Overexpression of Transforming Growth Factor (TGF) β1 Type II Receptor Restores TGF-β1 Sensitivity and Signaling in Human Prostate Cancer Cells

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Abstract

TGF-β1 is a potent negative regulator of cell growth that transduces signals through interaction with type I and type II receptors that form a heteroduplex. Abnormal expression and mutational alterations of these receptors have recently been shown in several human malignancies. In previous studies, we have demonstrated reduced expression of both types of transforming growth factor (TGF) β1 receptors in human prostate tumors. In this study, using the human prostate cancer cell line, LNCaP, which is refractory to TGF-β1 and lacks type II receptor (R-II), we investigated whether overexpression of the R-II receptor can restore sensitivity to the negative growth effects of TGF-β1. LNCaP cells were transfected with plasmid containing the full length of human TGF-β R-II receptor cDNA sequence. Stable transfectant clones were selected for R-II mRNA and protein expression by Northern and Western analyses, respectively. The effect of TGF-β on LNCaP R-II overexpressing clones was examined on the basis of: (a) growth inhibition (cell number); (b) DNA synthesis using the [3H]thymidine incorporation assay; (c) induction of cyclin-dependent-kinase inhibitors, p21WAF1/CIP1, p27Kip1, and p15; and (d) colony-forming ability in soft agar. Both cell the number and the rate of DNA synthesis of R-II-overexpressing clones were significantly suppressed by exogenous TGF-β1 in a dose-dependent manner, compared with control cell lines. Treatment of R-II cloned transfectants with TGF-β1 induced a G1 arrest, which was accompanied by a transient increase in p21WAF1/CIP1 and p27Kip1 expression at the mRNA and protein levels. Furthermore, the LNCaP R-II transfectants analyzed exhibited a markedly reduced colony-forming ability.

Our results indicate that overexpression of TGF-β1 R-II receptor in LNCaP prostate cancer cells caused tumor growth inhibition by restoring the TGF-β signaling mechanism and TGF-β1 sensitivity.

Introduction

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer mortality in American males (1). Prostate cancer varies in its clinical aggressiveness. Histologically detected localized prostatic cancers are heterogeneous, with only a small subset having undergone all of the malignant changes required to produce clinically aggressive tumors (2). Therefore, because not all histological prostate cancers are universally destined to produce clinical disease, there is an immediate need to develop new diagnostic methods for the identification of prostatic tumors that will progress to a stage that requires immediate surgical/therapeutic intervention. Development of such methods requires the characterization of the molecular and cellular changes associated with prostatic tumorigenesis and progression to metastatic disease.

In the normal prostate, androgens exert their growth-regulatory mechanisms via growth factor signaling pathways, escape from which would ultimately lead to tumorigenic growth (3). TGFβ is a negative growth regulator that has been postulated to play a significant role in the control of normal prostate growth via its ability to inhibit cell proliferation and induce apoptosis (4, 5). TGF-β1 has multifunctional properties in regulating cell growth, differentiation and apoptosis, control of extracellular matrix formation, and immunosuppression (6) in different cell types. TGF-β1 generates these biological responses by interacting with two major cell surface receptors, type I (R-I) and type II (R-II), which are serine/threonine kinases (7, 8). The signal transduction cascade mediated by TGF-β1 is initiated by the binding of receptor R-II to the ligand, TGF-β1 (8). Once bound to TGF-β1 receptor R-II, TGF-β1 can recruit receptor R-I to form a ternary complex (9). The direct involvement of both TGF-β receptors in the TGF-β1 signal transduction pathway suggests that the loss of functional TGF-β receptors may contribute to the loss of growth-inhibitory activity of TGF-β.

An expanding body of evidence indicates that a dysfunctional TGF-β signaling mechanism, due to loss of TGF-β receptors, leads to the development and progression of several human malignancies, including breast cancer (10), T-cell
lymphomas (11), colon cancer (12), and head and neck squamous carcinoma (13). Recent studies from our laboratory (14), as well as by other investigators (15, 16), have demonstrated a significant decrease in the expression of both TGF-β R-I and R-II receptors in human prostate cancer and a correlation of this receptor loss with tumor progression. These observations implicate loss of TGF-β receptors as a potential underlying molecular mechanism via which prostate cancer cells escape the negative growth effects of TGF-β1.

In this study, we demonstrate that gene transfer-mediated overexpression of the TGF-β R-II receptor in prostate cancer cells LNCaP restored TGF-β sensitivity and reduced their tumorigenic behavior. These results indicate that LNCaP cells are refractory to TGF-β1 negative growth effects, due to the lack of R-II receptor expression.

Results

The spectrum of TGF-β1 sensitivity obtained for the four different human prostate cancer cell lines is shown in Fig. 1. The PC-3 and TSU-Prl cells were partially responsive, whereas DU-145 cells exhibited a weak sensitivity compared with the positive control cell line, Mv-1-Lu. LNCaP cells were totally resistant to the growth-inhibitory effect of TGF-β1, in accord with previous reports (17, 18).

To investigate whether the antiproliferative activity of TGF-β was directly associated with the presence of functional TGF-β R-I and/or R-II receptors, we examined the mRNA expression of the two TGF-β receptors in the four human prostate cancer cell lines by Northern hybridization and RT-PCR analysis. Fig. 2A is a Northern blot indicating that the prostate cancer cell lines, PC-3, TSU-Prl, and DU-145, express high mRNA levels of both TGF-β R-I and R-II receptors. The LNCaP prostate cancer cell line, however, had complete loss of TGF-β R-II receptor transcripts while expressing relatively high levels of R-I mRNA. A significant decrease in R-II but not R-I receptor mRNA was observed in three primary prostate cancer specimens analyzed (Fig. 2A). RT-PCR analysis, using specific primers for TGF-β R-I and R-II receptors, confirmed the loss of R-II receptor mRNA.

**Fig. 1.** Sensitivity profile of human prostate cancer cells to TGF-β1. Prostate cancer epithelial cells PC-3, DU-145, TSU-Prl, and LNCaP cells, and the TGF-β-responsive mink lung epithelial Mv-1-Lu cells were exposed to increasing concentrations of TGF-β1 as indicated; after 5 days, cell numbers were counted. Each point is the mean of triplicate determinations.

**Fig. 2.** Expression of TGF-β R-I and R-II receptor mRNA in human prostate cancer cell lines. A, Northern blot hybridization analysis of R-I and R-II mRNA expression in the human prostate cancer cell lines PC-3, DU-145, TSU-Prl, and LNCaP and three primary prostatic tumors, as indicated. Actin mRNA expression was used to normalize RNA loading and transfer. The size of specific transcripts is shown on the right. Note the complete loss of R-II mRNA transcript (but not R-I) in the LNCaP cells. B, RT-PCR analysis was performed using total RNA from the various lines: Mv-1-Lu, R-II receptor positive control, PC-3, TSU-Prl, and LNCaP human prostate cancer cell lines. The conditions for the RT-PCR are described in "Materials and Methods." Lane 1, 100 bp molecular weight markers; Lane 2, negative control (cDNA omitted); Lanes 3–6, R-II receptor expression; Lanes 7–10, R-I receptor expression; Lanes 11–14, GAPDH expression (to confirm the integrity and equivalent loading of the cDNA products). The size of the RT-PCR products as shown, at 730 bp for R-I, 861 bp for R-II, and 983 bp for GAPDH. Note the complete absence of the fragment corresponding to TGF-β R-II receptor, but not R-I, in LNCaP cells.
expression in LNCaP cells. Fig. 2B shows the ethidium bromide-stained gel of RT-PCR products using RNA obtained from the different prostate cancer cell lines. The fragment corresponding to the TGF-β R-II (861 bp) is absent in the LNCaP cells.

To investigate whether loss of TGF-β sensitivity of LNCaP cells was conferred by the loss of expression of TGF-β receptor R-II, LNCaP cells were cotransfected with a TGF-β R-II expression plasmid and a neomycin-resistant plasmid. Selected G418-resistant colonies were cloned and expanded into cell lines and were subsequently examined for TGF-β R-II receptor expression at the mRNA and protein level. Northern blot analysis revealed different expression levels of R-II mRNA transcripts among the individual clones (Fig. 3A). The presence of variable expression of the R-II-transfected gene in the cloned transfectants and lack of expression in parental LNCaP cells was confirmed by RT-PCR analysis. Fig. 3B indicates a representative RT-PCR profile for R-II expression, using mRNA from LNCaP, neo control transfectants, and four cloned R-II transfectant cell lines. GAPDH coamplification (using the same RT mixture), confirmed the integrity and equal loading in all of the samples. Western blot analysis demonstrated that the parental LNCaP cells have undetectable TGF-β R-II protein expression (Fig. 4A). All LNCaP R-II transfectant lines thus analyzed, clones 8, 13, 26, 42, 61 and 64, exhibited different levels of TGF-β R-II expression. LNCaP R-II clone 61 was identified as the highest R-II receptor overexpressing clone, at both the mRNA (Fig. 3) and protein level (Fig. 4A). There was no change in the expression levels of R-I receptor protein in the R-II transfectants (Fig. 4B).

Because PSA is a powerful differentiation marker of the malignant phenotype of prostate cancer cells and because the LNCaP cells produce PSA (19), we also examined the effect of R-II receptor overexpression on PSA production by the transfectant clones. As shown in Fig. 3, there was a significant decrease in the PSA mRNA transcripts among all of the R-II receptor transfectants analyzed compared with the parental and neo control cell lines. These results were confirmed by analysis of the PSA protein in the medium of individual cell lines (data not shown).

To investigate whether overexpression of TGF-β receptor R-II in the LNCaP cells could restore the sensitivity of the TGF-β growth-inhibitory activity, we determined: (a) the cell number; and (b) the rate of DNA synthesis in the cloned TGF-β R-II transfectants in response to TGF-β. TGF-β treatment resulted in a significant growth inhibition among the R-II transfectants but not the parental LNCaP or the neo-control transfectants (as determined by cell number; Fig. 5A). Fig. 5B indicates that the rate of DNA synthesis in TGF-β R-II transfectants (clones 8, 13, and 61) was significantly inhibited in a dose-dependent manner by TGF-β1, compared with the parental and neomycin-control cells (P < 0.05). The LNCaP R-II clone 61 (highest R-II expression) exhibited the highest inhibition of DNA synthesis by TGF-β1.

In subsequent experiments, we examined the effect of TGF-β on cell cycle distribution of parental LNCaP and R-II receptor clones. The flow cytometric profile of propidium iodide-stained cells shown in Fig. 6 indicates that TGF-β (1 ng/ml) causes a G1 arrest in the R-II LNCaP receptor clones but not in the LNCaP parental cells after 24 h of treatment (Fig. 6A). The increase in the G1 population was accompanied by a decrease in the number of cells in S phase (from ~30 to 7.4%), with no significant changes in the G2 population (Fig. 6B).

The cyclin-dependent kinase inhibitors p15INK4a, p21WAF1/Cip1, and p27Kip1 have been assigned roles as key downstream effectors for the intracellular transduction of TGF-β negative growth signals, thus mediating the cellular sensitivity to TGF-β1 (20–22). Northern and Western blot analyses were conducted during a time course of TGF-β treatment to investigate whether restoration of TGF-β sensitivity in the LNCaP cells involves induction of cdk inhibitors, p15INK4a, p21WAF1/Cip1, and p27Kip1, as potential downstream signaling events. The expression profile of the cdk inhibitors in the parental LNCaP and R-II-overexpressing clone 61, in response to TGF-β, is illustrated in Figs. 7 and 8. As shown in Fig. 7A, treatment of R-II receptor-expressing clone 61 with TGF-β1 resulted in a significant (5-fold) induction of p21WAF1/Cip1 mRNA expression as early as 3 h of treatment. This significant elevation of p21WAF1/Cip1 mRNA was maintained at 6 and 9 h after treatment; subsequently, there was a slight decline at 18 to 48 h of TGF-β treatment, but the mRNA levels remained above the low basal levels of untreated cells (Fig. 7A, Lane 9). For p27Kip1 mRNA expression, an initial increase (2-fold, following normalizing relative to 18S band from the ethidium bromide staining; Fig. 7B) was observed after 3 h of TGF-β1 treatment, and this significant induction was maintained over 48 h of treatment, compared with the untreated R-II transfectant cells (Fig. 7A). With longer treatment periods (72 h), p27 levels declined to baseline constitutive levels (data not shown). A significant induction (2-fold) of p15 mRNA transcript expression after TGF-β1 treatment was similarly detected after 3 h of TGF-β treatment in the R-II receptor clone 61. This was maintained during the first 18 h of exposure to TGF-β, and at 24 h, there was a slight decrease in the mRNA levels. In marked contrast, as also revealed in Fig. 7A, during the course of TGF-β1 treatment of parental LNCaP cells, no significant changes in the mRNA expression of any of the three cdk inhibitors were detected after normalization of each specific signal to the 18S band from the ethidium bromide staining (Fig. 7B).

Western blot analysis (Fig. 8) revealed an increase in the protein levels for p21Cip1 as early as 3 h after TGF-β treatment, and elevated expression was maintained for 18 h, with a subsequent slight decrease at 24–48 h after treatment, that temporally correlated with a decrease in the mRNA expression (Fig. 7A). For p27Kip1, the initial induction in protein expression in response to TGF-β was observed after 9 h, reaching a maximum expression level by 24 h of treatment; this peak in p27 protein levels was maintained after 48 h of TGF-β treatment (Fig. 8). A moderate but detectable increase in p15INK4a protein was detected after 18–24 h of TGF-β treatment in the R-II transfectants, compared with the untreated cells. In a parallel pattern with the mRNA expression profile (Fig. 7), no significant changes in the protein levels for any of the three cdk inhibitors were detected in the parental LNCaP cells in response to TGF-β treatment (Fig. 8).
The in vitro malignant growth characteristics of TGF-β1 receptor R-II transfectants were analyzed by examining the ability of the TGF-β R-II receptor clones to form colonies in soft agar. The data summarized in Table 1 indicate that all three TGF-β R-II-expressing clones, 8, 13, and 61, exhibited a substantial reduction in their colony-forming ability (40–70% decrease), compared with the parental and neomycin control cell lines. The level of TGF-β1 R-II expression in the clones correlated with the degree of suppression of the colony-forming ability (Table 1).

Discussion
Numerous studies have recently demonstrated that a dysfunctional TGF-β1 signaling pathway due to loss and/or mutational alterations of TGF-β1 R-II receptor plays an important role in the progression of several human malignancies, including prostate cancer (11, 14–16). The present results indicate that overexpression of TGF-β1 R-II receptor in the human prostate cancer cells, LNCaP, which are resistant to the negative growth effects of TGF-β1 due to loss of R-II receptor, can restore TGF-β1 sensitivity. TGF-β1 inhibited cell proliferation of the R-II receptor expressing LNCaP prostate cancer cells by arresting cell cycle progression in G1. In addition, there was a marked decrease in the PSA production by R-II-overexpressing clones compared with the pa-
Different transfectants, were cancer growth Fig. 

Effect of TGF-β R-II receptor overexpression on TGF-β mediated growth inhibition (A) and inhibition of DNA synthesis (B) in LNCaP prostate cancer cells. A, cells were grown in six-well culture plates in the presence and absence of increasing concentrations of TGF-β, and cell numbers were counted after 5 days of treatment. B, [3H]thymidine incorporation in response to increasing concentrations of exogenous TGF-β1 was examined in Mv-1-Lu (positive control), LNCaP parental, neomycin-control transfectants, and R-II transfectant clones B, 13, and 61. Points represent mean values from triplicate wells for both types of experiments.

breast cancer (10), as well as hepatoma cells (23), in which overexpression of TGF-β R-II receptor caused a significant inhibition of tumor growth by restoring sensitivity to TGF-β1 (10, 23).

Growth factor-induced signals must converge on the cell cycle clock, composed of cyclins, cdks, and their regulatory inhibitors to ensure the cell entry into S phase (24). Rapidly expanding evidence has implicated several cdk inhibitors as postreceptor targets transducing the TGF-β1 signal intracellularly. The growth-inhibitory effect of TGF-β1 has thus been directly associated with a temporal induction of expression of cdk inhibitors, p15Nκp, p21WAF-1/Cip1, and p27Kip1 (20–22). Our data demonstrate that, in response to TGF-β1, LNCaP R-II-overexpressing cells exhibited a transient and significant elevation of p21WAF-1/Cip1, p27Kip1, and p15Nκp expression at both the mRNA and protein level, which temporally preceded the antigrowth response. These results suggest that these cdk inhibitors may serve as intracellular effectors of TGF-β1 signal transduction in prostate cancer cells with restored TGF-β1 sensitivity.

The results described in the present study are consistent with numerous recent studies, indicating that loss of R-II receptor expression/function is a common event in human tumors from different origins (10–13), and this receptor loss directly correlates with insensitivity to growth-inhibitory signals of TGF-β1 in cancer cells (10, 23). Our current findings, however, seriously challenge a recent study by Kim et al. (25), reporting the exact opposite result, i.e., that loss of TGF-β1 receptor R-I and not R-II occurs in the LNCaP prostate cancer line. Our present observations are, however, strongly supported by others, who also found loss of R-II receptor expression in the LNCaP cells. The basis for this serious paradox remains a mystery. As a remote possibility as it might appear, one could argue that the obvious contradiction between our findings and those of Kim et al. (25) could be due to a difference in the clonal origin of the LNCaP cell line. Alternatively, because in their studies relatively late-passage LNCaP cells were used and under different culture conditions, it is possible that these cells may have acquired a different phenotype after serial passages in culture.

Mutational alterations of the TGF-β1 R-II receptor gene might be responsible for the loss of functional receptors. Mutations can occur by either destroying the R-II receptor kinase activity, thus preventing receptor R-I phosphorylation (26), or by affecting the ligand-binding domain of R-II, thus blocking the ligand binding (27). One can argue that even in the presence of normal levels of R-I, mutational changes in the R-II receptor gene would still abrogate the TGF-β1 negative growth effect on prostatic tumor cells. Southern blot analysis of the genomic organization of the TGF-β1 R-II receptor gene revealed no deletions or rearrangements of R-II gene in the LNCaP cells (data not shown). Single-strand conformational polymorphism analysis and direct DNA sequencing are presently being conducted to further investi-

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igate the possibility of genetic mutations in R-II receptor gene in the LNCaP cancer cells.

Loss of TGF-β1 receptor R-II expression in the LNCaP cells could be due to either a decrease in the mRNA stability or a reduced transcription rate. Multiple mechanisms have been implicated in the regulation of message stability involving either the 3' or 5' untranslated regions or the coding region itself (28, 29). Nuclear run-on assays are presently performed to determine whether loss of the R-II mRNA expression is due to an increased rate of mRNA degradation. Alternatively, one could speculate on a potential loss of transcriptional regulation of TGF-β R-II gene in the TGF-β-resistant LNCaP cells. Because both Northern and RT-PCR analyses indicated complete loss of mRNA expression for R-II receptor, it is possible that loss of R-II expression is under transcriptional regulation. It is well established that interactions between transcription factors and specific sequences within the 5' untranslated region of the gene, i.e., cis-acting elements, are important in the regulation of the promoter activity of a given gene. The identification of both positive and negative regulatory regions in the human TGF-β R-II receptor gene promoter (30) indicates the complexity of the transcription regulation of the R-II receptor gene. One could argue that changes in the sequence within these regulatory elements might result in decreased transcription of the R-II gene. A direct PCR-derived DNA sequencing of LNCaP genomic DNA may be necessary to determine whether mutations occurring within the TGF-β1 R-II receptor gene promoter cause loss of binding of putative transcription factors. Such mutations leading to loss of R-II gene expression have been described recently in a squamous carcinoma cell line (31).

In conclusion, we have demonstrated that overexpression of TGF-β1 receptor R-II gene in TGF-β-resistant prostate cancer cells, LNCaP, renders them sensitive to the growth-inhibitory effect of TGF-β by restoring functionality of the TGF-β1 signaling pathway. These observations support a role for TGF-β1 R-II receptor as a potential tumor suppressor gene in prostate cancer.

Materials and Methods

Cell Culture. The three androgen-independent human prostate cancer cell lines PC3, TSU-Pr1, DU-145 and the androgen-sensitive cell line, LNCaP, used in these studies were obtained from American Type Culture
**Fig. 7.** Time course of mRNA induction of cdk inhibitors, p21\(^{WAF1} / \text{Cip1}\), and p27\(^{KIP1}\) expression in response to exogenous TGF-β. A, Northern blot. Cells were exposed to TGF-β (1 ng/ml) for various periods of time, and RNA was isolated and subjected to Northern hybridization analysis. Lanes 1–8, LNCaP cells after 0, 3, 6, 9, 18, 24, 48, and 72 h of treatment with TGF-β, respectively. Lanes 9–15, LNCaP R-II clone 61 transfec tant after 0, 3, 6, 9, 18, 24, and 48 h of TGF-β treatment, respectively. B, ethidium bromide-stained gel indicating the 28S and 18S mRNA, which was used to normalize for equivalent transfer and loading of the RNA samples.

**Fig. 8.** Western blotting to illustrate the induction of cdk inhibitors at the protein level in response to TGF-β in the R-II receptor transfec tant clones. Cell lysates were obtained from LNCaP parental and R-II transfec tant cells after various periods of TGF-β treatment and were subsequently subjected to Western blot analysis using antibodies against the p15\(^{NKP}\), p21\(^{WAF1/Cip1}\), and p27\(^{KIP1}\) proteins, as described in "Materials and Methods." Lanes 1–7, LNCaP cells after 0, 3, 6, 9, 18, 24, and 48 h of TGF-β treatment, respectively. Lanes 8–14, R-II receptor transfec tant cells (clone 61) after 0, 3, 6, 9, 18, 24, and 48 h of TGF-β treatment (1 ng/ml), respectively. Right, molecular weights (in thousands; kd) of the respective cdk inhibitor proteins.

Collection (Rockville, MD). The TGF-β-responsive mink lung epithelial cell line Mv-1-Lu, used as a positive control, was also obtained from American Type Culture Collection. The growth medium for the PC3, TSU-Prl, and DU-145 cells was RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% FBS (HyClone, Logan, UT) and penicillin/streptomycin (BioWhittaker, Walkersville, MD). LNCaP (passage 10) and Mv-1-Lu cells were cultured in DMEM and MEM media (Life Technologies, Inc.), respectively, containing 10% FBS and antibiotics. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂.

**In Vitro Response of Prostate Cancer Cell Lines to TGF-β1.** Cells were plated in six-well plates (50,000 cells/well). After 24 h, cells were rinsed with HBSS, and growth medium was added in the presence and absence of TGF-β1 (R&D Systems, Minneapolis, MN) at increasing concentrations as follows: 0, 0.05, 0.1, 0.5, 1, and 5 ng/ml. After 5 days in culture, cells were counted using a hemocytometer, and the mean values from triplicate wells (for each treatment) were determined.

**Transfection.** The LNCaP cells were cotransfected with the expression vector for TGF-β1 R-II receptor (a gift from Dr. X-F. Wang, Duke University Medical Center) and with the plasmid encoding for neomycin resistance (provided by Dr. J. Taylor, Imperial Cancer Research Fund, London, United Kingdom), using the calcium phosphate precipitation method as described previously (32). The cells were exposed to neomycin selection using medium containing 500 μg/ml G418 (Life Technologies, Inc.), and G418-resistant colonies were cloned and expanded into cell lines.

**Northern Blot Hybridization Analysis.** RNA was isolated from human prostate cancer cells and was size-fractionated through 1.2% agarose/formaldehyde gel as described previously (32). RNA samples were trans-
ferred onto nylon membranes (Hybond-N; Amersham International, Arlington Heights, IL) and cross-linked by UV irradiation. Blots were hybridized with a 32P-labeled cDNA probe, and after successive washes, under high stringency conditions (32), blots were exposed to Kodak X-AR-5 film using intensifying screens at ~80°C. Equivalent loading of RNA was confirmed by subsequent hybridization with the β-actin cDNA probe and/or inspection of the ethidium bromide-stained gel under UV illumination. All cDNA probes were labeled with [32P]dCTP using the multiprime labeling kit (Amersham International). The relative expression of specific transcripts was determined by densitometric analysis of the autoradiographs, using a scanning densitometer.

**RT-PCR Analysis.** RNA was extracted from the various prostate cancer cells as described above. RT-PCR was performed using 1 μg of total cellular RNA and the Ribo Clone cDNA synthesis kit (Promega Corp., Madison, WI) in a Stratagene thermal cycler (La Jolla, CA). The following primers were used for: (a) TGF-β1 receptor R-I (sequences obtained from Dr. Joan Massagué, Sloan-Kettering Medical Center, NY), primer 204 (5'-CCGAAAGTTACGGGTTACAGTTCG-3'; sense) and primer 102 (5'-CCGCTGAGCTGAGTCAGGCAATCTAGTGC-3'; antisense); and (b) TGF-β1 receptor R-II (sequences obtained from Dr. Sanford Markowitz, Case Western Reserve University, OH), primer 297 (5'-CCGCTGAGCTGAGTCAGGCAATCTAGTGC-3'; sense) and primer 55 (5'-CCACTGTCCTCAACGTCTGCT-3'; antisense). The following conditions were used for RT-PCR: (identical for both R-I and R-II primers): 95°C for 30 s; 60°C for 1 min, 70°C for 3 min; 35 cycles; and 70°C for 10 min, 1 cycle, final extension. The integrity of the RNA used for reverse transcription was confirmed using the GAPDH synthesis as a positive control reaction. The primers for the human GAPDH were obtained from Clontech (Palo Alto, CA), and the sequences are as follows: sense (5'-TGAAGGTGGTGAT-CAACGGATTGTG-3'; antisense (5'-CATGTTGGCATGAGTCACCAC-3'). The amplified RT-PCR products were electrophoretically analyzed through 1.0% agarose gels and visualized by ethidium bromide staining. RNA from the Mv-1 Lu cells was used as a positive control for the expression of both receptor types.

**Western Blot Analysis.** Cell lysates, prepared as described previously (32), were subjected to electrophoretic analysis through SDS-PAGE gel (80 μg/well). After electrophoresis, proteins were transferred to nitrocellulose membrane (Hybond C; Amersham International); complete transfer was assessed using prestained protein standards (Bio-Rad, Melville, NY). After blocking 1% BSA (in TBS), the membrane was incubated with the primary antibody against TGF-β1 R-I or R-II receptor protein at a concentration of 1 μg/ml for 1 h (at room temperature). The primary antibodies used were the rabbit polyclonal IgG raised against the peptide sequence, amino acids 158–179, for TGF-β-R-I and a rabbit polyclonal raised against the peptide sequence 550–565 for TGF-β-R-II receptor. Both antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes were incubated with the biotinylated anti-rabbit secondary antibody (1:200) for 30 min (Santa Cruz Biotechnology) and were subsequently treated with the biotin-avidin horseradish peroxidase complex using the ABC kit (Santa Cruz Biotechnology) for the detection of antibody complexes (with diaminobenzidine). The antibodies against the cdk inhibitors, p21CIP1/p27KIP1, and p27KIP1, were obtained from Santa Cruz Biotechnology. Specifically, they were following: (a) a mouse monoclonal antibody (157) against the full-length human p21CIP1/p27KIP1 protein; (b) a goat polyclonal antibody (C-20), against the epitope corresponding to 118–137 amino acids mapping at the COOH terminus of human p21CIP1/p27KIP1; and (c) a goat polyclonal antibody (C-19), against the epitope corresponding to 181–198 amino acids at the COOH terminus of human p21CIP1/p27KIP1 protein.

**[3H]Thymidine Incorporation Assay.** Cells were plated in 24-well plates at a density of 3 × 103 cells/well in the presence of increasing concentrations of TGF-β1 (0, 0.1, 0.5, 1, and 5 ng/ml). After 4 days, cells were pulsed with [3H]thymidine (1 μCi/ml; Amersham International) at 37°C for 2 h. DNA was precipitated with 10% (w/v) trichloroacetic acid, and the amount of [3H]thymidine incorporated was analyzed by liquid scintillation counting.

**Cell Cycle Analysis.** Cell cycle distribution was measured by flow cytometry. Exponentially growing cultures of LNCaP and R-II transfient cells were exposed to TGF-β1 (1 ng/ml) for 24 h. Cells were harvested and were subsequently stained with propidium iodide. Stained cells were subjected to flow cytometric analysis using the FACSscan (Becton Dickinson). G1, S, and G2-M populations were quantitated using Multicycle analysis software (Phoenix Flow Systems).

**Effect of TGF-β1 Treatment on the Expression of cdk Inhibitors.** Total cellular RNA and cell lysate were isolated from parental LNCaP cells and cloned TGF-β1 R-II transfient cells (clone 61) after TGF-β1 treatment (1 ng/ml) for various time periods (0, 6, 18, 24, and 48 h). RNA samples were subjected to Northern analysis as described above, and blots were sequentially hybridized with p21WAF1/CIP1, p27KIP1, and p15-labeled cDNA probes.

**Colony-forming Ability Assay.** Parental LNCaP cells, neomycin-control, and TGF-β R-II transfient clones (b, c) were subjected to 3 ml of 0.36% noble agar in growth medium containing 10% FBS and plated on top of 3% agar-underlayer in six-well plates at a density of 5 × 103 cells/well (triplicate wells for each cell line). After 4 weeks of incubation, colonies larger than approximately 30 cells were scored.

**Statistical Analysis.** Statistical analysis of significance was performed by conducting a paired Student’s t test. All numerical data were expressed as the mean values obtained, and ± SE was calculated when possible. A probability that the means were significantly different was reached at a P level of 0.05.

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**References**


