centrifuged at 6000 rpm for 1 minute and washed 2 times with 1 ml lysis buffer without inhibitors. The beads were then washed 2 times

with 1 ml kinase assay buffer (10 mM $MgCl_2$, 5 mM $MnCl_2$, 50 mM HEPES pH 7.0). Beads were resuspended in 25 µl kinase assay buffer with DTT and ATP (1 mM DTT, 5 µM ATP). Five µl of ^{32}P labeling mix (2 µg histone H1 (Boehringer Mannheim) or 2 µg GST/RB, 10 µCi/µl ^{32}P-γ-ATP, 2.5 µl kinase assay buffer with DTT and ATP) was added to each sample and incubated 30 minutes, 34°C. The reaction was stopped by adding 4X sample buffer. Proteins were boiled for 10 minutes, centrifuged at 6000 rpm for 2 minutes, and then loaded onto a 16% polyacrylamide gel. The gel was stained with 2% Commassie blue dye, destained (20% methanol, 10% HOAc, 70% dH_2O), dried in a gel dryer, and exposed to film using an intensifying screen for 30 minutes to 48 h at either room temperature or -80°C.

Immunoprecipitation/^{35}S Metabolic Labeling

PC3 cells were washed 2 times with PBS^- and then incubated in medium with a ratio of 1:5 consisting of 1 part DMEM medium plus 5% FBS and 5 parts cysteine and methionine free DMEM medium. In addition, 0.1 mCi final of ^{35}S-methionine/cysteine was added per 5 ml of medium. The same procedure was followed for PN cells except the medium contained 1 part prEBM medium and 5 parts cysteine and methionine free DMEM medium with prEBM supplements. Cells were incubated for 24 hours at 37°C. Media was removed and cells washed 2 times 5 ml per 100 mm plate or 10 ml per 150 mm plate with cold PBS^-. Cells were lysed and used for immunoprecipitation. Ten µl of immunoprecipitated protein was loaded and run on a 16% polyacrylamide gel. The gel was fixed in 10% MeOH/5% HOAc for 30 minutes and then incubated in Fluoro-Hance (Research Products International) for 30 minutes. The gel was dried in a gel dryer (BIORAD) and exposed to film using an intensifying screen for 72 hours to 2 weeks at

either room temperature or -80°C.

Reverse-transcription (RT) -polymerase chain reaction (PCR)

RT

For reverse transcription reactions, the following final concentrations were added in a microcentrifuge tube for a 20 µl final volume: 1X RT Buffer (GIBCO/BRL Laboratories), 0.25 µg/µl of either anti-sense Cdk2 primer 5'-AAGCCCACACTGGAGGAGAG-3' or anti-sense p21 primer 5'-GATGTAGAGCGGGCCTTTGAG-3' (GIBCO/BRL Laboratories), 10 mM DTT (GIBCO/BRL Laboratories), 1 µg PC3 RNA prepared with TRIzol reagent (GIBCO/BRL Laboratories), 200U/µl RTase Superscript II (GIBCO/BRL Laboratories), 0.8 mM dNTP Mix (Invitrogen), 40U/µl rRNAsin (Promega), and DEPC-H_2O. The samples were incubated at room temperature for 10 minutes, 42 °C for 1 hour, boiled at 95 °C for 5 minutes, and then immediately chilled on ice. Two additional reaction mixes were prepared as controls. Both control mixes contained all components except one contained no RNA and the other no RTase Superscript II.

First Run PCR

The following final concentrations were added in a PCR tube for a 100 µl final volume: 1X PCR Buffer (GIBCO/BRL Laboratories), BSA 10 µg/µl (SIGMA), 4.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.25 µg/µl of sense Cdk2 primer 5'-TATACTGCGTTCCATCCCGA-3' and 0.25 µg/µl of anti-sense Cdk2 primer 5'-AAGCCCACACTGGAGGAGAG-3' (GIBCO/BRL Laboratories) for Cdk2 cDNA amplification or 0.25 µg/µl of sense p21 primer 5'-GTCAGTTCCTTGTGGAGCCGG-3' and 0.25 µg/µl of anti-sense p21 primer 5'-GATGTAGAGCGGGCCTTTGAG-3'

(GIBCO/BRL Laboratories) for p21 cDNA amplification (GIBCO/BRL Laboratories), 0.8 mM dNTP Mix (Invitrogen), 1 µl of respective cDNA from the RT reactions, and DEPC-H_2O. The samples were hot started with 0.5 µl of AmpliTaq (Promega) per reaction and cycled in a thermocycler version 9600 (Perkin-Elmer) at 94 °C (30 seconds), 58 °C (30 seconds), and 72 °C (1 minute) for Cdk2 and 94 °C (30 seconds), 52 °C (30 seconds), and 72 °C (1.5 minutes) for p21.

Nested PCR

The following final concentrations were added in a PCR tube for a 100 µl final volume: 1X PCR Buffer (GIBCO/BRL Laboratories), BSA 10 µg/µl (SIGMA), 4.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.25 µg/µl of sense Cdk2 primer 5′-AGAGCCAGGGTCCGCCTTCT-3′ and 0.25 µg/µl of anti-sense Cdk2 primer 5′-TAGGGCTGGGGGCTTCAAGA-3′ (GIBCO/BRL Laboratories) for Cdk2 or 0.25 µg/µl of sense p21 primer 5′-GAGGCACTCAGAGGAGGCGC-3′ and 0.25 µg/µl of anti-sense p21 primer 5′-GGCTTCCTGTGGGCGGATTA-3′ (GIBCO/BRL Laboratories) for p21, 0.8 mM dNTP Mix (Invitrogen), 1 µl of respective cDNA from the first strand reactions, and DEPC-H_2O. The samples were hot started with 0.5 µl of AmpliTaq (Promega) per reaction and cycled in a thermocycler (Perkin-Elmer) at 94 °C (30 seconds), 59.2 °C (30 seconds), and 72 °C (2 minutes) for Cdk2 and 94 °C (30 seconds), 58 °C (45 seconds), and 72 °C (1.5 minutes) for p21. For further amplification of the Cdk2 and p21 cDNA, an second run of Nested PCR was performed as described above using a 1:10 dilution of the first Nested PCR products. All samples were run on a 0.1% agarose gel to determine purity of DNA fragments.

Sequencing

Cdk2 and p21 PCR products were run on a 1% agarose gel and the appropriate sized DNA bands were cut from the gel. The DNA was then purified using standard phenol/chloroform extraction followed by ethanol precipitation. Samples were resuspended in 20 µl of dH_2O. The DNA concentrations were determined at $O.D._{260}$ using a spectrophotometer. To prepare the samples for dye terminator labeling, reaction mixes were prepared for Cdk2 and p21 in the following final concentrations and volumes: 8 µl of dRhodamine Ready Reaction Mix (Perkin-Elmer Dye Terminator Sequencing Kit), 3.2 pmole of sense Cdk2 primer 5'-AGAGCCAGGGTCCGCCTTCT-3', anti-sense Cdk2 primer 5'-TAGGGCTGGGGGCTTCAAGA-3', sense p21 primer 5'-GAGGCACTCAGAGGAGGCGC-3', or anti-sense p21 primer 5'-GGCTTCCTGTGGGCGGATTA-3', 200 ng of PCR product, and brought to a volume of 20 µl with dH_2O. Another set of reaction mixes were prepared using 300 ng of DNA, addition of 4 µl of 50% glycerol, or both. All samples were dye terminator labeled using a thermocycler (Perkin-Elmer) set at 96 °C (10 seconds), 50 °C (5 seconds), and 60 °C (4 minutes). Unincorporated nucleotides were washed from the samples. This was accomplished by adding 2.0 µl of 3M NaAc pH 6.8 and 50 µl of 95% ethanol to the samples, vortexing, incubating on ice for 10 minutes, centrifugation at 13,200 for 30 minutes, and removal of the supernatant. Next, 250 µl of 70% ethanol was added to each sample and then poured off. Samples were vacuum dried and stored in a -20 °C freezer until they were run on a sequencing gel and the sequence read using an AVI Image System (Perkin-Elmer).

Densitometry and Data Analysis

Each experiment was performed at least three times. Densitometric scanning was used to quantitate mRNA and protein blots using an Ambis image analysis system (San Diego). Numeric scanning data from specific bands of mRNA or protein from at least three blots were averaged for each experiment. Data from β-actin bands were also averaged. Averaged mRNA or protein data were divided by the averaged β-actin data for each time point. These numbers were plotted as fold of induction versus time of treatment.

RESULTS

Effect of TGF-β1 on epithelial cell growth

To determine the effect of TGF-β1 dosage and time of treatment on prostate epithelial cell growth, standard growth curves were performed using four well-characterized cell lines (HaCaT, DU145, PC3, and LNCaP) and prostate primary cell cultures in the presence and absence of TGF-β1. HaCaT, immortalized human keratinocytes, served as a positive control as they are known to be growth inhibited by TGF-β (Hannon and Beach, 1994). LNCaP cells served as a negative control as these cells have a defective TGF-β type I receptor (Kim et al., 1996). Both DU145 and PC3 have normal TGF-β receptors (Wilding et al., 1989). Our results showed significant growth inhibition by TGF-β1 in HaCaT and PN cells at a dosage of 20 pM to 80 pM (Fig. 2). At 20 pM TGF-β1, growth inhibition was 80% for HaCaT cells, 70% for PN1, and 85% for PN2. A similar growth inhibition was observed at 40 and 80 pM of TGF-β1 (Fig. 2). The other three cell lines PC3, DU145, and LNCaP were not or only minimally growth inhibited by TGF-β1 as compared to normal cells regardless of the dosage (Fig. 2). Growth curves were performed using a dosage as high as 100 pM with no additional inhibitory effect (data not shown). Based on the dosage growth curve results, 80 pM TGF-β1 was chosen as the optimum dose for growth inhibition and used for subsequent experiments. However, in TGF-β1 timing experiments, HaCaT and PN2 cells were growth inhibited by treatment with 80 pM TGF-β1 on days 3, 4, and 5 (Fig. 3A and B). Prostate carcinoma cell lines DU145, PC3, and LNCaP did not demonstrate any statistically significant growth inhibition when treated with 80 pM TGF-β1 for the period of 1 to 5 days studied (Fig. 3C, D, and E). Therefore, these results show that normal

prostate cells are growth inhibited by TGF-β1 while prostate carcinoma cells are not or only minimally responsive to growth inhibition.

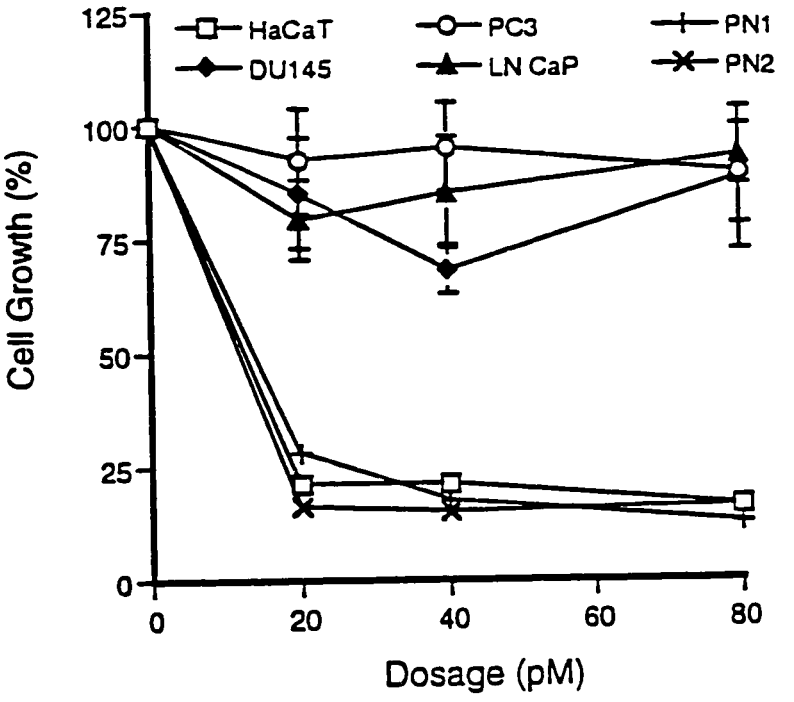


Fig. 2 Dosage effect of TGF-β1 on the growth of normal and tumor cells. Asynchronized cells were seeded in 24-well plates and 24 hours later treated with 0, 20, 40, or 80 pM TGF-β1. Cells were re-supplemented with medium containing the appropriate amount of TGF-β1 every other day. Cells were collected and counted on day 5. All experiments were done in triplicate and three independent experiments were performed. The bars indicated represent standard deviation.

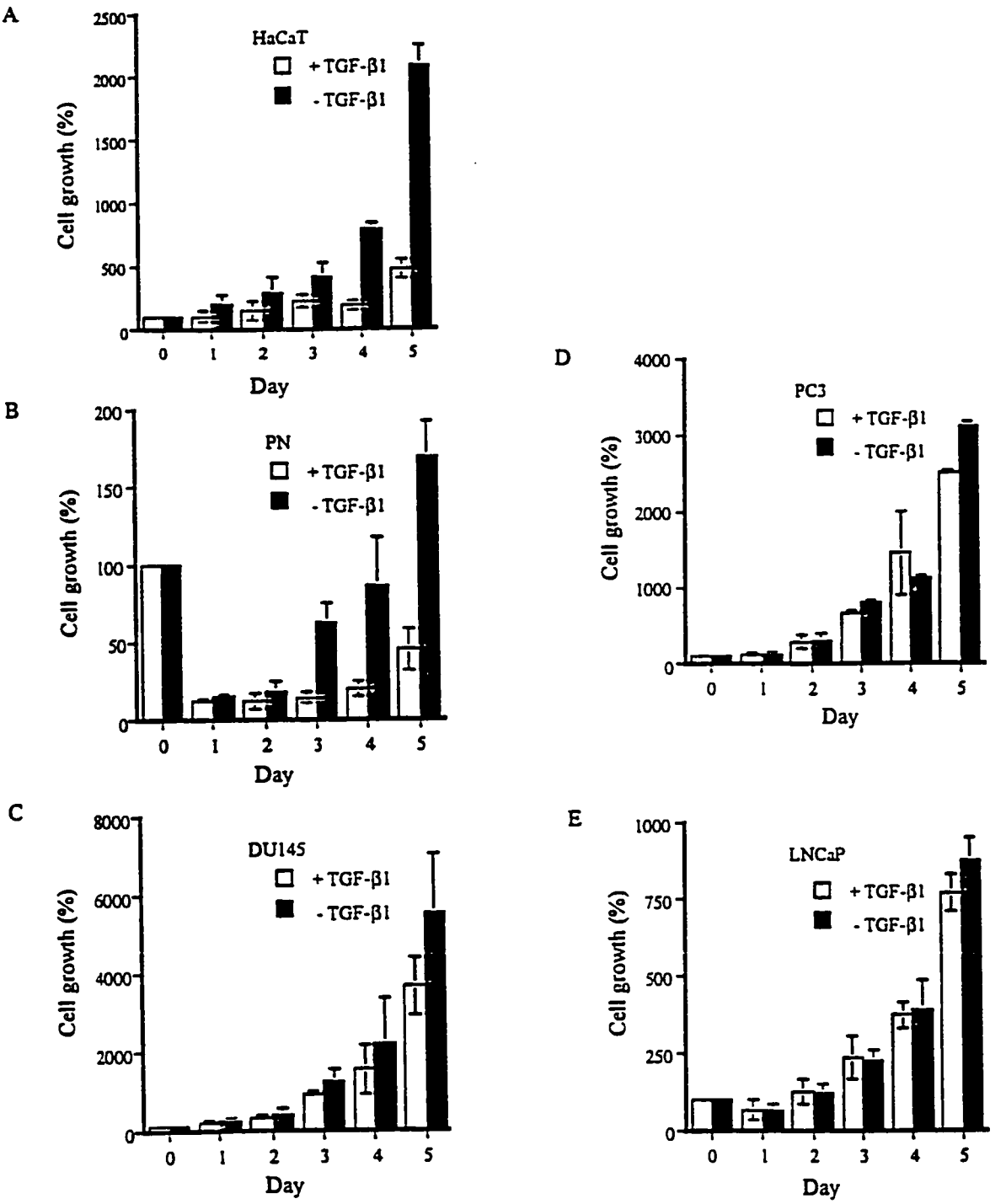


Figure 3 Time effect of TGF-β1 on the growth of normal and tumor cells.
'Asynchronized cells as indicated were seeded in 24-well plates and 24 hours later treated with either 0 pM or 80 pM TGF-β1. Cells were re-supplemented with medium containing either 0 or 80 pM TGF-β1 every other day. Cells were collected and counted daily for a period of 5 days. All experiments were done in triplicate and three independent experiments were performed. The bars indicated represent standard deviations. A: HaCaT, B: PN, C: DU145, D: PC3, E: LNCaP

Effect of TGF-β1 on CKI expression

To determine whether this loss of TGF-β1 mediated growth inhibition in prostate tumor cells correlates with a lack of G_1 CKIs expression, northern and western blot analyses were performed. p15, p21, and p27 mRNA and protein levels were determined in cell lines HaCaT, DU145, PC3, and PN in the presence and absence of TGF-β1. Due to limited availability of PN cells, HaCaT, instead of PN cells were used for Northern blot analysis.

p21 mRNA was significantly induced in cell lines HaCaT, PC3, and DU145 in the presence of 80 pM TGF-β1. Induction was seen in the time period studied, i.e. 3 to 12 hours. In contrast, p21 mRNA was not significantly induced in LNCaP cells at any time point with or without treatment (Fig. 4A and B). While p21 expression at the transcriptional level remained elevated for prostate tumor cells, these cells were not or only minimally growth inhibited by TGF-β (Fig. 2 and 3).

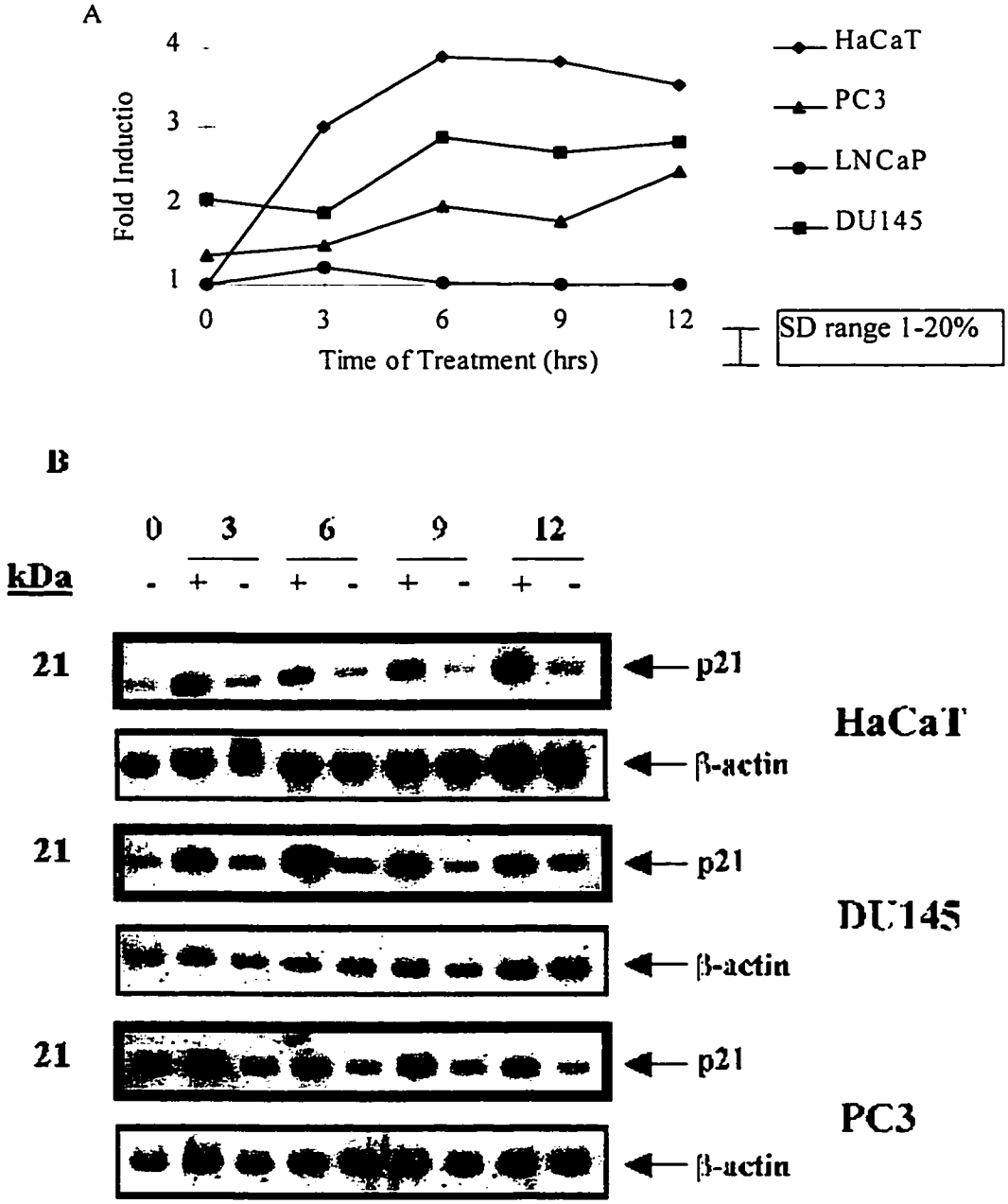


Fig. 4 Induction of p21 mRNA by TGF-β1

A: Quantitation of three independent Northern blot experiments. Asynchronized cell lines indicated were treated with either 0 or 80 pM TGF-β1 for 0, 3, 6, 9, and 12 hours. Values are expressed relative to the control (0 pM treatment).

B: Northern blot analysis of 10 μg of total RNA from the cell lines indicated hybridized with a p21 cDNA probe and a β-actin cDNA probe as an internal control. Data not shown for LNCaP.

Similarly, p21 protein levels were increased by TGF-β1 treatment in HaCaT, DU145, and PC3 but not in LNCaP cells. Induction was highest for PC3 cells peaking at approximately 16-fold at 12 hours post-treatment. Unexpectedly, p21 protein was not induced by TGF-β1 in PN2 cells at any time point with or without treatment (Fig. 5A and B). Induction of p21 in prostate tumor cells at the translational level was significantly elevated. However, this induction did not correlate with growth curve results which showed lack of growth inhibition by TGF-β1 in tumor cells (Fig. 2 and 3).

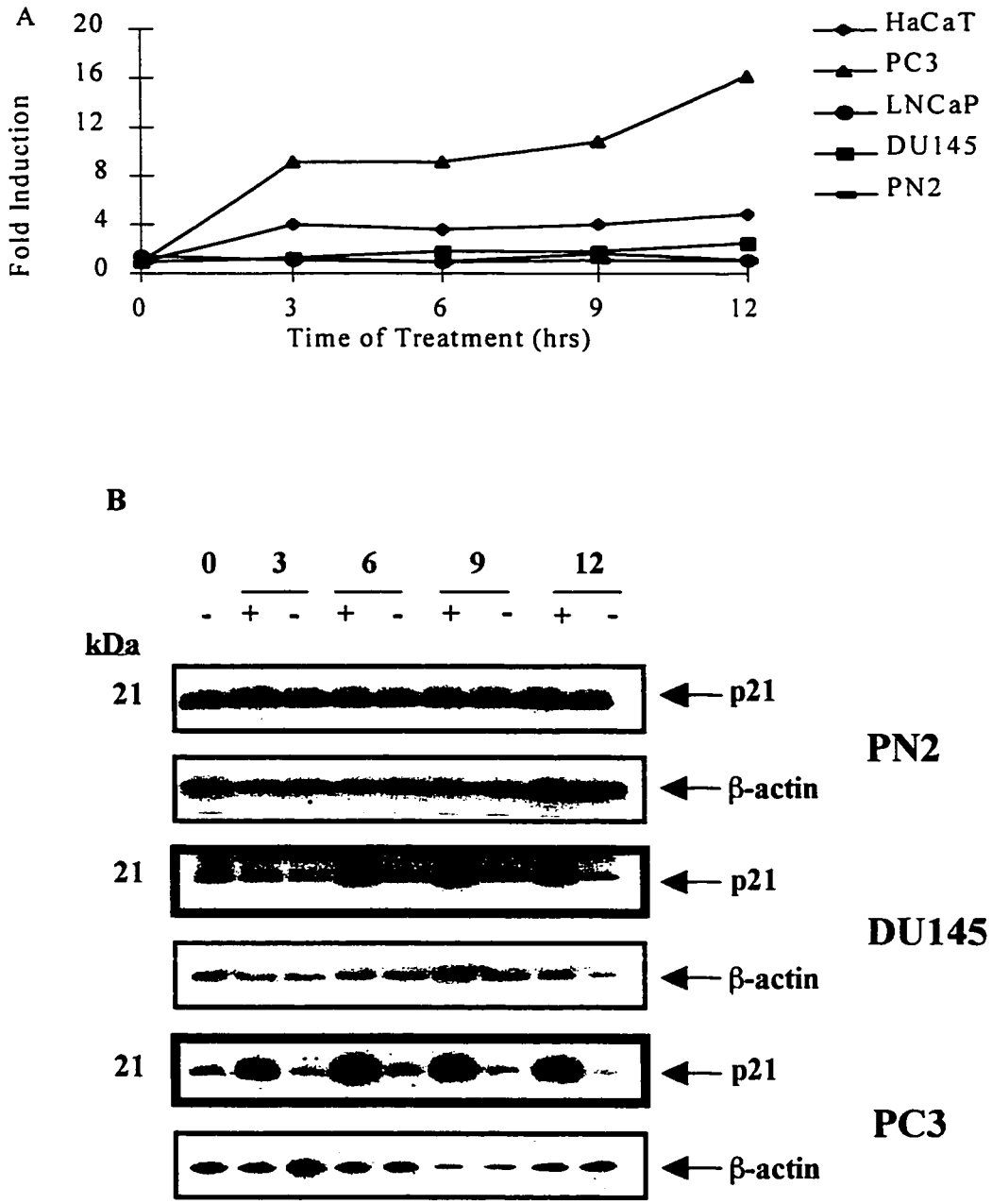


Fig. 5 Induction of p21 protein by TGF-β1

A: Quantitation of three independent Western blot experiments. Asynchronized cell lines indicated were treated with either 0 or 80 pM TGF-β1 for 0, 3, 6, 9, and 12 hours. Values are expressed relative to the control (0 pM treatment).

B: Western blot analysis of 40 μg of total protein from the cell lines indicated blotted with anti-p21 antibody, stripped, and re-blotted with an anti-β-actin antibody as an internal control. Data for HaCaT and LNCaP were not shown.

In the case of p15, its mRNA and protein levels were significantly increased in HaCaT cells in the presence of 80 pM TGF-β1 (Fig. 6 and 7). p15 induction in HaCaT cells correlated with growth inhibition by TGF-β1 seen previously (Fig. 2 and 3). However, no induction of p15 mRNA or protein was seen DU145 or PC3 cells (Fig. 6 and 7). Three bands were seen on Northern blot analysis for each cell line. This may be explained as alternative splicing of the mRNA. This three band pattern was consistent with blots showing p15 induction in HaCaT cells following TGF-β1 treatment (Hannon and Beach, 1994). The center band was used for scanning and quantitating blots.

We expected similar results for PN cells. However, there was no significant induction of p15 protein in PN cells (Fig. 7). p15 in these cells may be expressed at a low level and difficult to detect with Western blotting. Multiple bands indicated on the Western blot for p15 in DU145 cells may be due to phosphorylation.

Using Northern and Western blot analysis, p27 levels in HaCaT, DU145, LNCaP, and PC3 were not detectable at 0, 3, 6, 9, or 12 hours with or without TGF-β1 treatment. One possible explanation for the lack of detection of p27 may be that it is expressed at a low level not detectable by Western or Northern blot analyses.

In summary, p21 mRNA and protein levels were induced in HaCaT, PC3, and DU145 cells. However, p21 protein was not induced in PN cells. In addition, p15 mRNA was induced only in HaCaT cells. p27 was not detectable at the transcription or translation levels for any cells tested.

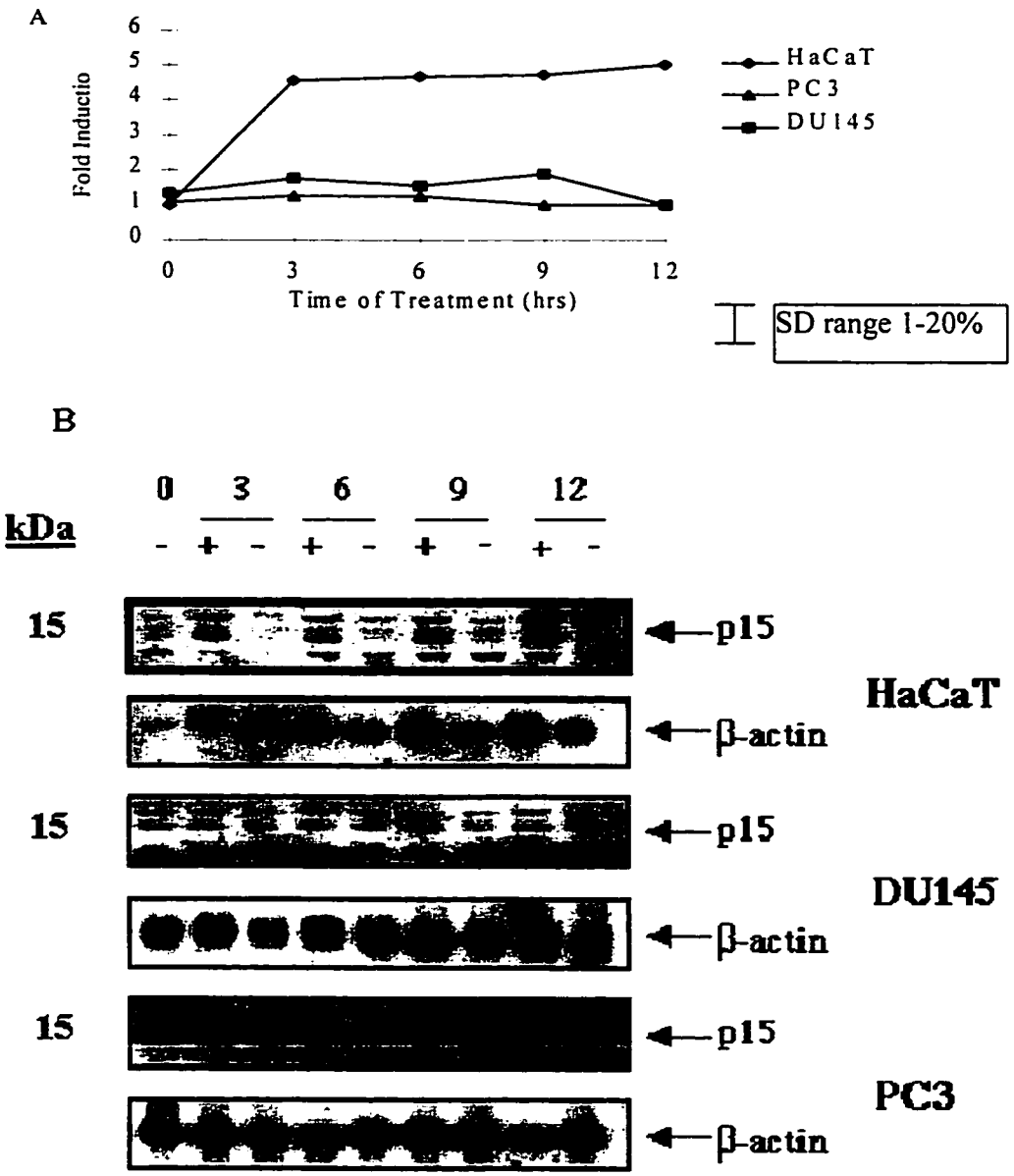


Fig. 6 Effect of TGF-β1 on p15 mRNA levels

A: Quantitation of three independent Northern blot experiments. Asynchronized cell lines indicated were treated with either 0 or 80 pM TGF-β1 for 0, 3, 6, 9, and 12 hours. Values are expressed relative to the control (0 pM treatment).

B: Northern blot analysis of 10 μg of total RNA from the cell lines indicated hybridized with a p15 cDNA probe, stripped and re-blotted with a β-actin cDNA probe as an internal control.

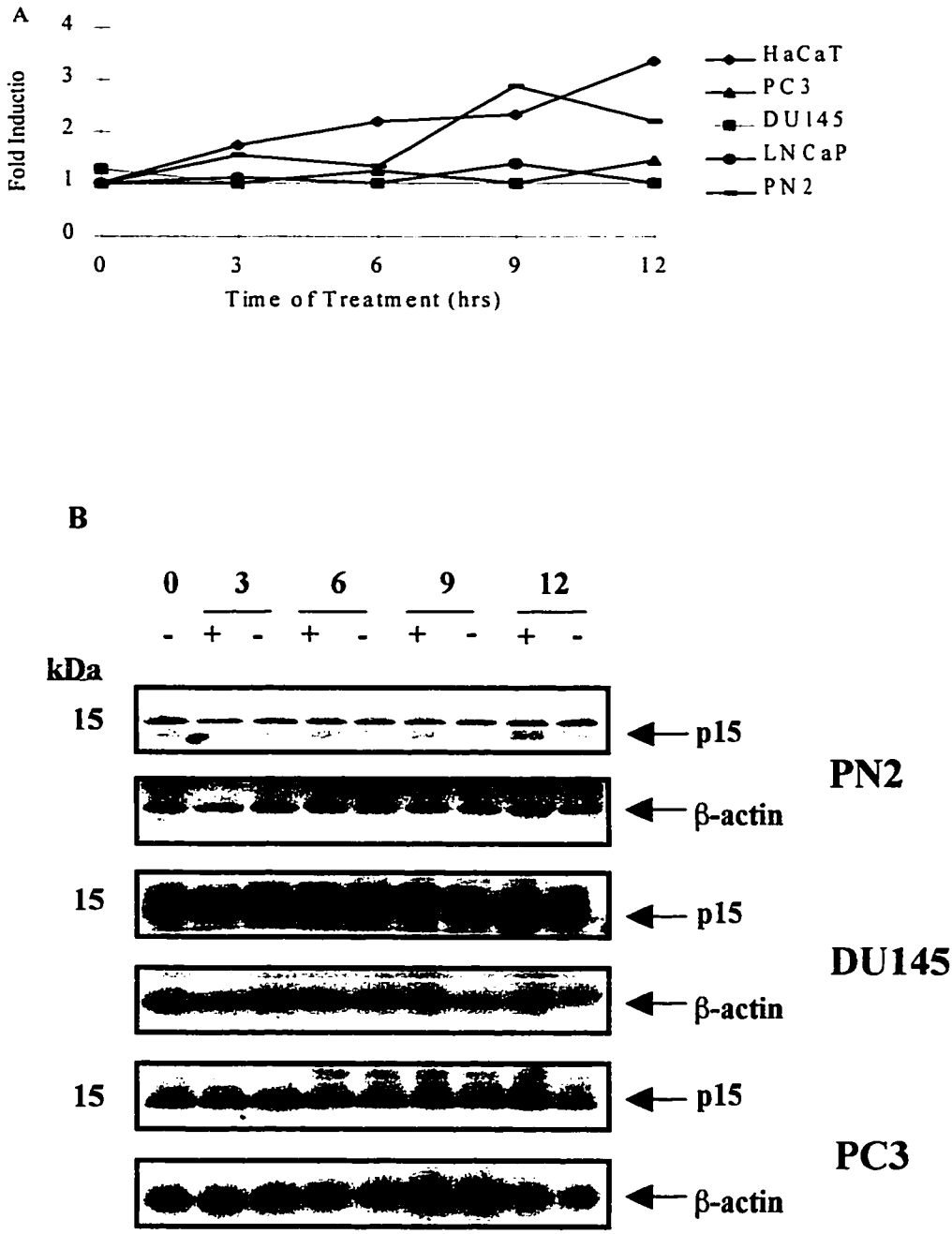


Fig. 7 Effect of TGF-β1 on p15 protein levels

A: Quantitation of three independent Western blot experiments. Asynchronized cell lines indicated were treated with either 0 or 80 pM TGF-β1 for 0, 3, 6, 9, and 12 hours. Values are expressed relative to the control (0 pM treatment). B: Western blot analysis of 40 μg of total protein from the cell lines indicated blotted with anti-p15 antibody, stripped and re-blotted with an anti-β-actin antibody as an internal control. Data for HaCaT and LNCaP not shown.

We asked whether lack of p15 induction in prostate tumor cells PC3 and DU145 could be due to methylation of the p15 promoter. To address this question, we performed a de-methylation experiment. De-methylation of a methylated p15 promoter region could lead to re-expression of the p15 mRNA product. The p15 promoter in HaCaT cells has been previously reported to be un-methylated. Addition of a de-methylating agent would not be expected to increase p15 mRNA expression in this cell line. Therefore, as expected HaCaT cells showed no increase in p15 mRNA induction in the presence of the de-methylating agent alone (Fig. 8A). These cells served as a control. Induction of p15 was seen in samples treated with TGF-β1 alone and TGF-β1 plus the de-methylating agent (Fig. 8A). In DU145 and PC3 cells, treatment with the de-methylating agent alone also did not result in p15 induction (Fig. 8B and C). Nor was p15 significantly induced in the presence of TGF-β1 alone or TGF-β1 plus the de-methylating agent (Fig. 8B and C). Control lanes indicated by DMSO, NaAc, and no treatment (tx) showed no p15 induction for any of the cells tested. Therefore, these results indicate that the p15 promoter in DU145 and PC3 cells is probably not methylated. Therefore, the lack of p15 induction by TGF-β1 and hence lack of growth inhibition by TGF-β1 is probably due to alterations other than promoter methylation.

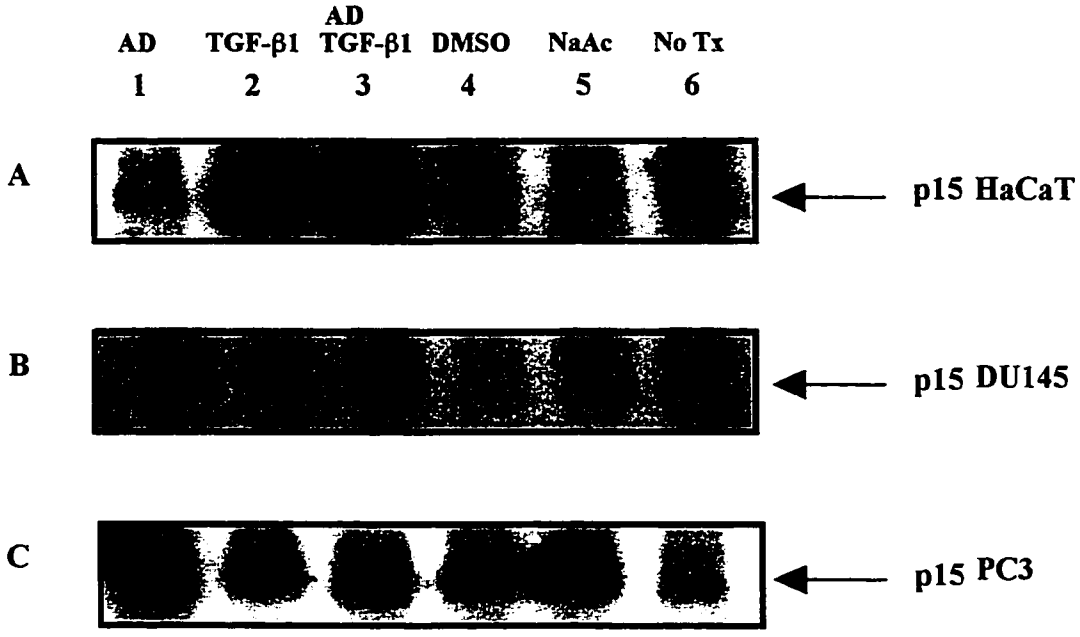


Fig. 8 Effect of methylation inhibitor on p15 mRNA
HaCaT (A), DU145 (B), and PC3 (C) cells were treated with either 5-aza-2′-deoxycytidine (AD) alone, TGF-β1 alone, both, DMSO, NaAc, or no treatment. Results show Northern blot analysis of 10 μg of total RNA from the cell lines indicated hybridized with a p15 cDNA probe.

Effect of TGF-β1 on association of CKIs with cyclins and Cdks

In addition to determining the effect of TGF-β1 treatment on levels of CKI expression in normal and prostate tumor cells, importantly Cdk-cyclin/CKI complex formation was also determined. While p21 is induced at a high level in prostate tumor cells, we asked whether p21 was actually binding to the Cdk-cyclin complex. Once bound, p21 can inhibit Cdk activity and therefore cell proliferation. If p21 is not binding to the protein-protein complex, it may not result in cell cycle arrest. To address this question, cell lysate from PN(3-5), DU145, and PC3 cells were immunoprecipitated with either Cdk2, Cdk4, or Cdk6 antibody followed by Western blot analysis for their associated cyclins and Cdk inhibitors. In all cell lines, p15, p21, p27, cyclin D1, D2, D3, and E were detected in immunoprecipitates.

In PN cells treated with 80 pM TGF-β1 for 96 and 120 hours, there was no increase in the amount of p15, p27, cyclin D1, D2, D3, or E associated with Cdk2, Cdk4, or Cdk6 (Fig. 9A, B, and C). Nor, was there an increase in the amount of p21 associated with Cdk4 or Cdk6 (Fig. 9B and C). However, there was an increase in the amount of p21 bound to Cdk2 in the presence of 80 pM TGF-β1 versus no treatment (Fig. 9A). Therefore, in normal prostate cells p21 appears to be bound to the Cdk2-cyclin complex in the presence of TGF-β1. This correlates with our growth curve results showing inhibition of normal cell growth by TGF-β1.

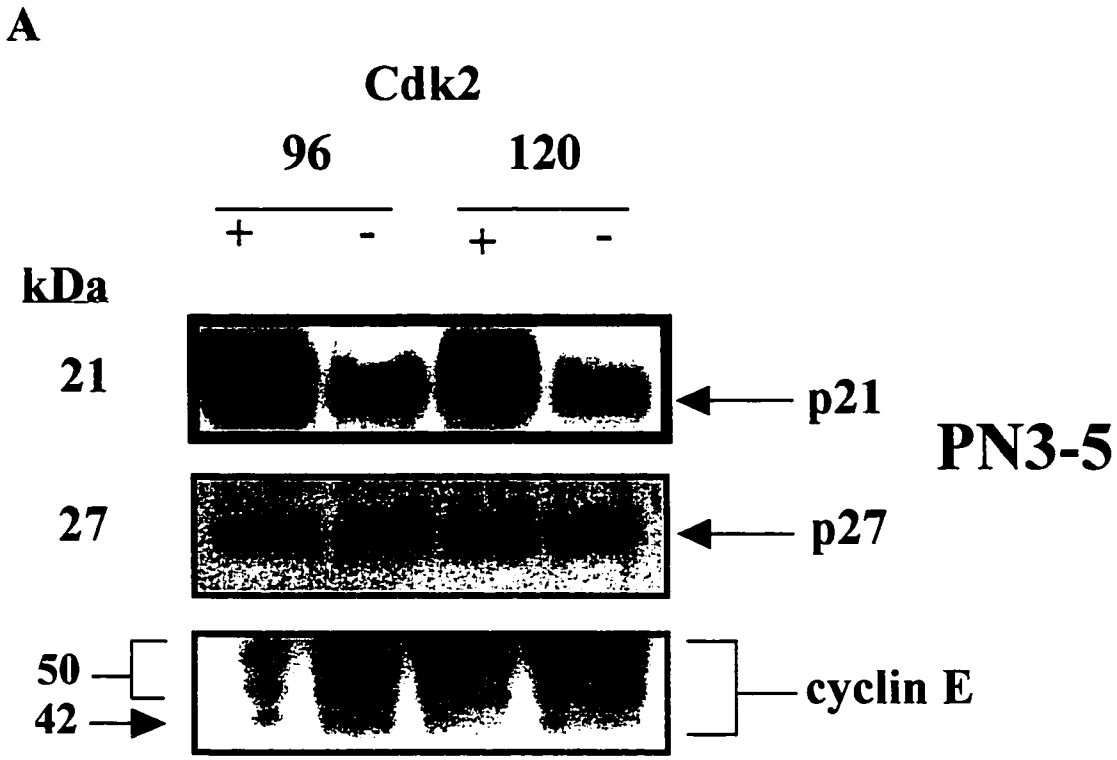
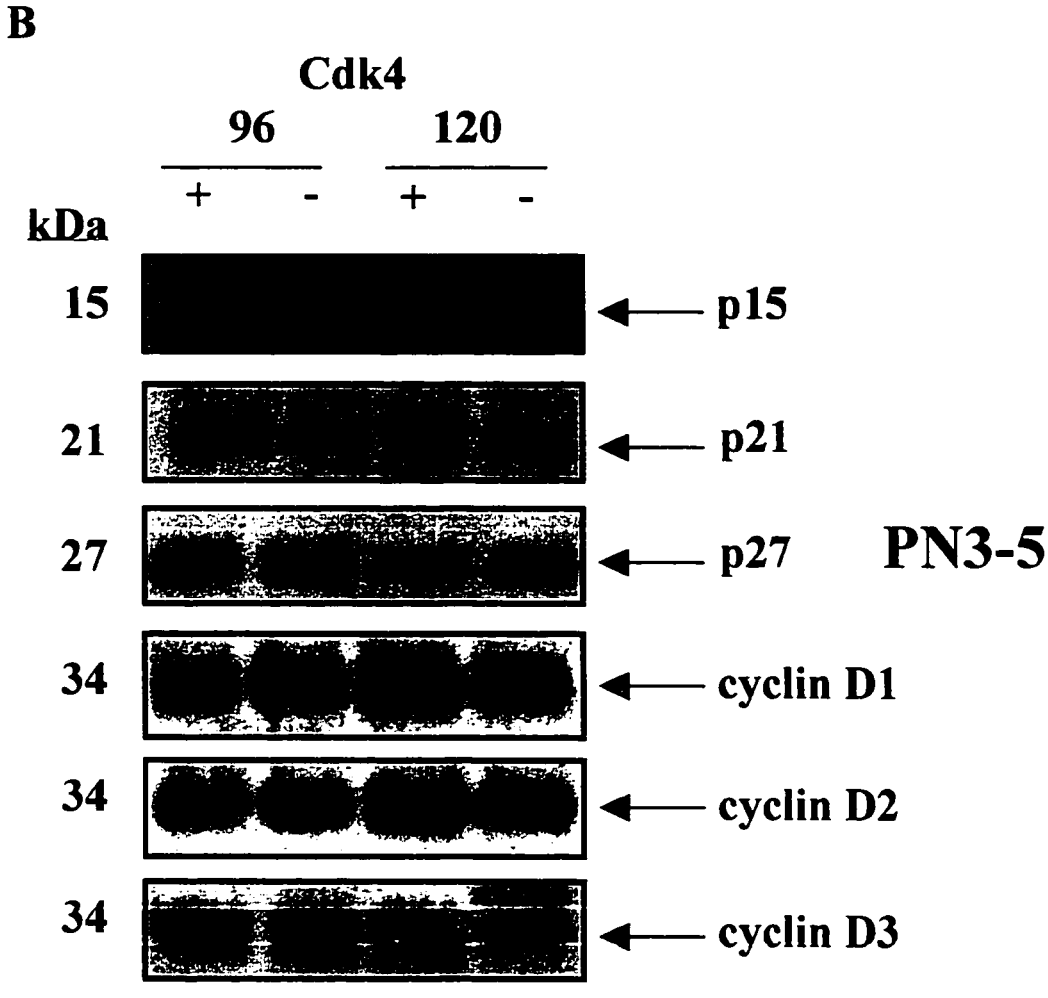


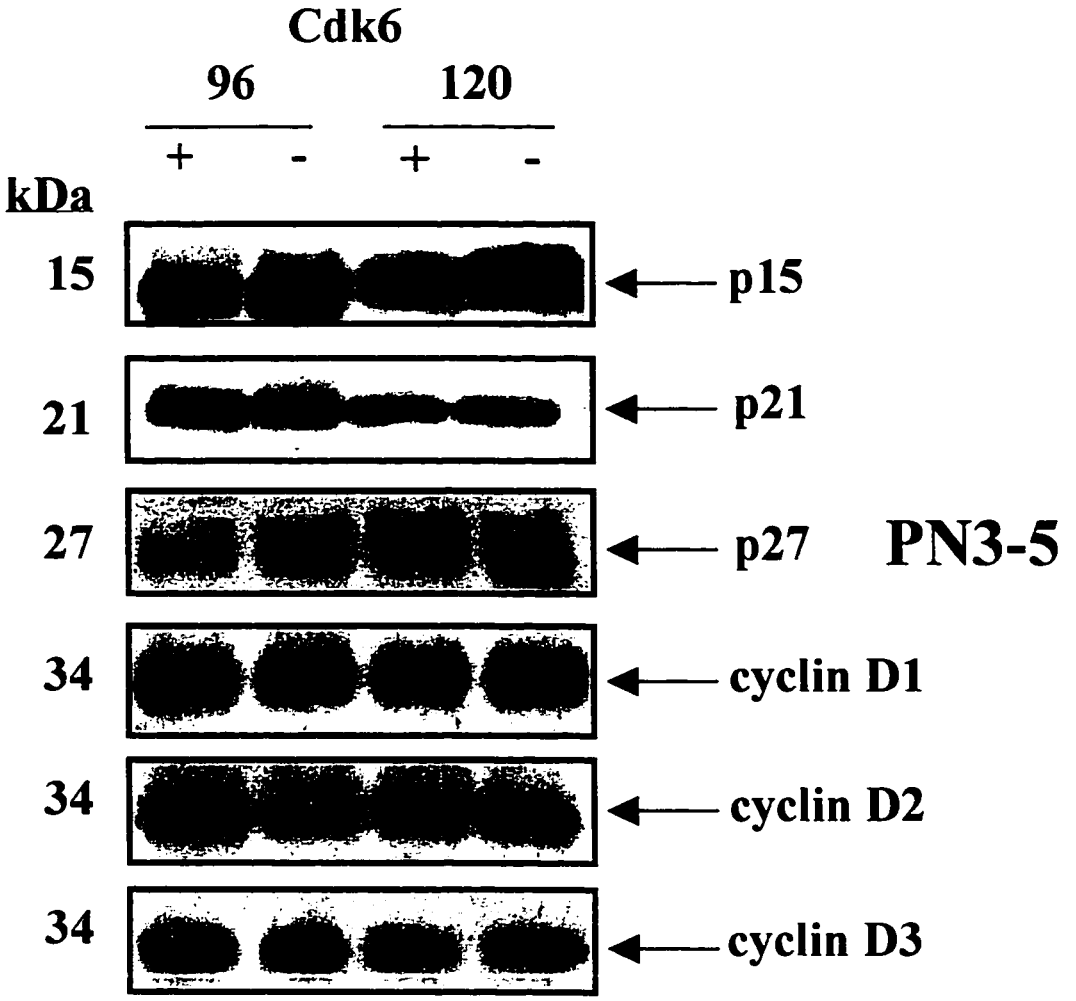
Fig. 9 Cyclin, Cdk, and CKI proteins and their complexes following treatment with 80 pM TGF-β1 in PN cells

A: Asynchronized PN cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-Cdk2 antibody. This was followed by Western blot analysis with either anti-p21, 27, or cyclin E antibody.



B: Asynchronized PN cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-Cdk4 antibody. This was followed by Western blot analysis with either anti-p15, p21, 27, cyclin D1, D2, or D3 antibody.

C



C: Asynchronized PN cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-Cdk6 antibody. This was followed by Western blot analysis with either anti-p15, p21, 27, cyclin D1, D2, or D3 antibody.

In PC3 cells, there was an increase in association of p21 bound to Cdk2 in the presence of 80 pM TGF-β1 at time points indicated (Fig. 10A and B). The 2 bands of equal intensity for p21 associated with Cdk2 could be a result of phosphorylation on p21 (Fig. 10A). The amount of p27 and cyclin E bound to Cdk2 did not increase regardless of dose or time of treatment (Fig. 10A and B).

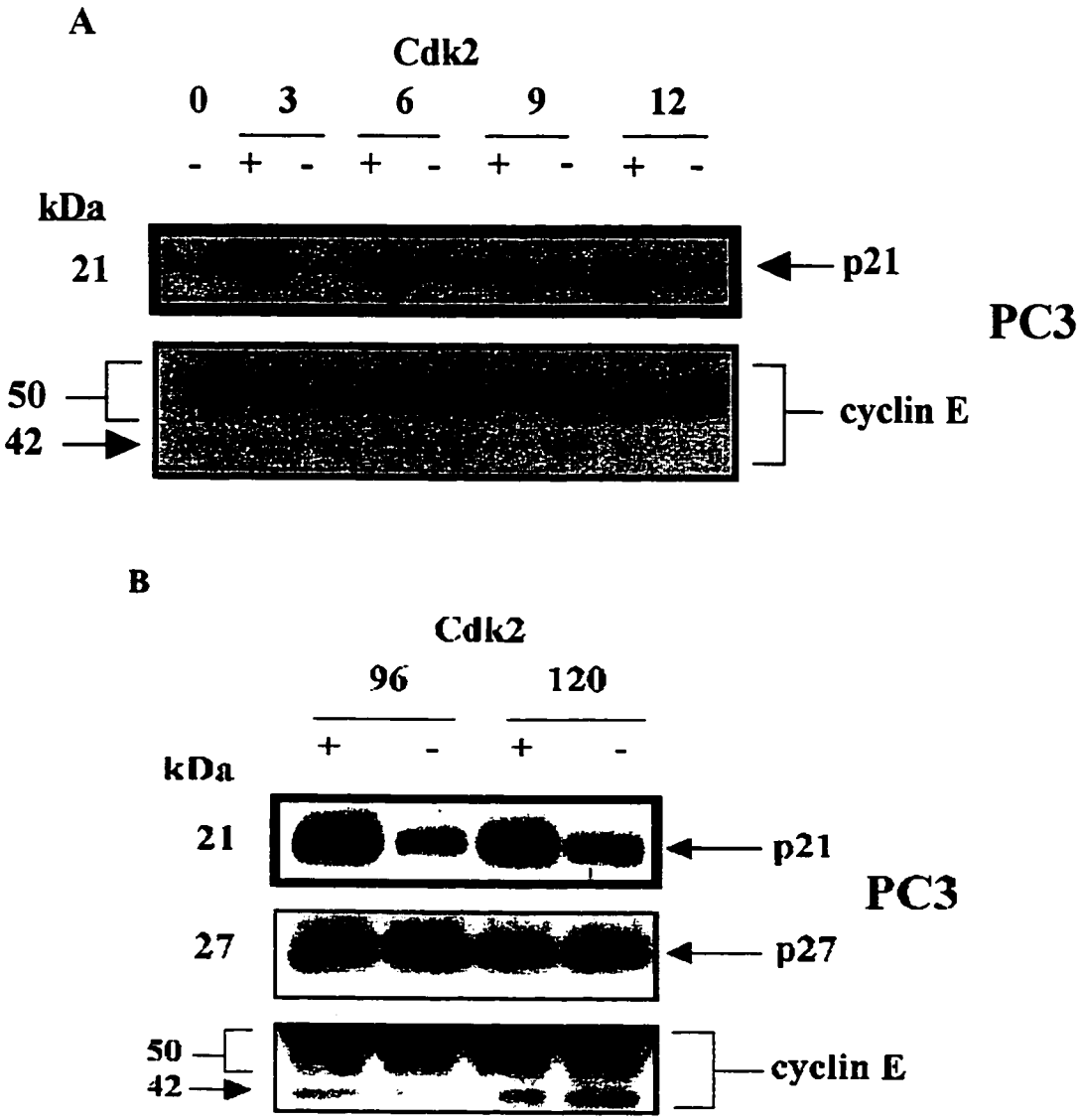


Fig. 10 Cyclin, Cdk2, and CKI proteins and their complexes following treatment with 80 pM TGF-β1 in PC3 cells

Asynchronized PC3 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 0, 3, 6, 9, and 12 hours (A) or 96 and 120 hours (B), and immunoprecipitated with anti-Cdk2 antibody. This was followed by Western blot analysis with either anti-p21 or anti-cyclin E antibody.

Immunoprecipitates of Cdk4 (Fig. 11A and B) from TGF-β1 treated PC3 cells from 3 to 120 hours revealed an increase in p21 bound to Cdk4. In these same cells, there was an increase in the amount of p21 bound to Cdk6 from 3 to 12 hours in the presence of TGF-β1 (Fig. 11C). However, no increase was observed at 96 and 120 hours (Fig. 11D). Furthermore, the amount of cyclin D1, D2, D3, and p15 bound to Cdk4 and 6 did not increase in the presence of 80 pM TGF-β1 (Fig. 11A, B, C, and D).

Since results were similar for short term (0 to 12 hours) and long term (96 and 120 hours) treatment, 96 and 120 hour time points were chosen for all subsequent experiments. These two time points also correlate with the maximal growth inhibition of normal cells by TGF-β1 shown previously in growth curve experiments. Interestingly, although our results show that there is an increase in association of p21 with Cdk2 and Cdk4 in PC3 cells in the presence of TGF-β1 (Fig. 10 and 11), no significant growth inhibition was observed (Fig. 2 and 3).

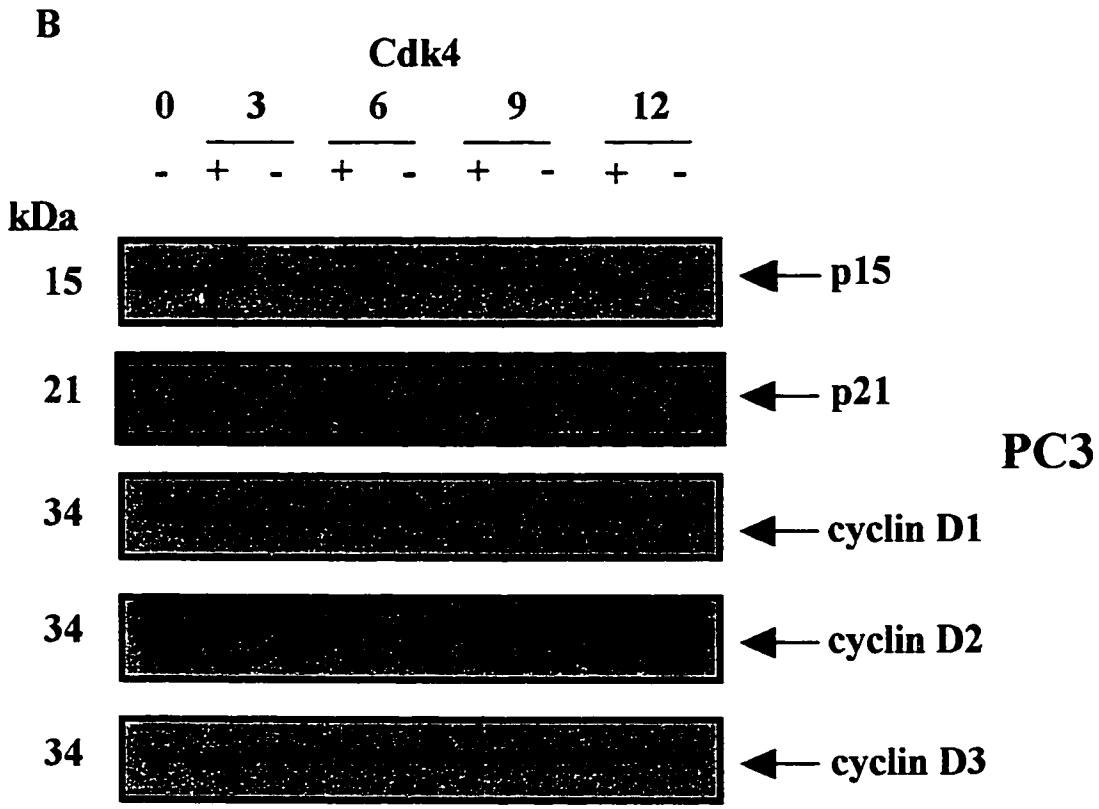
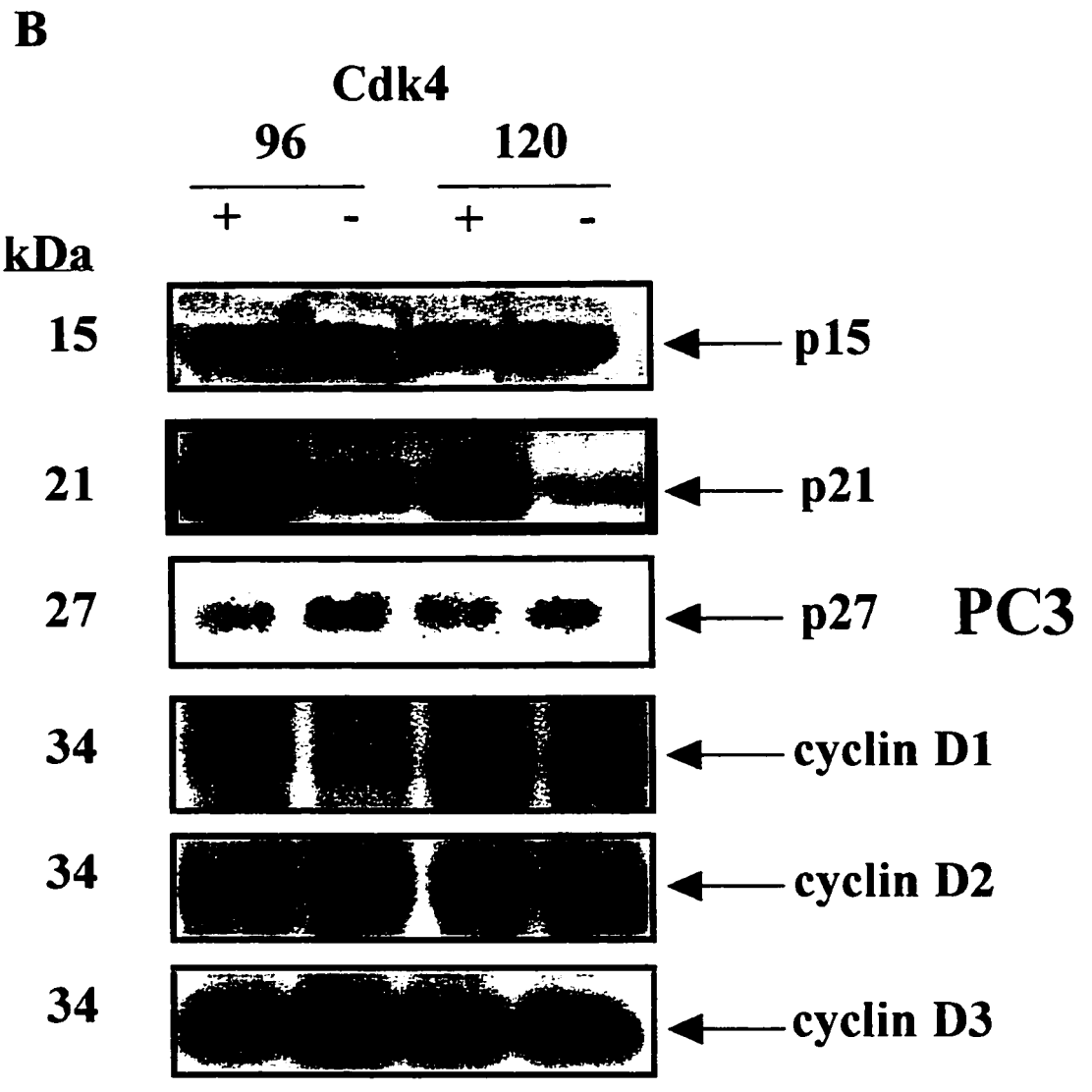
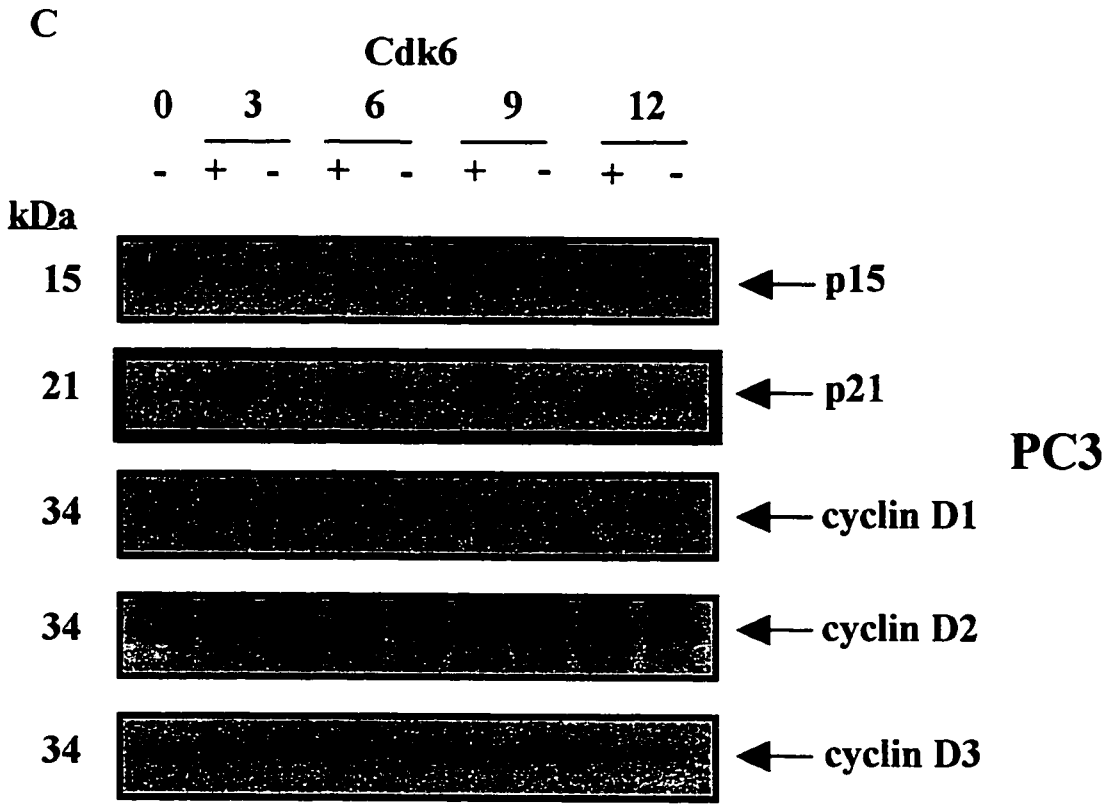


Fig. 11 Cyclin, Cdk4 and 6, and CKI proteins and their complexes following treatment with 80 pM TGF-β1 in PC3 cells

A: Asynchronized PC3 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 0, 3, 6, 9, and 12 hours, and immunoprecipitated with anti-Cdk4 antibody, followed by Western blot analysis with either anti-p15, p21, cyclin D1, D2, or D3 antibody.

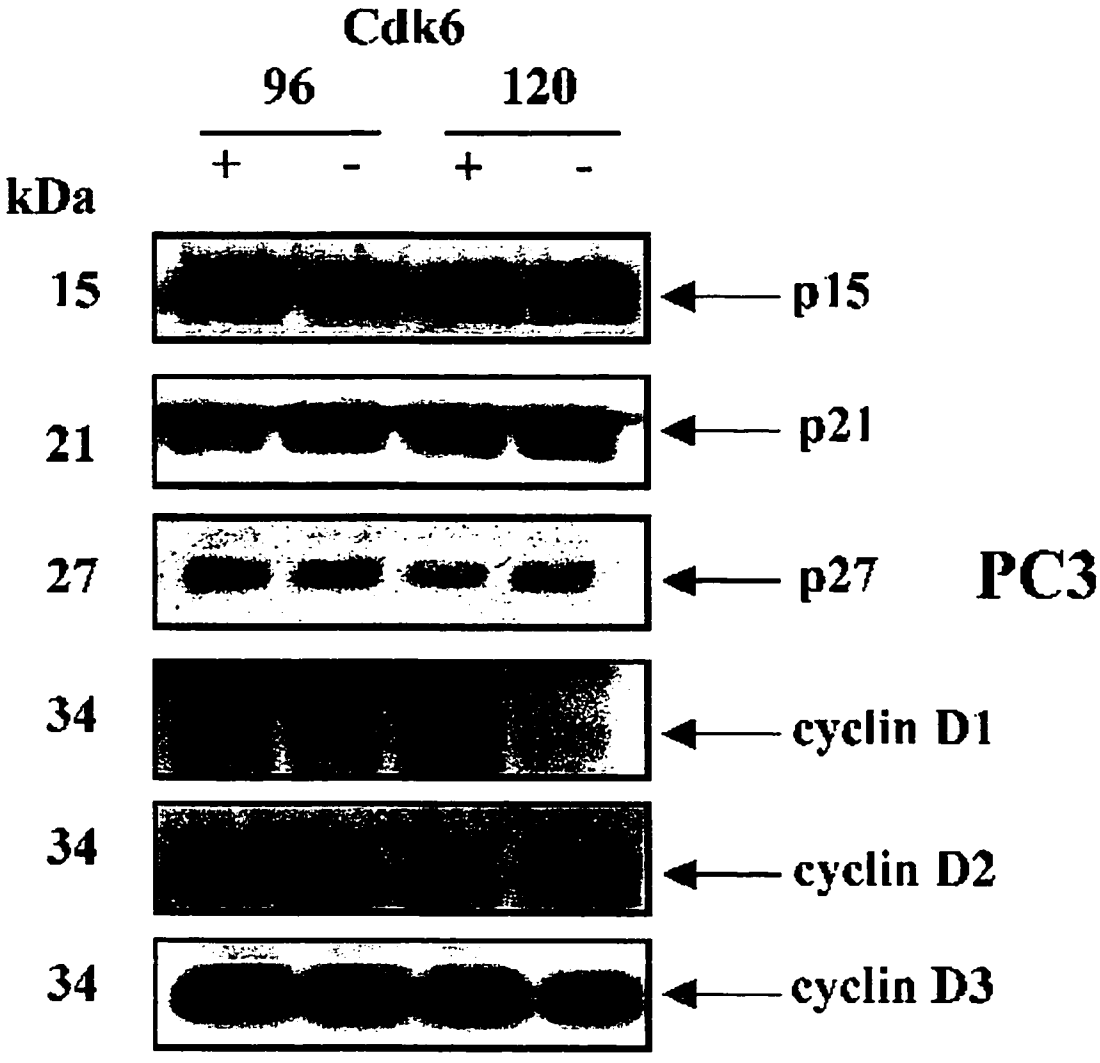


B: Asynchronized PC3 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-Cdk4 antibody, followed by Western blot analysis with either anti-p15, p21, 27, cyclin D1, D2, or D3 antibody.



C: Asynchronized PC3 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 0, 3, 6, 9, and 12 hours, and immunoprecipitated with anti-Cdk6 antibody, followed by Western blot analysis with either anti-p15, p21, cyclin D1, cyclin D2, or cyclin D3 antibody.

D



D: Asynchronized PC3 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-Cdk6 antibody, followed by Western blot analysis with either anti-p15, p21, 27, cyclin D1, D2, or D3 antibody.

Similar results were obtained for DU145 cells. The amount of p21 bound to Cdk2 increased in the presence of 80 pM TGF-β1 in DU145 cells (Fig. 12A). However, the amount of cyclin E bound to Cdk2 did not increase regardless of treatment (Fig. 12A). In addition, the amount of cyclin D1, D2, D3, p15, p21, and p27 bound to Cdk4 and 6 did not increase in the presence of 80 pM TGF-β1 (Fig. 12B and C). Similar to PC3 cells, increase in association of p21 with Cdk2 in DU145 cells in the presence of TGF-β1 did not correlate with our growth curve results (Fig. 2 and 3).

The conclusions from these sets of experiments include an increase in p21 association with Cdk2 in PN and DU145 cells at 96 and 120 hours. Also, in PC3 cells there was an increase in bound complex of p21 with Cdk2 and Cdk4 from 0 to 120 hours and Cdk6 from 0 to 12 hours. In contrast, there was no increase in association of p15, p27, cyclins D1, D2, D3, or E with Cdk2, 4, or 6.

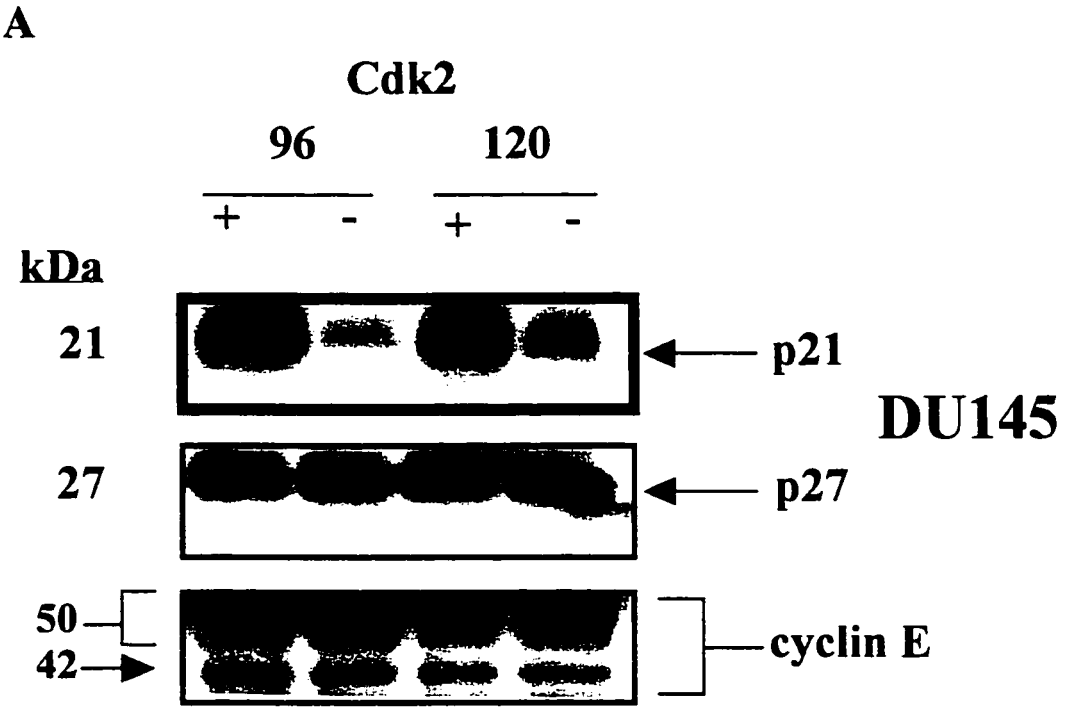
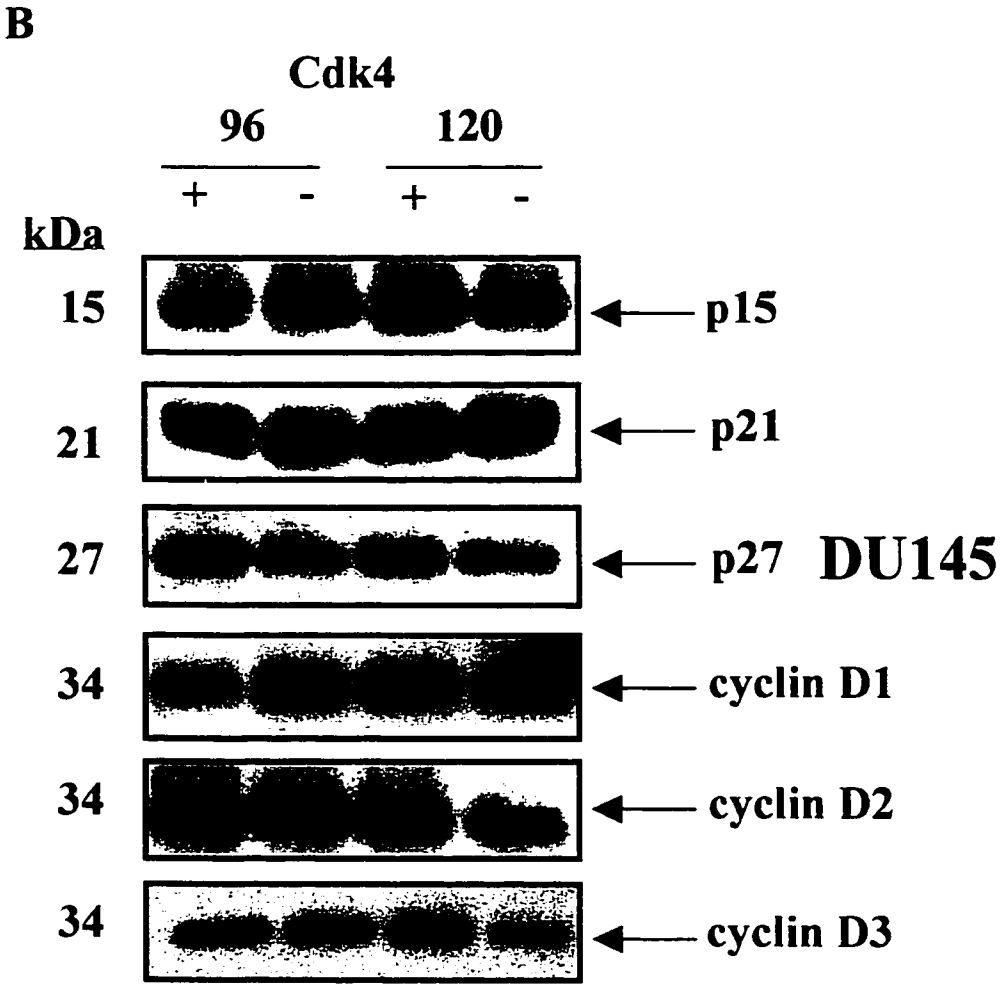


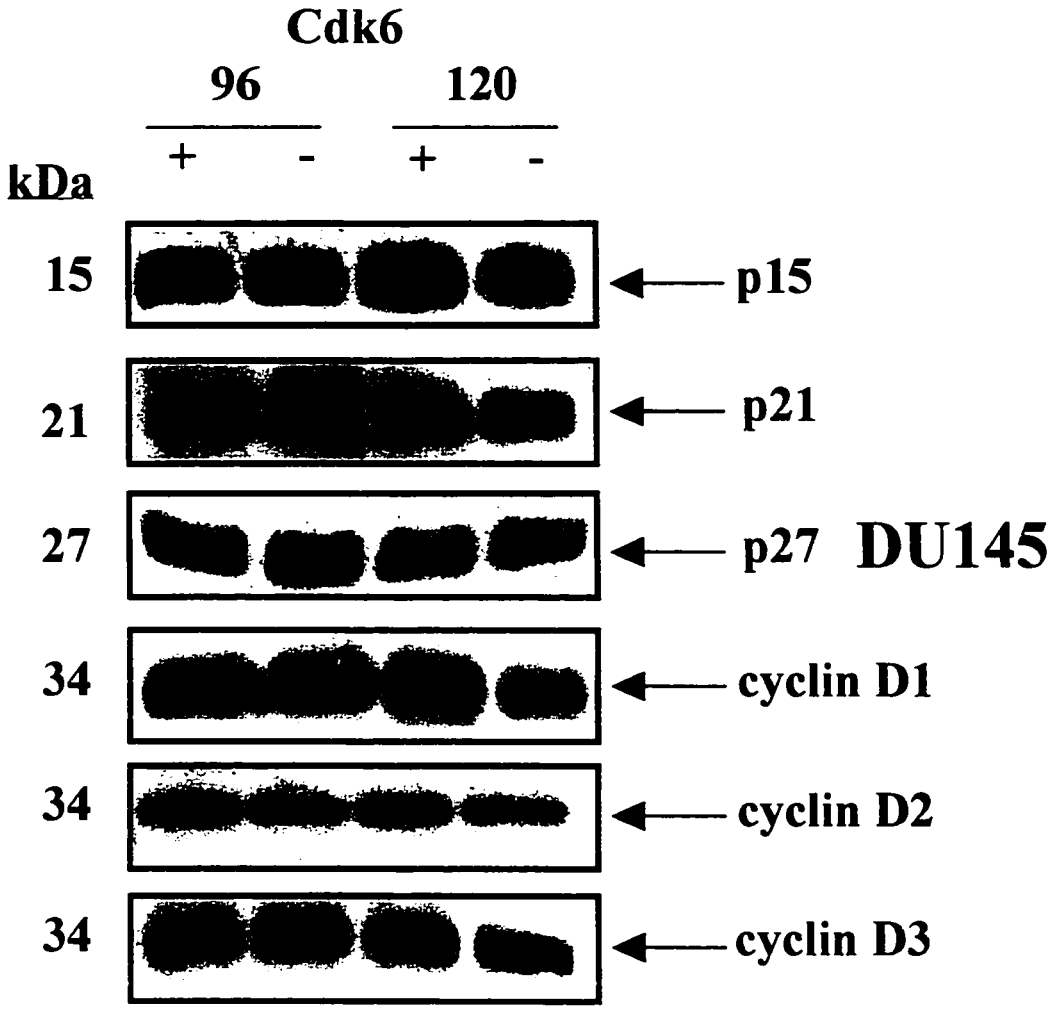
Fig. 12 Cyclin, Cdk, and CKI proteins and their complexes following treatment with 80 pM TGF-β1 in DU145 cells

A: Asynchronized DU145 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-Cdk2 antibody, followed by Western blot analysis with either anti-p21, 27, or cyclin E antibody.



B: Asynchronized DU145 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-Cdk4 antibody, followed by Western blot analysis with either anti-p15, p21, 27, cyclin D1, D2, or D3 antibody.

C



C: Asynchronized DU145 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-Cdk6 antibody, followed by Western blot analysis with either anti-p15, p21, 27, cyclin D1, D2, or D3 antibody.

Effect of TGF-β1 treatment on G_1 Cdk kinase activity

Binding of cyclin E to Cdk2 or cyclin D1, D2, and D3 to Cdk4 or Cdk6 results in an increase in the respective Cdk kinase activity leading to G_1 to S phase transition. Cdk inhibitors such as p15, p21, or p27 can bind to specific cyclin-Cdk complexes inhibiting kinase activity and thus cell proliferation. While we have previously determined the levels of cell cycle protein-protein complex formations, importantly we wanted to determine the resultant kinase activity of these complexes. To further define this functional role of TGF-β1, immunoprecipitation-kinase assays were performed.

Following treatment with 80 pM TGF-β1, the Cdk2 activity was inhibited in asynchronized PN cells (Fig. 13A). This result correlates with our previous data which showed an increase in the amount of Cdk inhibitor (p21) bound to Cdk2 after treatment with TGF-β1 (Fig. 9A). This increase in p21 as expected decreased Cdk2 activity. Similarly, Cdk2 activity was inhibited in asynchronized HaCaT cells in the presence of TGF-β1 (data not shown). Cdk4 and Cdk6 activity remained unchanged regardless of treatment in PN6 cells respectively (Fig. 13B and C). This was expected since there was no increase in the amount of p21, p15, or p27 bound to Cdk4 or Cdk6 in the presence of TGF-β1 (Fig. 9B and C).

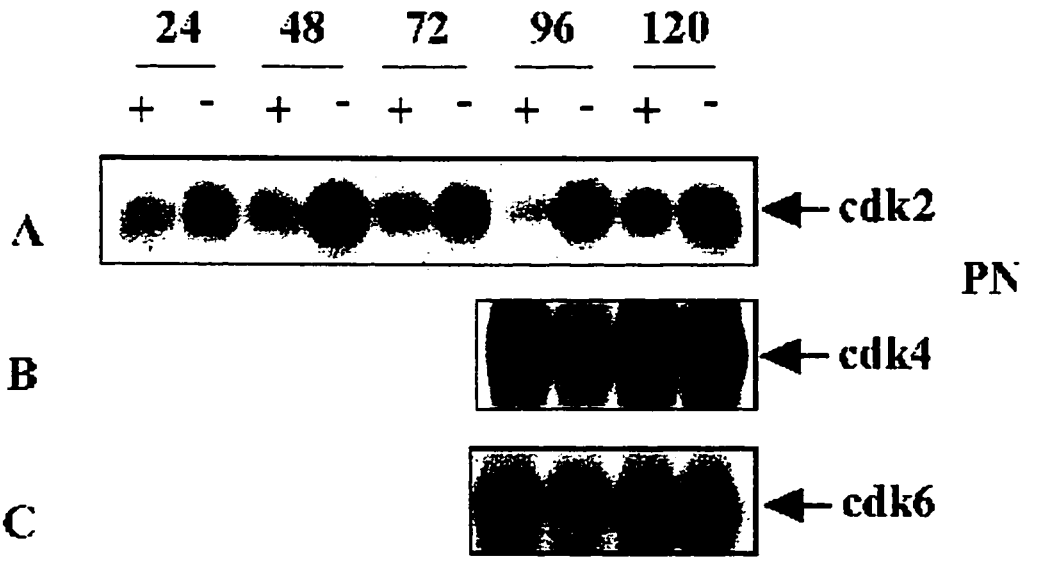


Fig. 13 Effect of TGFβ1 on G_1 Cdk kinase activity in PN cells
Asynchronized PN6 cells were either not treated (-) or treated (+) with 80 pM TGF-β1 and collected after 24, 48, 72, 96, or 120 hours. Cell lysate was immunoprecipitated with anti-Cdk2 antibody and used for kinase activity assay. (A) anti-Cdk2; (B) anti-Cdk4; (C) anti-Cdk6 antibodies

In the presence of TGF-β1, Cdk2 activity in DU145 cells was minimally inhibited (Fig. 14A). This correlates with our previous results which showed that DU145 cells were minimally growth inhibited by TGF-β1 (Fig. 2 and 3) and slightly more responsive than PC3 cells to inhibition of growth. Since no increase in p21, p15, or p27 bound to Cdk4 or 6 following treatment with TGF-β1 was observed (Fig. 12B and C), Cdk4 and 6 activity remained unaffected (Fig. 14B and C).

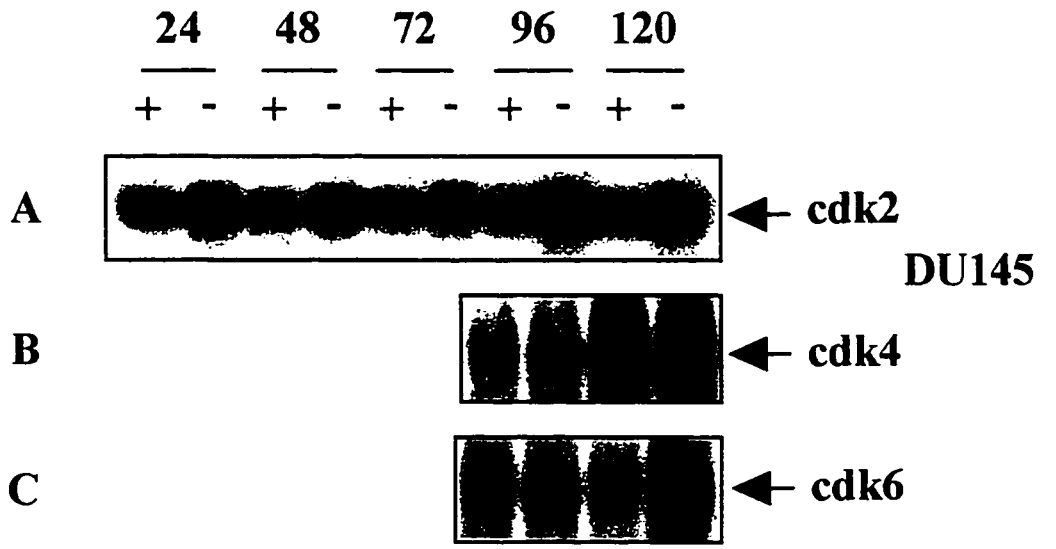


Fig. 14 Effect of TGFβ-1 on G_1 Cdk kinase activity in DU145 cells
Asynchronized DU145 cells were either not treated (-) or treated (+) with 80 pM TGF-β1 and collected after 24, 48, 72, 96, or 120 hours. Cell lysate was immunoprecipitated with anti-Cdk2 antibody and used for kinase activity assay. (A) anti-Cdk2; (B) anti-Cdk4; (C) anti-Cdk6 antibodies

Following treatment with 80 pM TGF-β1, Cdk2 activity was not inhibited in PC3 cells regardless of dose or time of treatment (Fig. 15). This was unexpected as previously we showed an increase in p21-bound Cdk2 in these cells (Fig. 10A and B). However, this lack of Cdk2 inhibition in the presence of TGF-β1 may partially explain PC3 cells lack of growth inhibition (Fig. 2 and 3). The activity of Cdk4 and Cdk6 remained unchanged in PC3 cells regardless of the time of treatment (Fig. 15). These kinase activity results were quite unexpected as previously we showed an increase in association of p21 with Cdk4 (Fig. 11 A and B).

In summary, a significant increase in the level of Cdk2-bound p21 proteins was seen in TGF-β treated PC3 and DU145 cells. Yet, no inhibition or a minimal inhibition of Cdk2 activity were detected. This may explain these cells lack of responsiveness to TGF-β1.

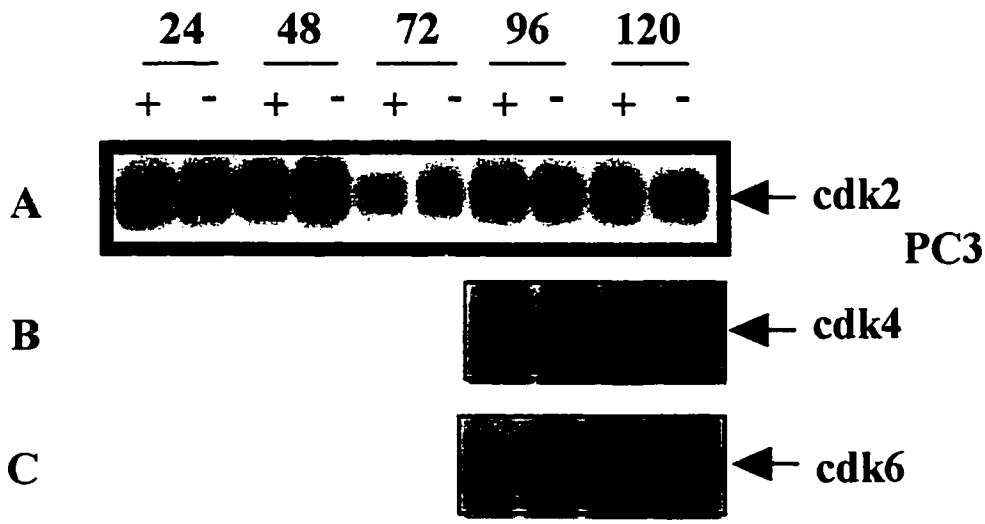


Fig. 15 Effect of TGFβ1 on G_1 Cdk kinase activity in PC3 cells
Asynchronized PC3 cells were either not treated (-) or treated (+) with 80 pM TGF-β1 and collected after 24, 48, 72, 96, or 120 hours. Cell lysate was immunoprecipitated with anti-Cdk2 antibody (A) anti-Cdk4 antibody (B), or anti-Cdk6 antibodies (C) and used for kinase activity assay.

We asked whether the Cdk2-cyclin complexes alone may be present in excess in prostate tumor cells. An elevated level of total Cdk2-cyclin E complexes in comparison to p21 may explain the inability of p21 to inhibit Cdk2 kinase activity. To determine if increased levels of Cdk2, 4, or 6 bound to their respective complexes can explain the lack of inhibition of Cdk kinase activity, immunoprecipitation experiments were performed. Our results showed no increase in association of Cdk4 or 6 with cyclin D3 in DU145, PC3, nor PN cells (Fig. 16A, B, and C). In addition, no increase in association of Cdk2 with cyclin E was observed in all cells tested (Fig. 17A, B, and C).

Therefore, the lack of inhibition of Cdk2, 4, and 6 activity in PC3 cells in the presence of TGF-β1 appears not to be explained by an increase in association of Cdks with cyclin/CKI complexes. This lack of inhibition of kinase activity may explain the lack of growth inhibition in tumor cells. We proposed three possible mechanisms to explain this lack of responsiveness to TGF-β1 in prostate tumor cells.

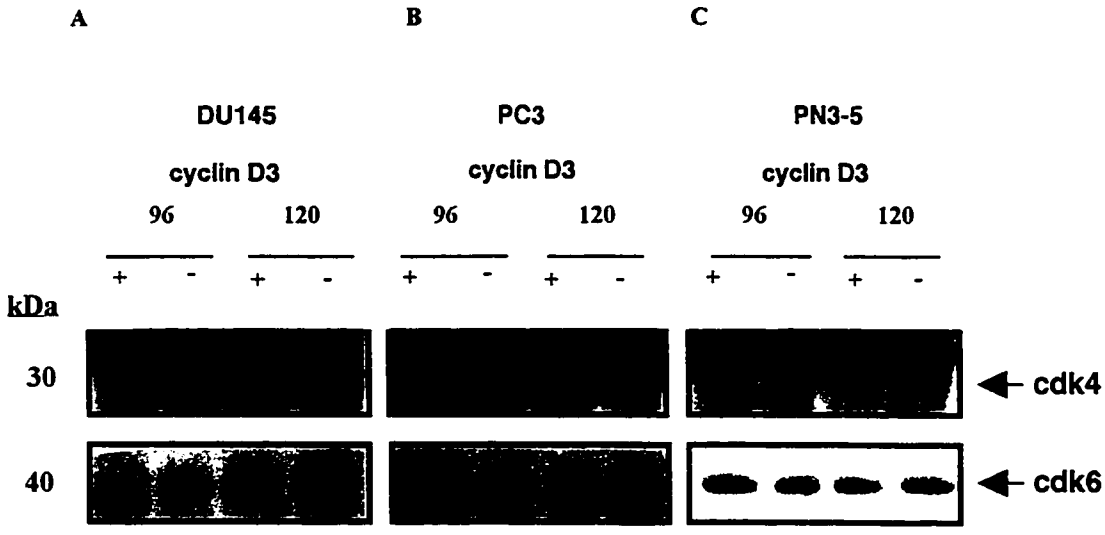


Fig. 16 Cyclin D3 complex formation with Cdk4 and Cdk6 following treatment with 80 pM TGF-β1 in (A) DU145, (B) PC3, and (C) PN cells.
Asynchronized DU145 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-cyclin D3 antibody, followed by Western blot analysis with either anti-Cdk4 or anti-Cdk6 antibody.

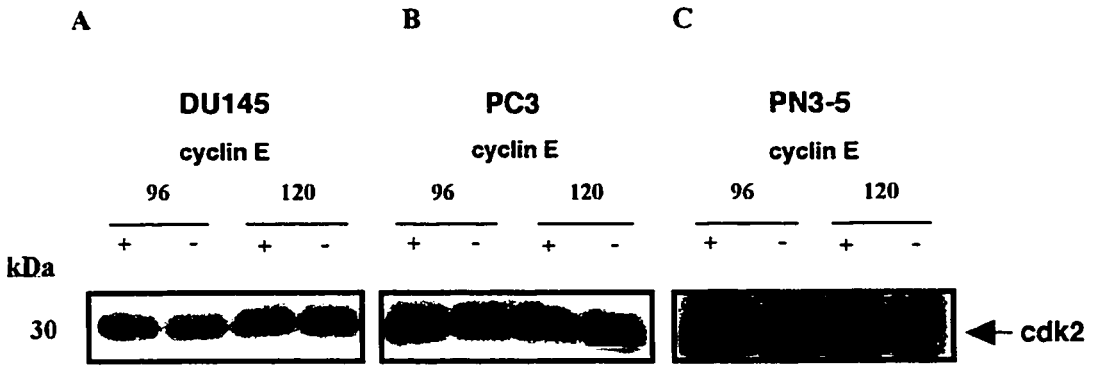


Fig. 17 Cyclin E and Cdk2 complex formation following treatment with 80 pM TGF-β1 in (A) DU145, (B) PC3, and (C) PN cells.
Asynchronized DU145 cells were either not treated (-) or treated (+) with 80 pM TGF-β1, collected after 96 and 120 hours, and immunoprecipitated with anti-cyclin E antibody, followed by Western blot analysis with either anti-Cdk2 antibody.

Metabolic Labeling

Despite increased levels of association of p21 with Cdk2 in the presence of TGF-β1 in PC3 prostate tumor cells, Cdk2 kinase activity remains unaffected. One possible mechanism is that an as yet unidentified protein, may bind to p21 in the presence of TGF-β1 preventing it from inhibiting Cdk2 kinase activity. To determine if such a protein exists, metabolic labeling and immunoprecipitation experiments were performed. If this protein was induced by TGF-β1, we would have expected to detect an additional band in PC3 cells plus treatment lanes. However, our results showed that no obvious additional protein was found to be bound to the Cdk2/cyclin E/p21 complex in PC3 or PN cells (Fig. 18A and B). One possible reason for lack of detection is if the protein synthesis rate was slow it may not have been detectable within the 24 hour labeling period.

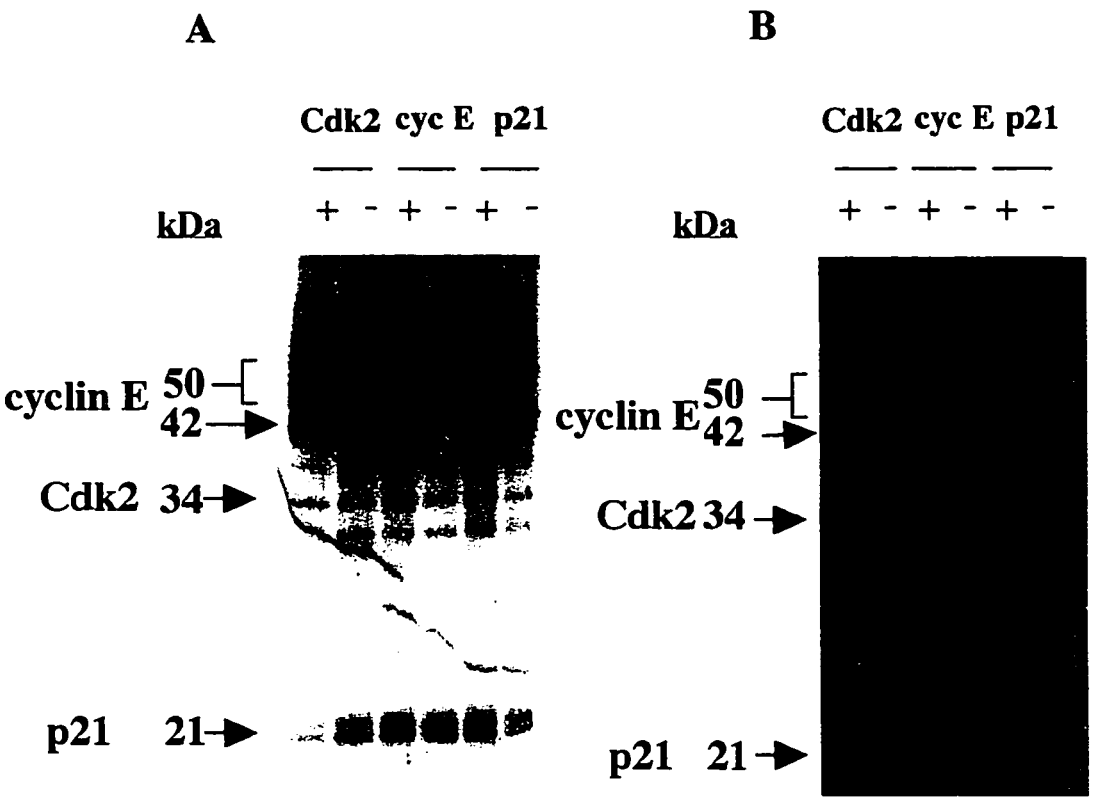


Fig. 18 Metabolic labeling to identify proteins complexed with Cdk2, cyclin E, and p21 in PN7-8 (A) and PC3 (B) cells. Asynchronized cells were either not treated (-) or treated (+) with 80 pM TGF-β1 and in the presence of ^{35}S-methionine,cysteine, collected after 24 hours, and immunoprecipitated with anti-Cdk2, cyclin E, or p21 antibody.

Analysis of p21 for mutations

Another possible mechanism which might explain PC3 tumor cells insensitivity to TGF-β1 is a mutation of p21. A mutation could render this protein non-functional. So, while the level of p21 protein associated with Cdk2 remain elevated in PC3 cells, a mutant p21 may not be able to inhibit Cdk2 kinase activity. To determine whether such a mutation exists in p21, we performed reverse-transcription/polymerase chain reaction followed by sequencing. Our results showed that PC3 cells contain the wild-type p21.

Analysis of Cdk2 for mutations

An as yet additional possible mechanism is a mutation in Cdk2 in PC3 cells which would render this protein constitutively active. This may result in continuous cell proliferation regardless of the amount of p21 present to bind to the Cdk-cyclin complex. If such a mutation existed, we would have expected it to be present in the catalytic domain of Cdk2. Alternatively, a mutation in the p21 binding domain of Cdk2 may alter that site's function. To determine whether PC3 tumor cells contain a Cdk mutation, we performed reverse-transcription/polymerase chain reaction followed by sequencing. Our results indicated that PC3 cells contain the wild-type Cdk2.

DISCUSSION

Inhibition of cell proliferation by TGF-β results in growth arrest in the G_1 phase of the cell cycle (Massague, 1990; Roberts and Sporn, 1992; Lyons and Moses, 1990). This has been proposed to be mediated by inhibition of G_1 Cdk activity (Sherr, 1993; Koff et al., 1993; Geng and Weinberg, 1993). In the present study, the effects of TGF-β1 on growth of prostate normal and carcinoma cells were determined. Normal prostate epithelial cells (PN) were found to be growth inhibited by of TGF-β1, while prostate tumor cells DU145, PC3, and LNCaP were not or minimally inhibited. The degree of inhibition of PN cells was comparable to those of HaCaT cells, a known TGF-β sensitive cell line (Hannon and Beach, 1994). To the best of our knowledge, this is the first study demonstrating that normal prostate epithelial cells indeed respond to growth inhibition by TGF-β1. The insensitivity of DU145 and PC3 cells to dosages of 20 pM and higher TGF-β has also been noted by other groups (Wilding et al., 1989; Franzen et al., 1993).

There are multiple points along the TGF-β signaling pathway in which aberrant regulation could ultimately lead to lack of cell cycle arrest. Access of TGF-β ligand to TGF-β receptors or (a) defect(s) in TGF-β cell membrane receptors are among them. The LNCaP cell line has a defective type I TGF-β receptor which explains its lack of response to growth inhibition (Kim et al., 1996). However, no mutations have been identified regarding the type I or type II TGF-β receptors in PC3 and DU145 cell lines (Wilding et al., 1989). Thus, the lack of responsiveness to TGF-β1 in these two cell lines is likely not due to loss or mutation at the receptor level.

Another possible point of aberration is in the signal transducers. Recently, a group of signal transduction proteins called Smad has been identified and shown to be

components of cell signaling pathways. In particular, Smad2, Smad3 and Smad4 have been shown to be involved in TGF-β signaling. Loss of Smad4 expression has been identified in a variety of TGF-β resistant cancer cells. In addition transfection of Smad4 into these cells restored their responsiveness to TGF-β (Zhang et al., 1996). We investigated the possibility whether loss of expression of Smad2, Smad3, and/or Smad4 in prostate tumor cells may explain their lack of response to TGF-β1. Expression of Smad2, 3, and 4 were determined by Northern blotting in PN, PC3, and DU145 cells. Our results showed no decrease in mRNA expression of these three Smads in prostate tumor cell lines PC3 and DU145 compared to that in PN cells (data not shown). Therefore, we directed our attention to regulatory components that are further downstream in the TGF-β1 signaling pathway.

Cyclin-dependent kinase inhibitors (CKIs) are a family of proteins which bind to Cdks and inhibit kinas activity. These CKIs, i.e. p15, p21, and p27 are known to be induced by TGF-β. Induction of these CKIs by TGF-β has been observed in many normal cell types. For example, p15 protein level was found to be induced 10-30 fold following treatment with TGF-β of exponentially growing HaCaT keratinocytes (Hannon and Beach, 1994) and Mv1Lu mink lung epithelial cells (Reynisdottir et al., 1995). In HaCaT cells, p21 was also induced by TGF-β1 (Reynisdottir et al., 1995). TGF-β treatment induced the association of p27 with Cdk/cyclin complexes (Polyak et al., 1994). However, our results indicated that TGF-β1 treatment did not result in induction of p15 and p27 in human prostate cells.

One mode of gene inactivation is aberrant methylation. This has been shown to be associated with transcriptional loss in certain tumor suppressor genes (Herman et al.,

1995). Although it is known that p15 is frequently methylated in certain tumor cells types (Piacibello et al., 1991), the p15 gene does not appear to be methylated in DU145 and PC3 cells (Herman et al., 1996). Our own results showed that treatment of these cells with a methylation inhibitor, 5-aza-2′-deoxycytidine, did not affect the level of p15 transcription.

Unexpectedly, TGF-β1 treatment did not induce p21 in normal prostate cells but strongly induced p21 in both prostate tumor cell lines DU145 and PC3. These results suggest that the TGF-β signaling pathway is, at least partially, functional in DU145 and PC3 cells. Normal prostate epithelial cells have a relatively high basal level of p21. Although TGF-β1 treatment did not increase the whole level of the p21 protein in PN cells, it did increase the association of p21 with Cdk2/cyclin E complexes. DU145 and PC3 cells have a mutant p53 (Chen et al., 1994) which may explain the relatively low basal level of p21. TGF-β1 treatment increased the expression of p21 and the association of the p21 protein with the Cdk2/cyclin E complexes in DU145 and PC3 cells. Therefore, it appears that TGF-β regulates the expression and interaction of the p21 protein in prostate cells.

Since the regulation of the G_1 to S phase transition involves multiple G_1 cycle regulators, the functional protein-protein complex formation between all known G_1 Cdks, cyclins, and CKIs were studied by Immunoprecipitation-Western analysis. No significant alterations were seen in the level of cyclin E, D1, D2, D3, p15 and p27 associated with Cdk2, 4, and 6 in the presence of TGF-β1. However, TGF-β treatment increased association of the p21 protein with Cdk2/cyclin E complex in PN, DU145, and PC3 cells, as well as, Cdk4/cyclin D in PC3 cells. Thus, p21 appears to be a primary target of TGF-

β in human prostate cells.

Association of CKIs with cyclin/Cdk complexes inhibits Cdk kinase activity and hence cell proliferation. TGF-β1 treatment did not inhibit Cdk4 or Cdk6 kinase activity in PN, DU145, and PC3 cells. These results correlate with the findings that TGF-β1 treatment did not result in induction of p15 protein or increase association of p15 with Cdk4 and Cdk6 in PN, DU145, and PC3 cells. Cdk2 kinase activity was inhibited by TGF-β1 in PN cells. This correlated with our Immunoprecipitation-Western data showing an increase in association of p21 with the Cdk2/cyclin E complex and growth curve inhibition. However, Cdk2 kinase activity was not inhibited in PC3 cells and only minimally inhibited in DU145 cells despite an increased association of p21 with Cdk2/cyclin E upon TGF-β1 treatment. To the best of our knowledge, we are the first to describe a Cdk cell cycle de-regulation in TGF-β signaling in prostate tumor cells.

Prostate carcinoma cell line DU145 contains a point mutation that results in mutated pRB which lacks 35 amino acids (Bookstein et al., 1990). Analysis of seven primary prostate carcinomas revealed absence or reduced expression of the RB protein product in two of the seven carcinomas (Bookstein et al., 1990). Another study showed allele losses of RB in 21 of 43 patients with primary prostate cancer (Brooks et al., 1995). However, in PC3 and LNCaP cell lines there was no evidence of RB inactivation (Bookstein et al., 1990).

Currently, the mechanism for the lack of Cdk2 inhibition in tumor cells is not clear. There are several possibilities. Upon treatment with TGF-β1, there may be an as yet unidentified protein bound to the Cdk/cyclin complex in tumor cells. This protein may prevent p21 from inhibiting Cdk kinase activity and therefore cell proliferation.

This protein may be constitutively expressed but not induced. In this case, protein turnover rate may not be high enough to be detected by metabolic labeling within 24 hours. This may explain why we were unable to detect an additional protein. Alternatively, this protein could be induced upon TGF-β1 treatment. In this scenario, we would have expected to identify an additional protein only in PC3 cells treated with TGF-β1. This protein would bind to p21 in the presence of TGF-β1 and prevent p21 from inhibiting Cdk2 activity. To investigate this possibility, metabolic labeling and immunoprecipitation were performed. Our results showed that no obvious additional protein was identified in PC3 cells with or without TGF-β1 treatment.

A second possibility is the presence of a mutation on Cdk2 which renders this kinase constitutively active regardless of the presence of CKIs. The p21 gene could also be mutated resulting in a non-functional protein. Indeed we have found p21 mutations in human prostate tumor tissues (Gao et al., 1995). To determine whether PC3 cells possess either of these mutations, reverse-transcription/polymerase chain reaction followed by sequencing were performed. Our results showed that no p21 or Cdk2 mutation was detected in PC3 prostate tumor cells.

A third possibility involves a stoichiometric problem. p21 containing enzymes have been shown to transit between inactive and active states, probably through changes in the stoichiometry of the p21 subunit. In vitro evidence suggests that inhibition of Cdk-cyclin complex was achieved only after p21 levels reached a saturation point (Zhang et al., 1994). This same study showed that binding of more than one p21 subunit is necessary to inhibit Cdk activity and therefore cell proliferation. This poses the possibility that in prostate tumor cells there may be an insufficient number of p21

subunits bound to the Cdk2-cyclin complex. Yet, this has never been confirmed in vivo. Also, we showed that TGF-β1 treatment resulted in increased association of p21 with Cdk2 in normal and prostate tumor cells while Cdk2 and cyclin E levels remained unchanged. Therefore, this is probably an unlikely mechanism.

TGF-β has been shown to play a role in a number of biological processes such as stimulation of angiogensis and elevated expression in morphogenesis. In addition, sensitivity of prostate cancer cells to TGF-β diminishes as cells progress to a less differentiated state. Each isoform (1-5) of TGF-β is preferentially expressed in certain tissue and cell types. For example, β1 is primarily found in platelets and bone. In order to understand the role TGF-β may play in vivo, it would be important to consider each isoform individually in relation to its expression and function in certain tissues. One technique which has been successfully used to study the in vivo effects of TGF-β are gene knockouts. Specifically TGF-β1 knockout mice were born healthy with no obvious abnormalities. However, three to four weeks after birth, the mice died from a diffuse inflammatory syndrome accompanied by massive infiltrates of mononuclear cells (Shull, M.M. et al., 1992).

TGF-β has been shown to be an important mediator of information that enables cells and tissues to make appropriate responses to cell injury (Sporn, M. B. and Roberts, A. B., 1992). TGF-β seems to be highly expressed in many tumor tissues which may indicate its role in stimulating angiogensis of tumor tissue. Clearly, persistent over expression or dysregulation of TGF-β in vivo can elicit proliferative disease such as pulmonary fibrosis (Sporn, M. B. and Roberts, A. B., 1992). Insensitivity of prostate tumor cells to growth inhibition by TGF-β in vitro demonstrates a similar response.

Therefore, it is important to formulate an understanding of the complex role TGF-β signaling plays in regulating cell cycle proteins and their ability to induce cell cycle arrest.

In summary, we have shown that treatment with TGF-β1 in prostate tumor cells leads to elevated levels of p21 at the transcription and translation levels. TGF-β1 treatment increased association of p21 with Cdk2, yet no decrease in Cdk2 kinase activity occurred in PC3 cells. This lack of Cdk2 kinase inhibition may partially explain the lack of growth suppression by TGF-β1 in prostate tumor cells. These results will provide valuable insight into the role of cell cycle regulators in TGF-β downstream signaling in prostate cancer.

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ABSTRACT

MOLECULAR CELL CYCLE MECHANISMS OF HUMAN PROSTATE CARCINOMA INSENSITIVITY TO TGF-β

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TGF-β is a potent growth inhibitor of epithelial cells. However, many transformed cells have lost their sensitivity to this growth inhibitory effect. The molecular mechanism of such insensitivity is not yet understood. Here, we have studied the TGF-β1 effect on normal human prostate and carcinoma cells. Our results showed that normal cells were sensitive to growth inhibition, whereas tumor cells were not or only minimally inhibited regardless of the concentration of TGF-β1 (20 to 80 pM) or time of exposure (1-5 days). $p21^{WAF1/Cip1/Sdi1}$ and $p15^{INK4B}$ but not $p27^{KIP1}$ were detectable by Western blotting in normal and tumor cells. TGF-β1 treatment increased the association of $p21^{WAF1/Cip1/Sdi1}$ with the Cdk2/cyclin E complex in both normal and prostate tumor cells. However, there was no increase in the association of $p15^{INK4B}$ nor $p27^{Kip1}$ with the Cdk/cyclin complexes. In normal cells, the increase in the association of $p21^{WAF1/Cip1/Sdi1}$ with the Cdk2/cyclin E complex resulted in inhibition of the Cdk2 activity. In contrast, although there was an increase in the association of $p21^{WAF1/Cip1/Sdi1}$

with the Cdk2/cyclin E complex in tumor cells, there was no inhibition of the Cdk2 activity. These results indicate that a lack of inhibition of the Cdk2 activity correlates with the insensitivity to TGF-β1 in prostate tumor cells.

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