Prohibitin and Cofilin Are Intracellular Effectors of Transforming Growth Factor β Signaling in Human Prostate Cancer Cells

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Abstract

A proteomic analysis was pursued to identify new signaling effectors of transforming growth factor β1 (TGF-β1) that serve as potential intracellular effectors of its apoptotic action in human prostate cancer cells. The androgen-sensitive and TGF-β-responsive human prostate cancer cells, LNCaP TβRII, were used as *in vitro* model. In response to TGF-β, significant post-translational changes in two proteins temporally preceded apoptotic cell death. TGF-β mediated the nuclear export of prohibitin, a protein involved in androgen-regulated prostate growth, to the cytosol in the LNCaP TβRII cells. Cofilin, a protein involved in actin depolymerization, cell motility, and apoptosis, was found to undergo mitochondrial translocation in response to TGF-β before cytochrome *c* release. Loss-of-function approaches (small interfering RNA) to silence prohibitin expression revealed a modest decrease in the apoptotic response to TGF-β and a significant suppression in TGF-β-induced cell migration. Silencing Smad4 showed that the cellular localization changes associated with prohibitin and cofilin action in response to TGF-β are independent of Smad4 intracellular signaling. (Cancer Res 2006; 66(17): 8640-7)

Introduction

Transforming growth factor-β (TGF-β) regulates a diverse set of biological activities including apoptosis, proliferation, cell motility, and angiogenesis. The breadth of cellular responses is derived from the numerous genes and their encoded proteins regulated by TGF-β. Intracellular signaling by members of the TGF-β ligand superfamily, such as TGF-β, activin, and bone morphogenesis proteins, proceeds via transmembrane heterotetrameric complexes composed of two types of serine threonine kinase receptors, type I (TβRI) and type II (TβRII). On ligand binding, TβRII receptor phosphorylates and activates TβRI receptor, which initiates the downstream signaling cascade by phosphorylating the receptor-regulated Smads (1). Besides Smad-mediated transcription, TGF-β activates other intracellular signaling cascades, including extracellular signal–regulated kinase, c-jun NH_2-terminal kinase, and mitogen-activated protein kinase pathways (2).

In normal prostate epithelium, TGF-β acts as a tumor suppressor via induction of apoptosis and inhibition of cell proliferation, whereas in advanced cancer, TGF-β promotes tumor progression and metastasis via induction of tumor cell invasion, enhanced angiogenesis, and immunosuppression. Deregulation of the TGF-β signaling is frequently detected in numerous human malignancies due to either loss of expression or mutational inactivation of its membrane receptors or intracellular Smad proteins (3–7). TGF-β serves as a growth suppressor by inducing cell growth arrest and promoting apoptosis (8–10); during tumorigenesis, loss of growth inhibitory control permits tumor cells to use TGF-β for evasion of immune surveillance and gain of metastatic ability (11–13). The mechanistic network underlying the molecular switch from TGF-β acting as a tumor suppressor to functioning as a tumor promoter during the metastatic process is not clearly defined. Current gene therapy–based approaches targeting TGF-β must consider the cell cycle restoration effect of TGF-β and must counteract its immunosuppressive effect (14). Further understanding of the TGF-β signaling and its cross-talk with other intracellular mechanisms will facilitate the development of novel effective therapeutic modalities specifically targeting the tumor-promoting action of this cytokine without compromising its tumor-suppressive effects.

In human prostate cancer, overexpression of the ligand TGF-β (15), loss expression of transmembrane receptors TβRII, and intracellular effectors Smad4 (3, 16–18) have been widely documented. Moreover, genetically mediated overexpression of TβRII in human prostate cancer cells LNCaP restored their apoptotic sensitivity to TGF-β, ultimately resulting in tumor suppression (4, 5). Studies in a transgenic mouse model support that disruption of TGF-β signaling can promote prostate cancer metastasis (19). Attempts to target TGF-β signaling include induction of the apoptotic action by receptor-targeted drugs such as the quinazoline-based α1-adrenoceptor antagonists (20), restoration of TβRII expression via gene therapy (5), and antisense inhibition of TGF-β (21, 22).

Apoptosis is the predominant form of prostate tumor cell death induced by androgen ablation, radiotherapy, and chemotherapeutic agents (23). Acquisition of antiapoptotic pathways ultimately leads to treatment resistance that typifies advanced prostate cancer. The well-documented apoptotic actions of TGF-β in the prostate and the recognized significance of TβRII as a tumor suppressor call for further mechanistic characterization of the signaling events triggered by this player. Apoptosis, cell differentiation, and cell motility depend directly on the dynamics of actin cytoskeleton. The intracellular pathways linking external TGF-β signals to the regulation of actin cytoskeleton are poorly characterized. In this study, we used two-dimensional PAGE and a proteomics approach to determine novel effectors regulated by TGF-β and potentially involved in its signaling pathway in the androgen-sensitive, TGF-β-responsive LNCaP prostate cancer cells. Two proteins, cofilin and prohibitin, were identified to be targeted by TGF-β and possibly involved in its apoptotic and/or cell motility/migration regulatory functions in prostate cancer cells.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell Culture and Treatment

Human prostate carcinoma cells LNCaP are obtained from the American Type Culture Collection (Manassas, VA). The TβRII-overexpressing LNCaP cells (LNCaP-TβRII), harboring an intact TGF-β signaling, have previously been generated and characterized (4, 5). Cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY), supplemented with 10% charcoal-stripped fetal bovine serum, 100 units of penicillin, and 100 mg/mL streptomycin. Subconfluent cultures were treated with 5 ng/mL TGF-β1 (R&D Systems, Minneapolis, MN) and/or 1 nmol/L dihydrotestosterone (Sigma, St. Louis, MO) for various time periods.

Reagents

Immobilized pH gradient (IPG) strips and appropriate IPG buffers were purchased from Amersham Biosciences (Piscataway, NJ). Acrylamide (40%, 29:1) was obtained from Bio-Rad (Hercules, CA). Trypsin (modified, sequencing grade, lypholized) was obtained from Promega (Madison, WI). Protease inhibitor mixture, CHAPS, and DL-DTT were purchased from Sigma. Other commonly used reagents were purchased from Fisher Scientific (Hampton, NJ).

Cell Viability Assay

The effect of TGF-β1 and dihydrotestosterone on cell viability before and after Smad4 silencing was assessed at 72 hours posttransfection with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Absorbance was read at wavelength 570/690 nm with μQuant (Bio-Tek Instruments, Inc., Winouski, VT) and values were expressed as the mean percent of cell viability relative to control cells.

Apoptosis Evaluation

The BrdUrd assay was used to assess TGF-β-mediated apoptosis. After the various treatments and small interfering RNA (siRNA) transfections, prostate cancer cells LNCaP TβRII [TGF-β treatment (5 ng/mL) for 24 hours] were fixed with 2% paraformaldehyde and stained with BrdUrd and propidium iodide following the APO-BRDU protocol (BD Biosciences, San Diego, CA) and analyzed immediately with Partec Flow Cytometry (Munster, Germany).

Protein Preparation

Cytosolic soluble fractions were isolated from prostate cancer cells by a non-detergent-based method. Briefly, cells were washed with PBS and pellets were suspended in hypo-osmotic buffer [20 mmol/L HEPES (pH 7.5), 1.5 mmol/L MgCl, 10 mmol/L KCl] and subsequently sonicated and centrifuged at 4,000 × *g* (4°C, 15 minutes). After centrifugation (10,000 × *g*, 4°C, 10 minutes), supernatants were subjected to ultracentrifugation at 100,000 × *g* (4°C, 1 hour). The final pellets were resuspended in isoelectric focusing buffer [7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 1% amidosulfobetaine-14, 0.2% DTT, and 0.5% 100× Bio-Lyte (w/v, 3/10)].

Proteomic Analysis

Protein concentration was determined with the R&D C assay (Bio-Rad) and protein samples (300 μg protein) in isoelectric focusing buffer were loaded on IPG strips (pH 3-10 NL, 18 cm, Amersham Bioscience), rehydrated, and subsequently subjected to isoelectric focusing (500 V, 1 hour; 1,000 V, 1 hour; and 8,000 V, 6 hours) with IPG phor (Amersham Bioscience). After equilibration, strips were subjected to the second dimension of 12.5% (w/v) SDS-polyacrylamide gels followed by Sypro Ruby staining. Gel images were acquired with a Storm fluorescence scanner (Amersham Pharmacia Biotech, Piscataway, NJ).

Analysis of Two-Dimensional Electrophoresis Images

Two-dimensional gel images were analyzed by PDQuest software (Bio-Rad). For each time point of TGF-β treatment, three separate gels were run, and matching sets were established. Matching of two-dimensional images (three from LNCaP cell and three from LNCaP-TβRII) was done automatically, followed by manual matching. Protein spots were excised and in-gel digestion was done as previously described (24, 25). The resulting peptides were extracted with 0.02% heptafluorobutylic acid and 0.02% heptafluorobutylic acid/50% acetonitrile and subsequently subjected to liquid chromatography-tandem mass spectrometry (MS/MS) analysis followed by scanning with Mascot.

Western Blot Analysis

Treated and untreated control cultures of prostate cancer cells LNCaP TβRII were harvested at various time periods as indicated (0-48 hours) and total cellular protein was extracted by lysis in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 8.0), 1% NP40, 0.5% deoxycholate sodium salt, 1 mmol/L phenylmethyl sulfonyl fluoride, and 2 mg/mL aprotinin]. Protein samples of 30 to 40 μg of whole-cell lysates were loaded on 4% to 12% SDS-polyacrylamide gels and were subsequently transferred to nitrocellulose (Hybond-C Extra, Amersham Biosciences). After blocking in 2% bovine serum albumin (BSA) in TBS containing 0.05% Tween 20, blots were incubated with the respective primary antibodies. Human anti-prohibitin antibody was obtained from Abcam (Cambridge, MA); anti-cofilin and anti–cofilin-phospho(Ser^3) polyclonal antibodies were purchased from Cell Signaling (Beverly, MA); Smad4 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and anti-γ-actin antibody was from Calbiochem-EMD Bioscience (La Jolla, CA). After incubation with the specific primary antibody, membranes were incubated with the relevant horseradish peroxidase–labeled secondary antibodies. Membranes were exposed to the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) and the image was detected with Bioimaging System (UVP, Inc., Upland, CA).

siRNA Transfection

The siRNA targeting of Smad4 used the short RNA duplexes of 21 nucleotides with two nucleotide 3′ overhangs on each strand; the siRNAs assemble into endoribonuclease-containing complexes. LNCaP TβRII cells were seeded in six-well plates (Nunc, Roskilde, Denmark) and after 24 hours in antibiotic-free medium, siRNA targeting Smad4 (Santa Cruz Biotechnology) or prohibitin (Ambion, Austin, TX) at a dose of 500 pmol siRNA oligomer and Lipofectamine 2000 (Invitrogen, Grand Island, NY), respectively, in Opti-MEM I Reduced Serum Medium (Invitrogen) was added to the cells. Following incubation at 37°C for 24 hours, cells were treated with TGF-β1 and/or dihydrotestosterone (0-48 hours) and harvested for Western blot analysis. At 48 hours post siRNA transfection, apoptosis and cell migration (as biological end points) were evaluated in response to TGF-β treatment.

Cell Migration Assay

Wounding assay. LNCaP TβRII cells were transfected with 500 pmol prohibitin siRNA; at 48 hours posttransfection, the cell monolayer was wounded and cells were treated with TGFβ1 (5 ng/mL). Cells migrating to the wounded areas (at 48 hours post-wounding) were counted (under microscopy) and migration was defined as the mean number of cells in three random fields (200×) per well (26).

Immunoprecipitation Analysis

LNCaP TβRII cells were treated as described above and total cell lysates were prepared by direct lysis in immunoprecipitation buffer [10 mmol/L Tris-Cl (pH 8.0), 0.25% Triton X-100, 0.5% NP40, 10 mmol/L EDTA, 0.5 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride]. Cell lysates were precleared with protein A or G agarose/sepharose beads and incubated with immunoprecipitating antibody and protein A or G agarose/sepharose beads (4°C, overnight). The agarose/sepharose beads were subjected to pulse centrifugation (14,000 rpm), resuspended in sample buffer, boiled, and centrifuged again. Resulting supernatants were electrophoretically analyzed through 12.5% SDS-PAGE.

Confocal Laser Scanning Microscopy

LNCaP TβRII prostate cells were cultured on four-chamber culture slides (BD Falcon, Bedford, MA) and, at 50% density, were incubated with 500 nmol/L Mito Tracker Red (Molecular Probes, Eugene, OR; 15 minutes). Following exposure to TGF-β1, cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Nonspecific binding was blocked with 5% BSA (Sigma-Aldrich, St. Louis, MO) in PBS. Cells were exposed to the cofilin antibody (Cytoskeleton, Inc., Denver, CO),

incubated with fluorescein-conjugated goat anti-rabbit (Invitrogen), mounted, and then were scanned by confocal laser scanning microscopy (Leica Microsysterms, Wetzel, Germany) with constant intensity settings. Images were analyzed with Leica software (Leica Microsysterms).

Statistical analysis. Values represent the mean of three independent experiments and are expressed as mean ± SE. Statistical analysis of the data was done using the Student's *t* test. Values were considered statistically significant at $P < 0.01$.

Results

Novel signaling effectors of TGF-β identified by proteomic analysis. Proteomic approaches can characterize the information flow between the extracellular microenvironment and intracellular signaling mechanisms. To identify novel effectors of TGF-β signaling, we applied a proteomic approach to comparatively analyze the parental LNCaP cells and the LNCaP TβRII cells in response to TGF-β (5 ng/mL). After TGF-β treatment, cytosolic protein fractions were isolated from the two cell lines and subjected to two-dimensional gel electrophoresis. The two-dimensional gel images obtained were analyzed with the PDQuest software. More than 100 spots were identified as being differentially expressed (54.3% up-regulated, 45.7% down-regulated); 7 of the 100 spots were selected for identification by mass spectrometry (Fig. 1*A* and *B*, respectively). These spots were selected on the basis of their consistent occurrence and clearly visible changes in response to TGF-β. Areas corresponding to the spots were cut and subjected to protein sequencing by high-performance liquid chromatography-MS/MS. The identities of the seven selected spots (from the mass spectrometry analysis) are listed in Table 1. Dramatically notable changes involving new appearance and total loss were detected for two distinct proteins, prohibitin (Fig. 1*C* and *D*) and cofilin (Fig. 1*E* and *F*), respectively, in response to TGF-β. Sequences highlighted in red are prohibitin (Supplementary Fig. S1*A*) and cofilin (Supplementary Fig. S1*B*) and were identified with liquid chromatography-MS/MS sequence coverage of 70% and 63%, respectively.

Comparative analysis between total cell lysates from the LNCaP TβRII cells before and after TGF-β treatment was also done (data not shown).

Nuclear export of prohibitin in response to TGF-β1. Prohibitin is a protein that has been linked to diverse cellular functions including cell cycle progression, senescence, apoptosis, and stabilization of mitochondrial membrane proteins (27, 28). Two primary roles are assigned to prohibitin: as a cell cycle regulator and as a mitochondrial chaperone protein. Because this was one of the proteins identified to undergo significant increase in response to TGF-β in the cytosol of LNCaP TβRII prostate cells, we initially focused on the characterization of prohibitin and its potential contribution to the apoptotic action of TGF-β. Soluble cytosolic fractions from LNCaP TβRII cells (untreated and TGF-β1 treated) were subjected to Western blot analysis. Figure 2 shows a characteristic dose-response (Fig. 2*A*) and a time-course (Fig. 2*B*) analysis, indicating a dose- and time-dependent up-regulation of cytosolic prohibitin by TGF-β. Within 6 hours of treatment, a significant increase in prohibitin expression was detected (Fig. 2*B*). Concomitant to the increase in cytosolic prohibitin levels, there was a time-dependent depletion of prohibitin in the nuclear fraction (Fig. 2*C*). To confirm that a dynamic nuclear export is underlying this topological change of prohibitin, confocal microscopy was conducted. The results shown in Fig. 2*E* (representative of three independent experiments) indicate that within 6 hours of treatment and preceding apoptosis induction, TGF-β promoted prohibitin export from the nuclei to the cytosol. There were no significant differences in the basal levels of prohibitin expression in

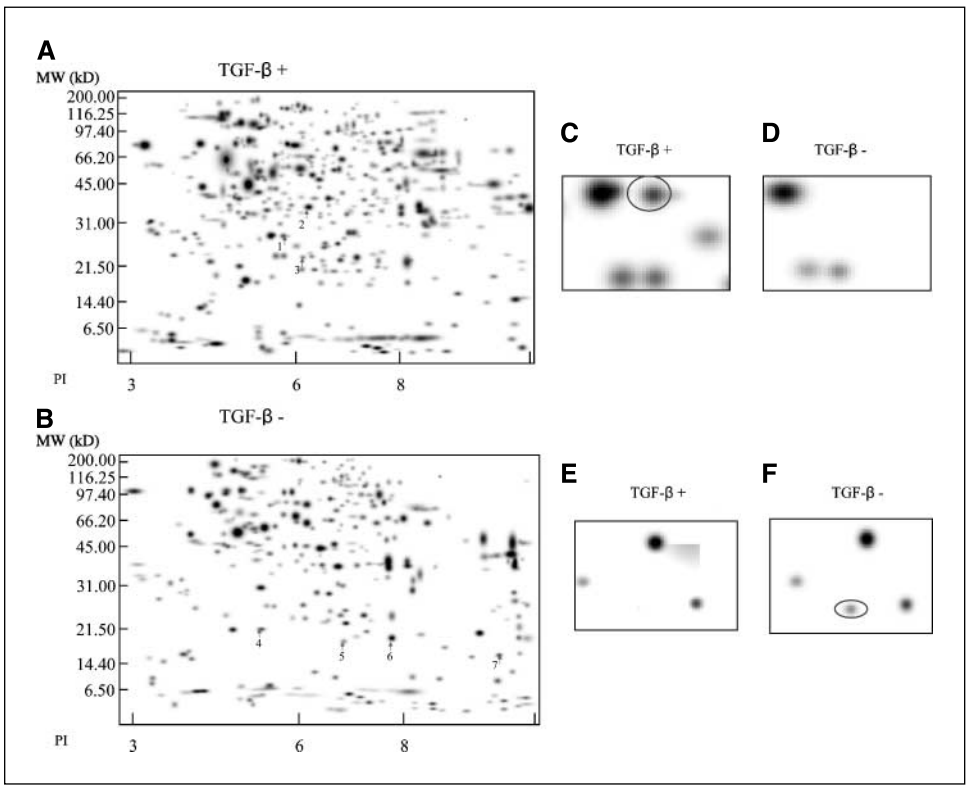

Figure 1. Two-dimensional gel image of soluble proteins from prostate cancer cells after TGF-β treatment. The LNCaP TβRII transfectants (*A*) and the TβRII negative parental LNCaP cells (*B*) were treated with 5 ng/mL TGF-β (24 hours). Cytosolic soluble fractions were isolated by the non-detergent-based method as described in Materials and Methods and protein samples (150 μg) were resolved by two-dimensional gel electrophoresis. *Arrows,* differential protein spots in response to TGF-β. The amplified image of spot 1 and spot 6 reveals changes resulting from cellular response to TGF-β characterizing prohibitin (*C* and *D*) and cofilin expression (*E* and *F*).

Table 1. Protein identified with MS/MS

Spot number*	Protein identification	Theoretical		Changes
		MW †	pI ‡	
1	Prohibitin	29,802	5.57	↑
2	L-Lactate dehydrogenase (EC 1.1.1.27) chain H	36,615	5.71	↑
3	Dodecenoyl-CoA δ isomerase	32,779	8.8	↑
4	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase	24,504	6.78	↓
5	Flavin reductase (EC 1.5.1.30)	21,974	7.31	↓
6	Cofilin	18,491	8.22	↓
7	Nucleoside diphosphate kinase (EC 2.7.4.6), chain A	17,156	8.55	↓

*Spots shown in Fig. 1.
† Molecular weight (kDa).
‡ Isoelectric point.

the various prostate benign and malignant cells [i.e., BPH-1 (benign epithelial), DU145, PC-3 (androgen independent), LNCaP (androgen sensitive), and LNCaP TβRII (androgen sensitive, TGF-β responsive) cells; Supplementary Fig. S2].

Effect of androgens and TGF-β on prohibitin expression and localization. We previously showed that dihydrotestosterone enhances TGF-β-induced apoptosis in hormone-sensitive human prostate cancer cells (29). To dissect the potential function of prohibitin in TGF-β signaling and the enhanced apoptotic action of this ligand by androgens, the expression profile of prohibitin was examined in response to TGF-β alone or in combination with dihydrotestosterone. LNCaP TβRII cells were treated with TGF-β1 (5 ng/mL) and/or dihydrotestosterone (1 nmol/L) for 3 to 24 hours. Soluble cytosolic protein fractions (40 μg/protein/well) were subjected to PAGE analysis and Western blotting. As shown in Fig. 3, 12 hours of dihydrotestosterone exposure (alone) resulted in a significant decrease in prohibitin protein levels whereas TGF-β resulted in increased prohibitin expression (Fig. 3*B*). Interestingly, dihydrotestosterone counteracted the TGF-β-enhanced prohibitin levels in the cytosolic fraction (Fig. 3*B*).

Association of prohibitin with bcl-2 and bax apoptosis regulators. Many apoptotic signals converge at the level of mitochondria and release mitochondrial proteins that promote apoptosis. Considering the ability of prohibitin to confer stabilization of mitochondrial membrane proteins (28), the effect of TGF-β on the association between prohibitin and two key apoptosis regulators, bcl-2 (apoptosis suppressor) and bax (apoptosis inducer), which are associated with mitochondria, was subsequently examined. Following treatment of LNCaP TβRII cells with TGF-β, total cell lysates were prepared and subjected to immunoprecipitation with the bcl-2 antibody and Western blotting with the prohibitin antibody (Supplementary Fig. S3*A*). The reverse immunoprecipitation with the prohibitin antibody and Western blotting with the bcl-2 antibody are shown in Supplementary Fig. S3*B*. Within 6 hours of TGF-β treatment, an enhanced association between prohibitin and bcl-2 was detected. No significant changes in the complex formation between prohibitin and bax were detected in response to TGF-β (not shown).

Mitochondrial translocation of cofilin. The actin-depolymerizing factor/cofilin family is a stimulus-responsive mediator of actin dynamics. In the cellular setting, cofilin emerges as a key regulator of actin dynamics at the leading edge of motile cells via its phosphorylation status; cofilin is inactivated by phosphorylation at Ser^3 and reactivated by dephosphorylation. *In vivo*, cofilin acts synergistically with the Arp2/3 complex to amplify local actin polymerization responses on cell stimulation and set the direction of cell motility (30). Cofilin translocates into the nucleus together with actin in response to various stress signals. In view of this evidence, we pursued the characterization of cofilin as a player in the TGF-β apoptotic signaling in prostate cancer cells. The initial two-dimensional analysis of soluble cytosolic factions indicated cofilin down-regulation by TGF-β1 (Fig. 1*E* and *F*). Subsequent subcellular fractionation and Western blot analyses confirmed that, indeed, cytosolic cofilin was down-regulated by TGF-β1, a change that was concomitant with a time-dependent, TGF-β1-mediated increase of cofilin in the mitochondrial fraction (Fig. 4*A*). This change in the subcellular localization of cofilin proceeded after Smad4 silencing, indicating a Smad-independent response (Fig. 6*D*). No changes in cellular cofilin distribution were detected when total cell lysates were used (data not shown). The phosphorylated cofilin expression profile revealed no significant changes in the translocation of phosphorylated cofilin to mitochondrial fraction of prostate cancer cells by TGF-β throughout the treatment (Fig. 4*A*).

Driven by the evidence that mitochondrial translocation of cofilin is an early step in apoptosis induction (31), we subsequently attempted to correlate the effect of TGF-β on the cellular distribution of cofilin in prostate cancer cells and apoptosis induction. Because cytochrome *c* release from the mitochondria (in the cytosol) is an early event in apoptosis triggering, we first evaluated cytochrome *c* levels in LNCaP TβRII prostate cancer cells in response to TGF-β. Figure 4*A* indicates a mitochondrial cofilin translocation occurring as an early event in response to TGF-β (within 3-6 hours of treatment), which temporally precedes its apoptotic action as assessed by cytochrome *c* release from the mitochondria.

To confirm that the topological translocation of cofilin was a direct consequence of the effect of TGF-β, confocal laser scanning microscopy was done on LNCaP TβRII cells after TGF-β treatment. The fluorescence photomicrograph in Fig. 4*B* illustrates the characteristic subcellular distribution of cofilin after continuous exposure of cells to TGF-β. This was an early event, with a

significant accumulation of cofilin in the mitochondria within 6 hours of treatment, preceding the apoptotic response elicited by TGF-β.

Prohibitin silencing interferes with TGF-β-mediated prostate cancer cell migration. To determine whether the nuclear/cytosolic translocation of prohibitin is a critical intracellular event in the apoptotic signaling of TGF-β, an siRNA approach was used to transiently knockdown prohibitin expression. As shown in Fig. 5*A*, endogenous prohibitin levels were efficiently and specifically reduced in the presence of a prohibitin-specific siRNA. Apoptosis evaluation indicated that prohibitin silencing had only a modest inhibitory effect on TGF-β-induced cell death (Supplementary Fig. S4). In response to TGF-β, LNCaP TβRII prostate cancer cells with normal levels of prohibitin exhibited a significant induction in their migration potential (Fig. 5*B* and *C*). The prohibitin-knockdown cells, however, displayed a significant suppression of TGF-β-mediated cell migration to a level comparable with the untreated control cells (Fig. 5*C*). Thus, prohibitin provides a protective effect against cell migration by TGF-β.

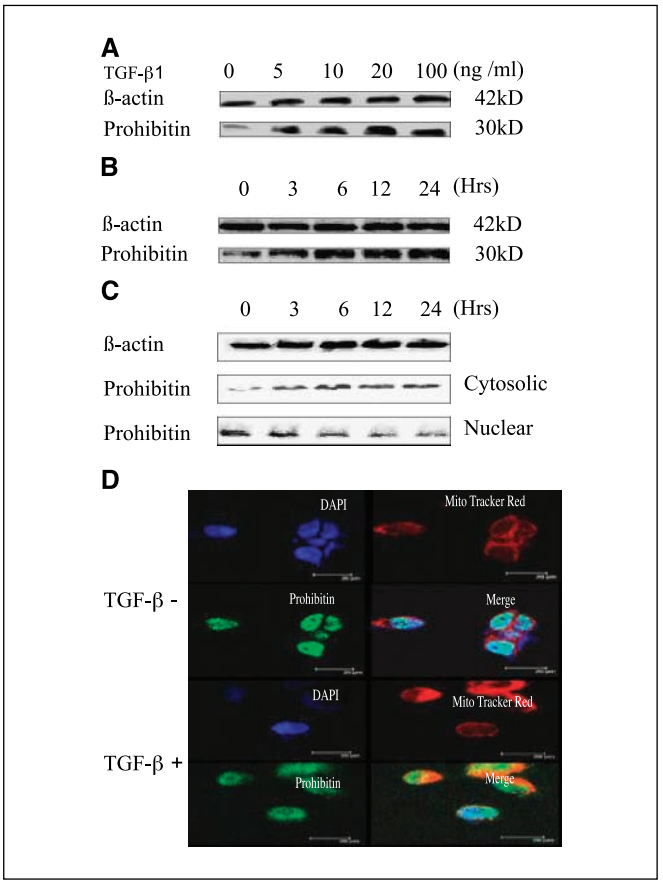

Figure 2. TGF-β mediates nuclear export of prohibitin in prostate cancer cells. *A,* dose-response effect of TGF-β on prohibitin cellular distribution. LNCaP TβRII cells were treated with increasing concentrations of TGF-β1 for 6 hours and total cell lysates (40 μg/lane of total cell lysates) were subjected to Western blot analysis. *B,* time course of prohibitin regulation by TGF-β. LNCaP TβRII cells were treated with 5 ng/mL of TGF-β1 for increasing periods of time and subjected to Western blotting. *C,* LNCaP TβRII cells were treated with 5 ng/mL TGF-β1 and subcellular fractions were isolated for Western blot analysis. *D,* confocal microscopy identifies prohibitin export from the nucleus to the cytosolic compartment (representative image). LNCaP TβRII cells treated with 5 ng/mL TGF-β1 (*bottom*) or untreated cells (no TGF-β1; *top*) were incubated with 500 nmol/L Mito Tracker Red. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. After exposure to the prohibitin antibody, signal was detected with a fluorescein-conjugated goat anti-mouse secondary antibody and slides were visualized with confocal laser scanning microscope (constant intensity settings).

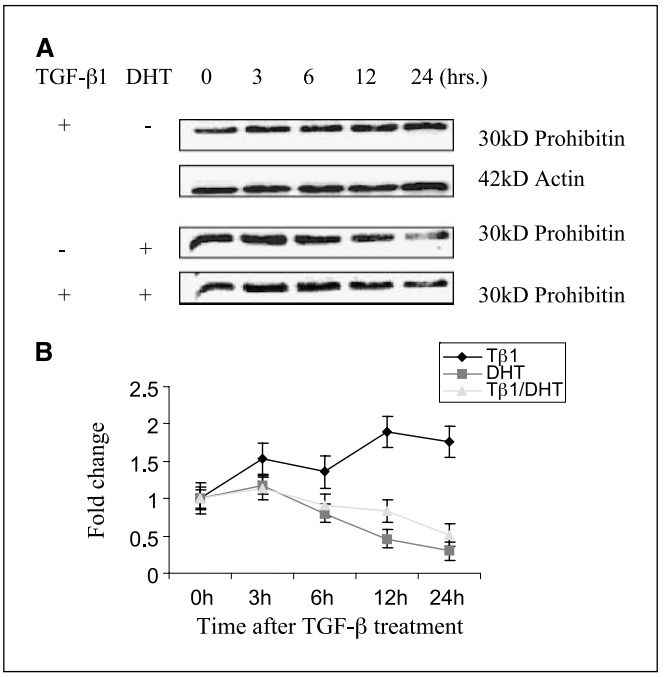

Figure 3. Combined effect of TGF-β1 and dihydrotestosterone on prohibitin expression. LNCaP TβRII cells were treated TGF-β1 (5 ng/mL) and/or dihydrotestosterone (*DHT*; 1 nmol/L) for various time periods (0-24 hours) as indicated. *A,* total cell lysates (40 μg protein/sample) were subjected to electrophoretic analysis and Western blotting. *B,* the fold change was determined and expressed as the relative prohibitin/actin intensity of respective bands.

TGF-β targeting of prohibitin and cofilin is independent of Smad4. LNCaP TβRII cells treated with 500 pmol siRNA targeting Smad4 exhibited a significant suppression of Smad4 expression (Fig. 6*A*). The effect of TGF-β1 on cell death was determined before and after Smad4 silencing. As shown in Fig. 6*B*, knockdown of Smad4 resulted in a significant decrease in TGF-β1-mediated cell death in LNCaP TβRII cells; there was no detectable effect (by Smad4 siRNA), however, on LNCaP cells (Fig. 6*C*). Exposure of LNCaP TβRII cells to TGF-β1, subsequent to Smad4 silencing (12 hours), suppressed the TGF-β-mediated changes in prohibitin and cofilin topological distribution (i.e., decrease in prohibitin and increase in cofilin in cytosolic fraction), indicating that their "targeting" is independent of Smad4 (Fig. 6*D*).

Discussion

TGF-β signaling is critical in apoptosis induction and inhibition of prostate cell proliferation (32–35). Growing evidence supports that in addition to the Smad family of proteins serving the primary role as intracellular effectors of TGF-β signaling, Smad-independent pathways also exist in the execution of TGF-β signals in target cells (36, 37). Integrin-mediated interactions of cytoskeletal structures with the extracellular matrix can generate the required forces for cell movement, and sandwiched between them is the plasma membrane that may influence cellular processes such as activation of growth factor receptors and interaction of transmembrane signaling molecules with the cytoskeleton and extracellular matrix (38).

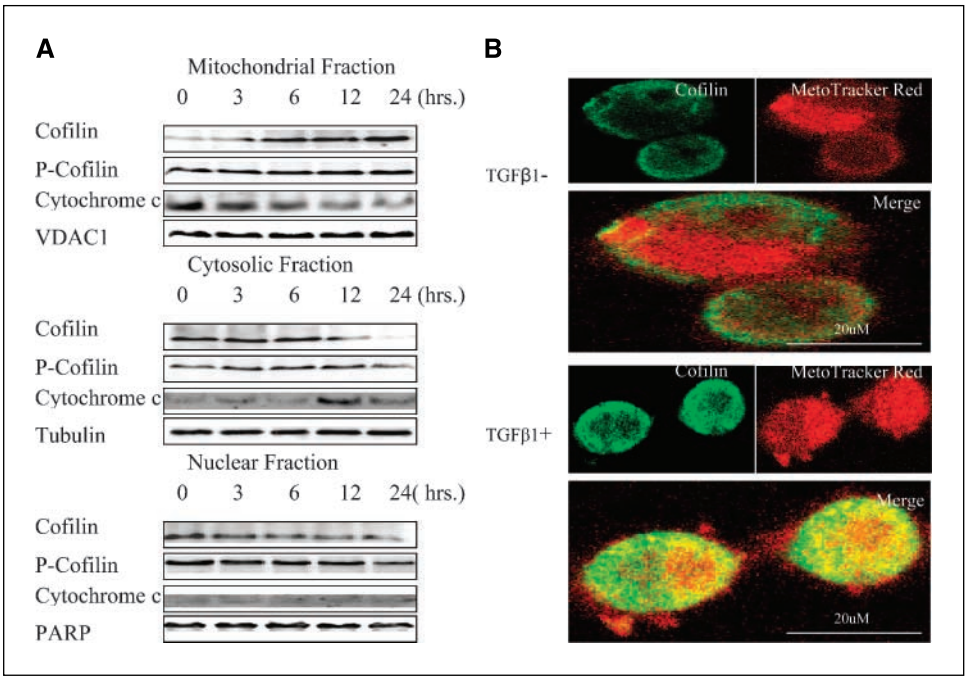

Figure 4. Mitochondrial translocation of cofilin in response to TGF-β. *A,* LNCaP TβRII cells were treated with 5 ng/mL of TGF-β1 for the indicated time periods and cells were subjected to cell fractionation. Protein samples (40 μg/lane) were subjected to SDS-PAGE electrophoretic analysis. Blots were incubated with ether phospho(Ser^3) polyclonal antibody or anti-cofilin polyclonal antibody and signal development was detected. *B,* confocal laser scanning microscopy. LNCaP TβRII cells, untreated controls (*top*) and following treatment with TGF-β1 (*bottom*), were incubated with 500 nmol/L Mito Tracker Red; cells were subsequently fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. After exposure to cofilin antibody, signal detection and visualization were conducted (see Materials and Methods).

In this study, a proteomic analysis led to the identification of two novel membrane proteins, prohibitin and cofilin, as effectors of TGF-β signaling in human prostate cancer cells responsive to TGF-β. To the best of our knowledge, this is the first evidence to implicate prohibitin and cofilin as intracellular effectors of TGF-β mechanism in human prostate cancer cells. Functional characterization studies (in other systems) indicate that prohibitin is primarily localized to the mitochondria, potentially playing a role in the maintenance of mitochondrial function and protection against senescence (39, 40). More recent studies showed a significant functional contribution by prohibitin to Ras-mediated epithelial cell migration (41). This evidence is in accord with our observations supporting a role for prohibitin in prostate cancer cell migration mediated by TGF-β. The Smad4 loss-of-function studies (siRNA silencing) indicate that prohibitin and possibly cofilin are involved in the apoptosis and/or cell migration elicited by TGF-β

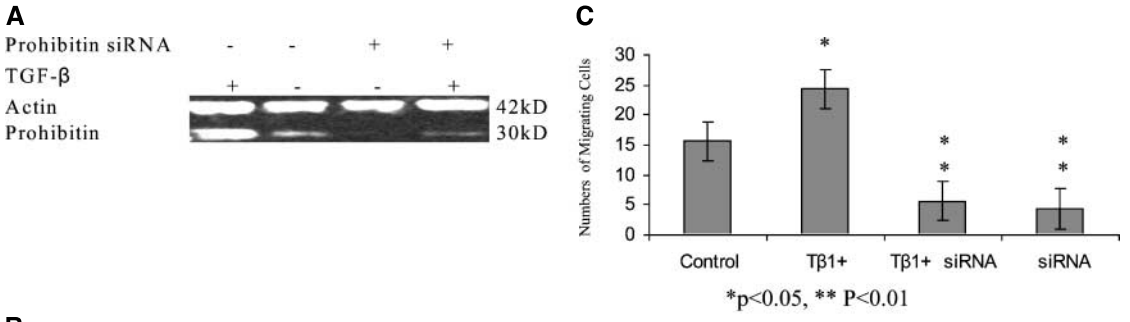

Figure 5. TGF-β induction of prostate cell migration requires prohibitin. LNCaP TβRII cells were transfected with 500 pmol prohibitin siRNA and, at 48 hours posttransfection, cell monolayer was wounded and exposed to TGFβ1 (5 ng/mL). Cells were subsequently harvested for Western blot analysis (*A*) or evaluated for their migration potential to the wounded areas (48 hours post-wounding) under a microscope (*B*); cell migration was defined as the average number of cells in three random fields per well (magnification, ×200). *C, columns,* average of three independent experiments. Statistical significance was achieved at $P < 0.01$. *, $P < 0.05$; **, $P < 0.01$.

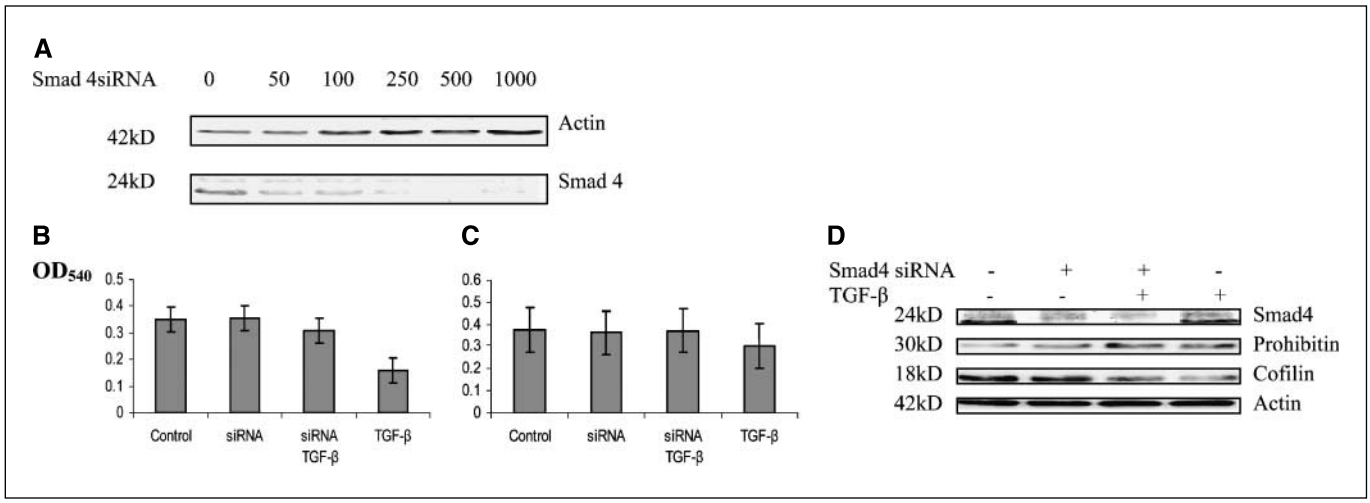

Figure 6. Impact of Smad4 silencing on prohibitin expression in prostate cells. LNCaP TβRII cells were transfected with increasing doses of Smad4 siRNA; 500 pmol of siRNA led to a significant suppression of Smad4 expression (*A*). The effect of TGF-β1 on cell viability before and after Smad4 siRNA silencing was assessed at 72 hours posttransfection. TGF-β1-mediated cell death was significantly decreased after Smad4 siRNA silencing in LNCaP TβRII cells (*B*) compared with the parental LNCaP cells (*C*). *Columns,* mean percent of cell viability from three independent experiments done in duplicate (500 pmol siRNA significantly blocked Smad4 expression). After 6 hours of TGF-β1 treatment, cytosolic fractions were subjected to Western blotting. TGF-β1 increases prohibitin (nuclear export) and decreases cofilin (mitochondrial translocation) protein levels in the cytosol despite the absence of Smad4 (*D*).

signaling in prostate cancer cells by bypassing Smad4 receptor activation. To dissect the functional involvement of prohibitin in the TGF-β/androgen interplay, we profiled prohibitin expression and localization during the combined apoptotic action of androgens and TGF-β in LNCaP TβRII cells. The evidence on the up-regulation of prohibitin by TGF-β as an early response, while dihydrotestosterone down-regulated prohibitin at a later period, points to the possibility of a negative feedback regulatory interplay between androgen receptor and TGF-β signaling. Support for this concept stems from reported studies by other investigators identifying Smad3 within the TGF-β signaling as a negative regulator of androgen receptor–mediated transcription in prostate cancer cells (42, 43).

Prohibitin forms a physical interaction with bcl-2 in response to TGF-β; this provides an intriguing mechanistic basis for the antagonistic role of bcl-2 in the apoptotic action of TGF-β signaling in prostate cancer cells (44). Temporary recruitment of bcl-2 to the mitochondria by prohibitin may diminish the ability of bcl-2 to suppress apoptosis in target cells, thus adding an alternative spatial-controlled regulatory layer. Indirect support for such a concept stems from our previous studies, documenting that bcl-2 suppresses the combined apoptotic effect of TGF-β and androgens in human prostate cancer cells by targeting cytochrome *c* release from the mitochondria (44). One could further argue that prohibitin nuclear export and cytoplasmic translocation is involved in the stabilization of adherent junctions in the TGF-β-treated prostate cancer cells, with important consequences on cell migration, anoikis, and metastasis. Such a function is supported by the present data indicating that loss of prohibitin function via siRNA silencing led to a dramatic inhibition of cell migration of LNCaP TβRII cells.

Other growth factors, such as insulin-like growth factor, epidermal growth factor and platelet-derived growth factor, can regulate cofilin phosphorylation (45, 46) and expression (47). Cofilin can serve not only as an actin-depolymerizing factor but can also promote actin polymerization and direct cell migration (45). Interestingly, mitochondrial translocation of cofilin in response to TGF-β was observed during apoptosis of prostate cancer cells, without requiring a functional Smad4. Cofilin dephosphorylation/activation emerges as a critical convergent point in an intracellular signaling network through which an extracellular stimulus, such as TGF-β, can potentially regulate actin cytoskeletal dynamics and potential apoptotic death of prostate cells. One must consider, however, that in other systems, diverse signaling stimuli are involved in the regulation of cofilin phosphorylation status. In insulin-stimulated cells, phosphatidylinositol 3-kinase and phosphatidylinositol-3,4,5-triphosphate are involved in cofilin dephosphorylation (48). In response to extracellular signals that elevate intracellular Ca^{2+} levels, calcium-induced cofilin dephosphorylation is mediated by Slingshot via calcineurin in HeLa cells (49), whereas in other cells, cyclic AMP and protein kinase C signals stimulate cofilin dephosphorylation (50). Considering the significance of changes in the intracellular Ca^{2+} in apoptosis induction, one could argue that cofilin dephosphorylation in response to TGF-β might be indirectly mediated via transient changes in Ca^{2+} levels. The essential role of cofilin in actin filament dynamics highlights the importance of dissecting signaling mechanisms that regulate cofilin phosphorylation and dephosphorylation to gain a better understanding of its targeting by TGF-β towards cystoskeletal remodeling in prostate tumors.

In summary, this study suggests a Smad4-independent mechanism involving topological distribution/cellular localization changes of prohibitin and cofilin that may functionally support a signaling network of TGF-β-mediated anoikis and/or apoptosis of prostate cancer cells. Concurrent loss of TβRII and cofilin may promote prostate cancer cell invasion by reducing apoptosis. Ongoing studies focus on defining the function of prohibitin and cofilin in anoikis, survival connections with extracellular matrix components, and metastasis of tumor epithelial and endothelial cells. Further understanding of the cross-talk between the androgen axis and TGF-β signaling in prostate cancer cells will enable the design of novel anoikis-modulating therapeutic strategies for advanced prostate cancer.

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