PTOV-1, a Novel Protein Overexpressed in Prostate Cancer, Shuttles between the Cytoplasm and the Nucleus and Promotes Entry into the S Phase of the Cell Division Cycle

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PTOV1 was recently identified as a novel gene and protein during a differential display screening for genes overexpressed in prostate cancer. The PTOV1 protein consists of two novel protein domains arranged in tandem, without significant similarities to known protein motifs. By immunohistochemical analysis, we have found that PTOV1 is overexpressed in 71% of 38 prostate carcinomas and in 80% of samples with prostate intraepithelial neoplasia. High levels of PTOV1 in tumors correlated significantly with proliferative index, as assessed by Ki67 immunoreactivity, and associated with a nuclear localization of the protein, suggesting a functional relationship between PTOV1 overexpression, proliferative status, and nuclear localization. In quiescent cultured prostate tumor cells, PTOV1 localized to the cytoplasm, being excluded from nuclei. After serum stimulation, PTOV1 partially translocated to the nucleus at the beginning of the S phase. At the end of mitosis, PTOV1 exited the nucleus. Transient transfection of chimeric green fluorescent protein-PTOV1 forced the entry of cells into the S phase of the cell cycle, as shown by double fluorescent imaging for green fluorescent protein and for Ki67, and also by flow cytometry. This was accompanied by greatly increased levels of cyclin D1 protein in the transfected cells. These observations suggest that overexpression of PTOV1 can contribute to the proliferative status of prostate tumor cells and thus to their biological behavior. *(Am J Pathol 2003, 162:897–905)*

Prostate adenocarcinoma is the second most prevalent cancer among males in Western countries, with an incidence that increases in direct proportion with age. A large body of evidence indicates that activation of the pathways regulated by androgen receptor plays a central role in the development and malignant progression of prostate cancer.[1] Because this is a physiologically regulated pathway, the question remains as to the underlying mechanisms that can direct the activation of the androgen receptor-signaling routes toward undue responses, eventually leading to malignant transformation of the prostate epithelial cells. In a few instances of primary tumors, mutations in the androgen receptor have been found that permit its activation by steroids other than androgens, or even other stimuli, independent of steroids.[2] Most frequently, however, androgen receptor mutations are a late event in the progression of prostate cancer,[3] and appear to be a major mechanism, along with androgen receptor gene amplification and overexpression,[4] by which these tumors become insensitive to androgens, and independent from these hormones for their growth.[5] This hormone-independent state is usually accompanied by an increase in the capacity of tumor cells to invade surrounding tissues and to form distant metastases.[6] In consequence, in the majority of prostate cancers other mechanisms must be invoked to explain the early events leading to the induction of the neoplastic phenotype.

Over the past few years, genetic and biochemical approaches have identified a number of molecular alterations that are associated with different stages of prostate tumorigenesis.[7–10] Systematic approaches using microarray-based global transcriptome analysis have also attempted to identify markers and pathways altered

in prostate cancer.[11–19] These studies have identified novel markers of early-stage prostate neoplasia,[14] as well as transcriptional signatures of advanced disease and poor prognosis.[18] In general, the possible biological significance of the altered expression of the genes identified in such analyses has not been addressed experimentally.

In our laboratory, we have recently identified a new protein, which we have designated PTOV1, that is overexpressed in a significant proportion of prostate adenocarcinomas.[20] Recent global transcriptional profile analyses by others[17] have found that *PTOV1* is one of the genes most discriminant between the normal and carcinomatous prostate. Importantly, we have found that PTOV1 is also overexpressed in early neoplastic lesions, the prostate intraepithelial neoplasias.[20] At present, the function of this protein is not known, nor can it be deduced or suggested from its sequence, because it does not contain any known protein motifs.[20] In this report we present an extended immunohistochemical study on samples from 38 prostate adenocarcinomas showing a correlation between proliferating tumor cells and levels of PTOV1 expression *in vivo*. We also describe cell-cycle-associated changes in subcellular localization of PTOV1 in prostate cancer cell lines, and the consequences on the cell cycle of its overexpression by transient transfection. Our observations suggest that overexpression of PTOV1 can have a direct influence on the proliferative state of prostate cells.

Materials and Methods

Antibodies

Rabbit antibodies to human PTOV1[20] were affinity purified using Sulfolink columns from Pierce (Rockford, IL), on which the immunizing peptide was covalently immobilized. Monoclonal anti-Ki67 antibody was from Immunotek (Marseille, France), anti-p27^{Kip1} antibody was from Novocastra (Edinburgh, UK) and anti-cyclin D1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-coupled anti-rabbit antibodies, tetramethyl-rhodamine isothiocyanate-coupled anti-rabbit antibodies were from Sigma (Alcobendas, Madrid, Spain) and fluorescein isothiocyanate-coupled anti-mouse antibodies were from DAKO (Glostrup, Denmark).

Immunohistochemistry

A total of 38 prostate adenocarcinomas from radical prostatectomies for T2 to T3 tumors were obtained from the files of the Department of Pathology of the Hospital Clinic of Barcelona. The mean patient age in this study was 63.5 ± 5.81 years (median, 64 years; range, 45 to 75 years). The mean preoperative serum prostate-specific antigen (PSA) was 11.50 ± 7.88 ng/ml (median, 9.6 ng/ml; range, 1.29 to 38 ng/ml). The Gleason sum score assigned to the radical prostatectomy specimens ranged from 4 to 9 and was evaluated as grade ≤6 (low, 17 patients) *versus* ≥7 (high, 21 patients). Normal or hyperplastic prostatic tissue could be evaluated in 29 of the

cases as well as 10 high-grade prostatic intraepithelial neoplasia (PIN) lesions. Also, normal prostatic tissue from six patients who underwent cystoprostatectomy for bladder tumors were included in the study. Two-μm sections from multitissue blocks[21] harboring 6-mm-diameter selected fragments were deparaffinized in xylene, and rehydrated with graded alcohols, water, and phosphate-buffered saline (PBS). Antigen unmasking was performed with pressure cooker heating in ethylenediaminetetraacetic acid buffer, pH 7, for 2 minutes, slides were allowed to cool down for 5 minutes and were sequentially incubated in vertical humid chambers with PBS (3 minutes) background suppressor (DAKO S3022, 30 minutes at room-temperature) and the primary antibody (2 μg/ml overnight, humid chamber at 4°C). Slides were then rinsed twice in PBS and endogenous peroxidase-quenched with 2% hydrogen peroxide in methyl alcohol for 30 minutes. After rinsing twice in PBS, slides were incubated for 30 minutes with Envision anti-rabbit antibody (Envision, DAKO). The staining was developed with substrate chromogen solution (Envision, DAKO) and diaminobenzidine for 3 to 5 minutes. The slides were counterstained with Gill-I hematoxylin for 1 minute, dehydrated, and mounted with DPX solution. Negative controls consisted in the use of 100 μl of the primary antibody previously incubated at 37°C for 1 hour with 0.16 mg of the purified peptide, the latter previously resuspended in 0.1 N of acetic acid, and used as above in parallel with similar slides with nonadsorbed primary antibody. The use of such preadsorbed primary antibody gave clean negative results in all cases tested (not shown). A semiquantitative gradation of the intensity was performed with intensities ranging from weak (intensity level 1) to very strong (intensity level 4). A case was considered positive when >10% of the cells showed unequivocal staining (levels ≥1). Both cytoplasmic and nuclear staining were recorded.

A similar procedure was followed for detection of Ki67 except that incubation of the primary antibody was for 30 minutes at room temperature and was preceded by antigen unmasking in citrate buffer. Ki67 was evaluated by counting the total of positive cells in all tumor fragments of the same size. A correction factor was used when the fragments were only partially involved by tumor. Staining for p27^{Kip1} was performed with the streptavidin-alkaline phosphatase method (Biogenex, San Ramon, CA) as previously described[22] and the percentage of positive cells was recorded.

Cell Culture

All cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cell lines PC-3, LNCaP, and COS7 were maintained in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) at 37°C in an atmosphere of 5% CO_2. The prostate cell line PZ-HPV-7 was maintained in Keratinocyte-Serum-Free Medium (Life Technologies, Inc.) supplemented with 5 ng/ml of human recombinant epidermal growth factor and 50 μg/ml of bovine pituitary extract.

Immunocytochemistry

Immunocytochemistry for confocal microscopy was performed as described.[20] Briefly, cells cultured on coverslips were washed with cold PBS, fixed with 4% paraformaldehyde for 10 minutes, incubated with 50 mmol/L of NH_4Cl for 30 minutes, and permeabilized with PBS/1% bovine serum albumin/0.1% saponin for 30 minutes. Samples were sequentially incubated with primary antibody diluted in permeabilization buffer for 2 hours, and secondary antibody for 1 hour, and mounted with Immuno-Fluore Mounting Medium (ICN, Costa Mesa, CA). Fluorescence was visualized by inverted fluorescence microscope DM IRBE (Leica, Wetzlar, Germany) and captured by a TCS-NT argon/krypton confocal laser (Leica).

Western Blotting

Cultured cells, washed with cold PBS, were lysed with Laemmli sample buffer [50 mmol/L Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol, β-mercaptoethanol] heated at 95°C and centrifuged at 10,000 $\times$ g. Fifty to 100 μg of cell lysate were electrophoresed and transferred to nitrocellulose. Membranes were blocked for 1 hour with blocking buffer (5% nonfat dry milk in PBS/1% Tween-20) and incubated for 2 hours with anti-PTOV1 (5 μg/ml in blocking buffer), washed, and incubated with horseradish peroxidase-conjugated goat anti-rabbit Ig. Reactivity was detected with a chemiluminescent substrate (Amersham, Buckinghamshire, UK). For sequential Western blotting, membranes were stripped at 50°C according to the manufacturer's instructions.

Transfections

Constructs for the expression of chimeric green fluorescent protein (GFP)-PTOV1 with GFP at the amino or carboxy termini of PTOV1 have been described previously.[20] Transient transfections were done with Lipofectamine (Life Technologies, Inc.). Twenty-four or 48 hours after transfection, cells were washed, fixed in 4% paraformaldehyde, processed for immunofluorescence with anti-PTOV1, mounted, and analyzed by confocal microscopy. Control transfections were performed with the vector pEGFP alone, for the expression of nonchimeric GFP, which consistently yielded a diffuse fluorescence distributed throughout the cells, without any subcellular specific localization.

Flow Cytometry

Cells were fixed in 70% ethanol in PBS at -20°C for at least 1 hour, washed several times with cold PBS, and treated with RNaseA (50 μg/ml) for 30 minutes at 37°C and incubated with propidium iodide (30 μg/ml). Cells were subsequently analyzed on a Coulter Epics XL flow cytometer (Coulter, Miami, FL) for GFP fluorescence (detection filter set at 525 nm) and DNA content (filter set at 675 nm). Cell aggregates were gated out, and 10,000 events were analyzed. For each cell type analyzed, untransfected cells were used to establish a threshold for green fluorescence (up to 10^2 arbitrary fluorescence units in a typical case), and which was taken as a threshold for positivity. GFP-positive and -negative populations were analyzed separately for DNA content and assigned to specific cell-cycle phases by applying the Multicycle cell-cycle analysis software (Phoenix Flow Systems, San Diego, CA).

Results

Immunohistochemical Analysis of Prostate Adenocarcinomas for the Expression of PTOV1 and Markers of Cell Proliferation

In our initial studies on tumor samples, most prostate adenocarcinomas analyzed by immunohistochemistry expressed high levels of PTOV1, without a correlation between these levels and clinicopathological parameters such as histological grade or clinical stage.[20] To test whether other parameters of *in vivo* biological activity, more directly associated with proliferative status, correlate with levels of PTOV1, we analyzed samples from 38 prostate adenocarcinomas and 6 normal prostates for the expression of PTOV1 and two other markers, the antigen Ki67, as an indicator of proliferating cells,[23] and the cyclin-dependent kinase inhibitor p27[Kip1], a regulator of the G_1 to S transition[24] that is frequently down-regulated in prostate cancer.[22,25,26] Immunohistochemistry for these markers was performed in serial or near-serial sections.

As previously reported, PTOV1 was mostly undetectable or showed a weak (level 1) cytoplasmic staining in normal prostate glandular epithelial cells (Figure 1). Interestingly, a more intense (level 3) staining was seen in sporadic luminal cells in isolated glands, which was mostly cytoplasmic but in some cases also nuclear. Staining of serial sections showed that these PTOV1-positive cells most likely corresponded to chromogranin A-positive cells, a marker for neuroendocrine cells (data not shown). Also, a very strong PTOV1 expression (levels 3 to 4) was observed in endothelial cells, erythrocytes (Figure 1A), and ganglion cells of vegetative ganglia. Therefore, most normal glandular epithelial cells usually do not express PTOV1, or do so at low levels, although several types of nonepithelial cells present in normal prostate structures can express PTOV1 at high levels.

Of the 38 adenocarcinoma samples analyzed, 27 (71%) overexpressed (ie, expression levels 2 to 3) PTOV1 in cells of carcinomatous appearance (Figure 1 and Table 1). The majority of the specimens positive for PTOV1 staining showed a homogeneous distribution of positive tumor cells throughout the carcinomatous areas, with >80% positive cells. Overexpression of PTOV1 was limited to the cytoplasm of carcinomatous cells in 16 cases, whereas it was strong (level 3) both in the nucleus and the cytoplasm in the remaining 11 cases (Table 2). Two cases were an exception to this situation in that tumor cells had a strong nuclear staining in combination with a weak cytoplasmic staining for PTOV1. There was no ob-
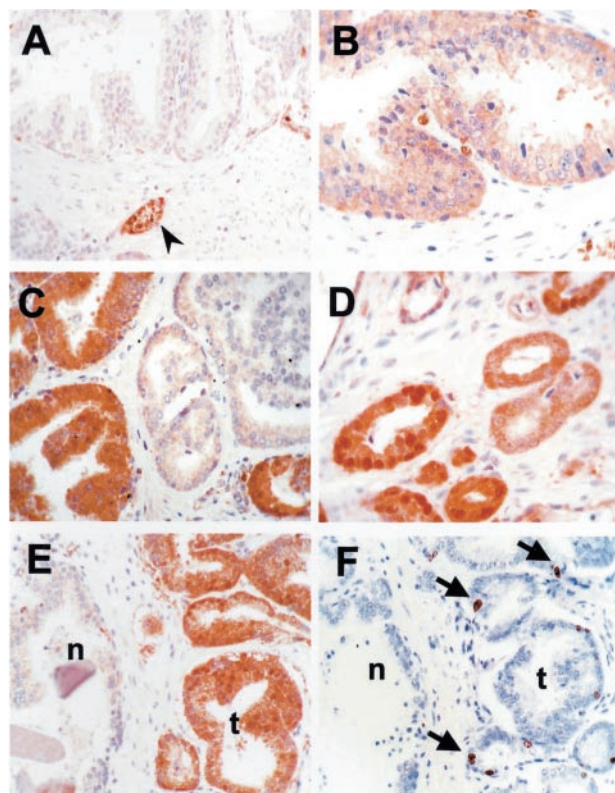
Figure 1. Immunohistochemical analysis of expression of PTOV1 and Ki67 in normal and tumor samples. **A:** Staining for PTOV1 of normal prostate epithelium, showing no detectable staining of glands, and a strong staining of blood vessels and red blood cells (**arrowhead**). **B:** High-grade PIN, showing a diffuse cytoplasmic staining for PTOV1 (level 2, see text and Materials and Methods). **C:** Staining for PTOV1 of a tumor sample, showing heterogeneous staining, with a strong positivity (level 3) in some glands, and faint to no staining in other tumoral glands. **D:** A tumor sample showing strong nuclear and cytoplasmic staining for PTOV1. **E** and **F:** Near-serial sections of a tumor sample containing glands with normal (n) and carcinomatous (t) morphology, stained for PTOV1 (**E**) and for Ki67 (**F**). **Arrows** indicate nuclei positive for Ki67 staining. Original magnifications, ×400.

Table 2. Expression of Ki67 and p27 in Relation to PTOV1 Levels and Subcellular Localization in Prostate Tumors

	PTOV1 level 1	PTOV1 level 2/3	*P*
Ki67 index*			
Total PTOV1	42.5	74.5	0.013
PTOV1 C only	42	77	0.015
PTOV1 C and N	0	83.6	0.010[†]
p27 (% positive cells)	26	27	0.714

*Total PTOV1, average of Ki67 index for all samples; PTOV1 C only, average of Ki67 index for samples with PTOV1 localized to the cytoplasm only; PTOV1 C and N, average of Ki67 index for samples with PTOV1 localized both to the cytoplasm and the nucleus.
[†]*P* value obtained by comparison with Ki67 values of tumors expressing low levels of PTOV1, with PTOV1 localized to the cytoplasm only.

a normal morphology (BPZ) were either very low (level 1) or undetectable (equal to or below background staining) (Figure 1 and Table 1).

The same samples were analyzed by immunohistochemistry for the expression of Ki67 and p27[Kip1]. Expression of Ki67 was assessed in the form of an index, calculated as a function of positive nuclei per total nuclei in sections of comparable size (see Materials and Methods). Normal prostate epithelium did not express Ki67 at significant levels (index <1). In tumor samples, areas of normal morphology did not show Ki67 staining or did so in very few cells (index <1), whereas the carcinomatous areas from the same samples had indexes that ranged from 4 to more than 100 (Figure 1F). When these indexes were compared with levels of expression of PTOV1, a significant correlation (*P* = 0.013) was found between Ki67 index and levels of PTOV1 (Table 2), indicative of an association of high levels of PTOV1 with an active proliferative status. Of interest, all of the tumor samples with nuclear localization of PTOV1 showed high levels (levels 2 to 3) of expression of PTOV1 and had elevated Ki67 indexes (Table 2). Expression of p27[Kip1] protein was evaluated in 18 of the 38 tumors. As shown in Table 2, no significant correlation was observed between levels of expression of PTOV1 and levels of p27[Kip1]. Patients with overexpression of PTOV1 (levels 2 or 3) showed higher PSA serum levels (12.62 ng/ml) compared to patients with low levels (level 1) (8.57 ng/ml), although this association did not reach statistically significant values (*P* = 0.056). When subcellular localization of PTOV1 was considered, independently of its levels of expression, pa-

vious association between gland morphology (differentiation) or topographical area of the tumor (periphery *versus* central) with the levels or the pattern of PTOV1 expression. Ten tumors also contained abnormal glands with morphological characteristics of PIN. Of these, eight showed strong cytoplasmic or cytoplasmic and nuclear staining for PTOV1 (Figure 1 and Tables 1 and 2) without significant differences in intensity between basal or luminal cells, and also contained a higher number of Ki67-positive cells. In the tumor samples analyzed, the expression levels of PTOV1 in the epithelial cells of glands with

Table 1. Summary of Immunohistochemical Analyses of PTOV1 Expression in Prostate Cancer and Normal Prostate

	PTOV1			*P*[†]
	Level 0* (%)	Level 1 (%)	Level 2/3 (%)	
Tumor				
Carcinoma	0	11/38 (29)	27/38 (71)	0.0001
PIN	0	2/10 (20)	8/10 (80)	0.0001
BPZ	6/29 (20.7)	22/29 (75.8)	1/29 (3.4)	
Normal	3/6 (50)	3/6[‡] (50)	0	

*Level 0, no detectable staining; level 1, low; and level 2/3, high levels of expression (see Materials and Methods).
[†]*P* values obtained by comparisons with BPZ.
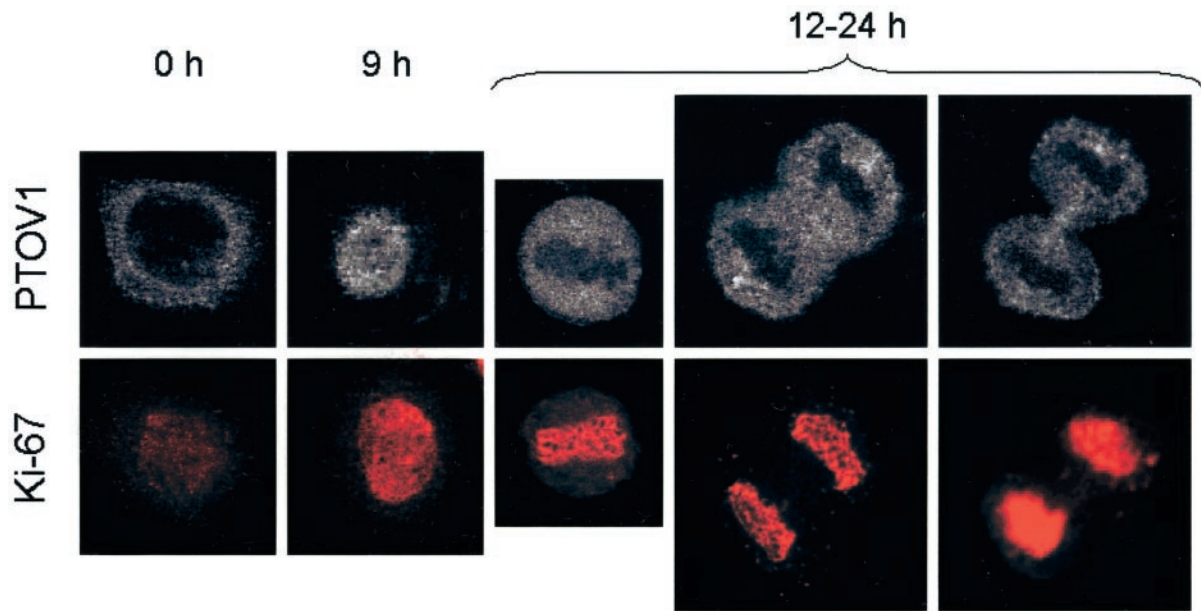[‡]Focal expression in isolated glands (<10% of cells).

Figure 2. Nuclear localization of PTOV1 in proliferating cells. PZ-HPV-7 cells were growth factor-deprived for 48 hours, stimulated with 10% fetal bovine serum for the indicated times, and stained for PTOV1 or Ki67. Growth factor-deprived cells (time 0) that did not show staining for Ki67 (quiescence) showed a cytoplasmic staining for PTOV1 with nuclear exclusion. Nine hours after serum stimulation, coincident with a nuclear staining for Ki67, PTOV1 translocated into the nucleus. In dividing cells (12 hours after stimulation), PTOV1 is excluded from Ki67-stained condensed chromosomes.

tients whose samples showed nuclear expression of PTOV1 had significantly ($P = 0.019$) higher preoperative serum PSA levels (13.83 ng/ml) than did patients whose samples had an exclusively cytoplasmic localization of PTOV1 (8.74 ng/ml). Increased levels of expression of PTOV1 were not associated with a higher Gleason score (≥ 7, not shown), confirming our previous observations.[20] In conclusion, high levels of expression of PTOV1 in prostate cancer correlated with an active proliferative status, as determined by elevated Ki67 indexes. Also, overexpression of PTOV1 was frequently associated with its nuclear localization, both in carcinomatous and PIN glands. Furthermore, nuclear localization of PTOV1 in the tumor was significantly associated with higher serum PSA levels.

Changes in Subcellular Localization of PTOV1 as a Function of the Cell Cycle

The above observations suggested the existence of a direct relationship between the proliferative status of prostate epithelial cells and the levels and subcellular localization of PTOV1. In our initial description, PTOV1 was found mainly in the cytoplasm but also in the nucleus of prostate cancer cells.[20] To study if the subcellular localization of PTOV1 was dependent on particular stages of the cell cycle, PC-3 and PZ-HPV-7 prostate cancer cells were serum-starved for 48 hours, allowed to re-enter the cell cycle by re-addition of serum to the culture medium, and analyzed by immunocytochemistry for the expression of PTOV1. To monitor the proliferative status of the cells in these experiments, we used the marker Ki67,[23] an antigen that is not expressed in quiescent cells, that shows a nuclear pattern in the first part of S and a perinucleolar pattern toward the end of S, and is

associated with condensed chromosomes throughout mitosis.[27] To further assess the cell-cycle status of the cells, double-fluorochrome immunocytochemistry was also performed for cyclin D1, which, after a mitogenic stimulus, exits the nucleus at the beginning of S.[28] After 48 hours of growth in serum-free medium, the majority of the cells were in proliferative quiescence as shown by lack of expression of Ki67, and PTOV1 was detected in a cytoplasmic localization, with nuclei clearly excluded from staining (Figure 2). Nine hours after the addition of serum, Ki67 was detected in the nucleus of the majority (50 to 80%) of the cells, indicating entry into the S phase of the cell cycle, and PTOV1 was seen both in the cytoplasm and in the nucleus (Figure 2). The nuclear localization of PTOV1 was maintained throughout the S phase, including the stage in which Ki67 concentrates around the nucleolus (Figure 2). In mitotic cells, with Ki67 associated with condensed chromosomes, most of the signal for PTOV1 excluded the chromosomes (Figure 2). Twenty-four hours after stimulation of quiescent cells with serum, PTOV1 was seen again mostly in the cytoplasm, although some nuclei retained positivity. This distribution of PTOV1 is equivalent to that originally observed in asynchronously growing cells.[20]

Cyclin D1, which is necessary for the progression of the cell cycle from G_1 to S phase,[28] is localized in the nucleus at late G_1, and is exported from the nucleus or degraded at the beginning of the S phase.[29] We studied whether the nuclear shift of PTOV1 in serum-stimulated cells followed a similar pattern. In quiescent PC-3 cells, little or no specific staining for cyclin D1 was detected (Figure 3). Nuclear translocation of the protein was observed at 3 hours after stimulation of the cells with serum, corresponding to approximately late G_1 phase and up to
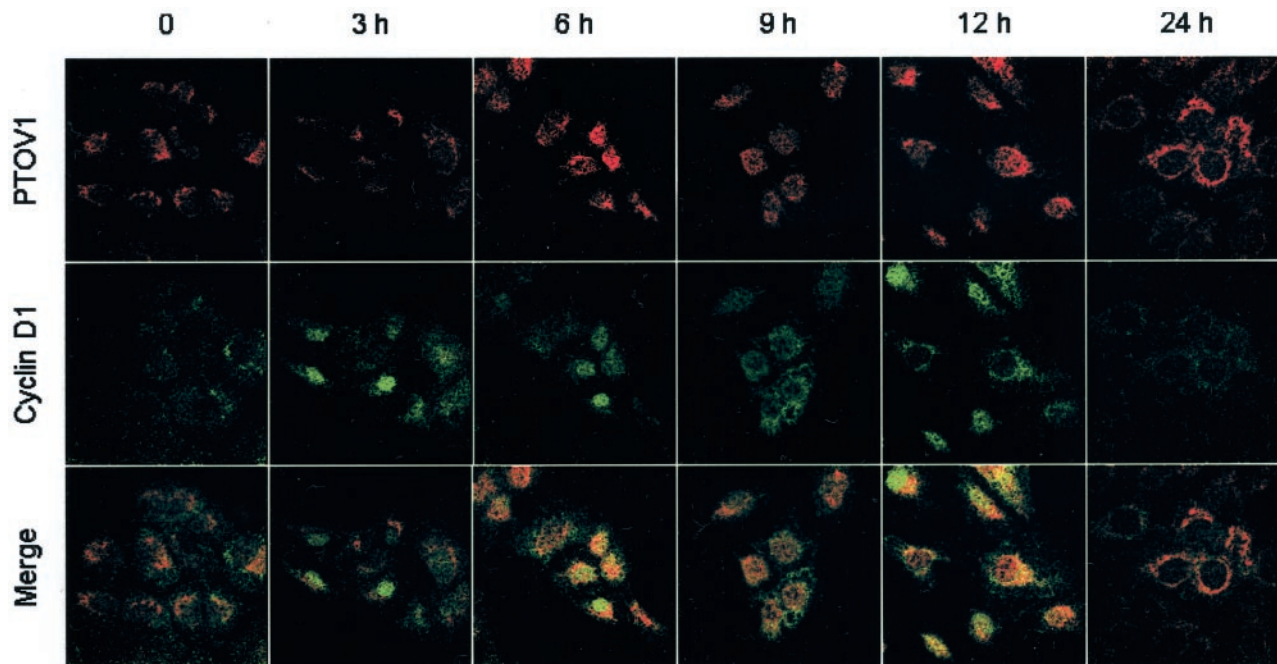
Figure 3. Nuclear entry of PTOV1 is concomitant with nuclear exit of cyclin D1. PC-3 cells were growth factor-deprived for 48 hours, stimulated with 10% fetal bovine serum for the indicated times, and stained by double fluorescence for PTOV1 and cyclin D1. In quiescent cells (time 0), PTOV1 is localized exclusively in the cytoplasm, whereas cyclin D1 is barely detectable. From 3 to 6 hours after stimulation with serum, cyclin D1 is detectable in the nucleus of most cells. PTOV1 is found in the nucleus at increasing proportion at 9 to 12 hours after serum stimulation, and its nuclear localization is most evident in cells in which cyclin D1 has been excluded from the nucleus (12 hours). At 24 hours after stimulation, cells show either a cytoplasmic or a nuclear localization of PTOV1, together with low levels of cyclin D1.

9 hours, corresponding to entry into S phase, in agreement with observations previously reported for other cell types.[29,30] Twelve hours after stimulation, a diffuse staining for cyclin D1 was observed in the cytoplasm, and weak or no staining was detectable at 24 hours (Figure 3). Again, specific cytoplasmic staining for PTOV1 was observed in quiescent cells and 6 to 9 hours after serum stimulation PTOV1 entered the nucleus. In the majority of cells, the nuclear localization of PTOV1 was coincidental with the exit of cyclin D1 from the nucleus (Figure 3). At 24 hours after serum stimulation, PTOV1 was observed mostly in the cytoplasm.

To determine whether these changes in subcellular localization of PTOV1 were accompanied by changes in protein levels, Western blotting was performed on serum-starved and serum-induced PC-3 and LNCaP cells. The levels of PTOV1 did not change significantly at any time after stimulation of these cells with serum (not shown). In conclusion, PTOV1 oscillates between the cytoplasm and the nucleus of prostate cancer cells in a cell-cycle-dependent manner, with nuclear concentration of the protein beginning in the S phase and peaking in mitosis. These changes in subcellular localization are not accompanied by changes in PTOV1 protein levels.

Effects of the Overexpression of PTOV1 on the Cell-Cycle Status of Prostate Cancer Cells

Because PTOV1 shows specific subcellular localizations that change according to the proliferative state, we reasoned that this protein could play a regulatory role in one

or more of the cell-cycle transitions and that the artificial expression of high levels of PTOV1 in cultured cells could produce a significant accumulation of cells in that phase of the cell cycle at which this protein exerts a predominant regulatory role. For this purpose, we used a construct for the expression of PTOV1 with the green fluorescent protein (GFP) in frame at the amino terminus of PTOV1 (GFP-PTOV1).[20] This construct was introduced in transient transfection experiments with cationic liposomes into the prostate cells lines PC-3 and PZ-HPV-7, and the green monkey kidney fibroblast line COS7, and analyzed after 24 hours by counting the number of cells positive for both Ki67 and PTOV1 (Figure 4). Controls for these experiments were untransfected cells and cells transfected with the vector pEGFP. As shown in Figure 4, in PC-3, PZ-HPV-7, and COS7 cells the number of cells positive for Ki67 is significantly higher in cells transfected with the fusion protein GFP-PTOV1 compared to cells transfected with GFP only, suggesting that expression of PTOV1 induces entry of the cells into a proliferative state.

To study this effect more precisely, the cell-cycle status of cells overexpressing PTOV1 was assessed by flow cytometry 24 or 48 hours after the transfections. Three types of controls were used in these experiments. First, untransfected cells; second, transfections with GFP expression constructs that do not carry PTOV1; and, third, cells from the same transfections with the GFP-PTOV1 construct that show levels of GFP below background (GFP-negative population), and thus correspond to cells that either have not been transfected in those experiments or to cells that do not express the fusion protein
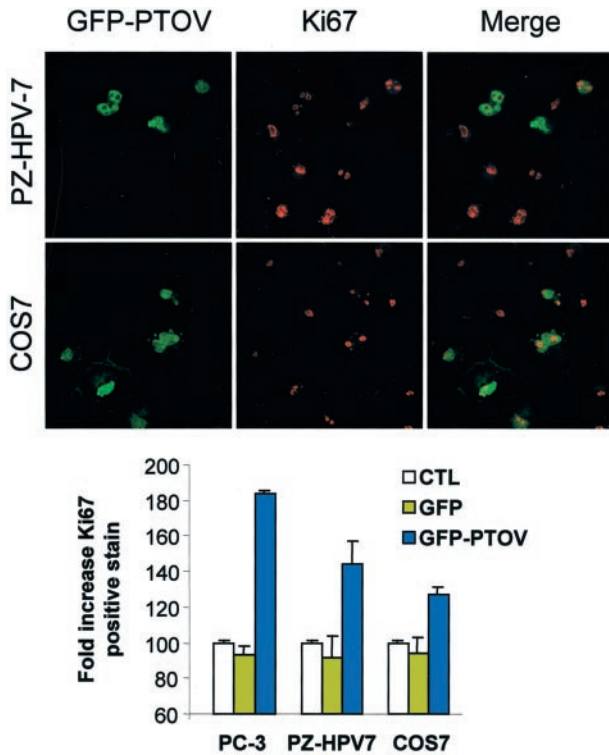
Figure 4. Overexpression of GFP-PTOV1 induces cell proliferation. PZ-HPV-7 and COS7 cells were transfected with GFP-PTOV1 (green fluorescence) and stained for Ki67 (red fluorescence). Cells positive for GFP-PTOV1, Ki67, or both were quantitated, and the relative numbers were compared to untransfected cells (histogram). As controls, cells were transfected with vector alone (GFP).

above the established threshold. The transfection efficiencies ranged from 28 to >90%, measured as the percentage of cells with a GFP signal above background. For each transfection, the distribution of DNA and cell-cycle profile were analyzed separately for GFP-positive and GFP-negative populations, thus providing the cell-cycle profiles for cells expressing, or not expressing, the transfected proteins in the same experiment. Untransfected PC-3, PZ-HPV-7, and COS7 cells; cells transfected with GFP vector alone; and GFP-negative cells from the GFP-PTOV1 transfections had similar cell-cycle distributions (Figure 5). For cells from transfection experiments, these distributions did not show significant differences between those harvested at 24 hours and those harvested at 48 hours after transfection. Therefore, the transfection procedure per se, or the expression of GFP alone, did not have significant effects on the cell-cycle profile of PC-3, PZ-HPV-7, or COS7 cells. In contrast, GFP-positive cells from transfections with GFP-PTOV1 showed a clear increase in the proportion of cells in the S phase when compared with any of the controls (Figure 5). This increase in the proportion of cells in S was accompanied by a corresponding decrease of the population of cells in G_1 (Figure 5). These results confirm that the overexpression of PTOV1 promotes the entry into the S phase of the cell cycle of PC-3, PZ-HPV-7, and COS7 cells. In parallel experiments, we determined that cells forced to entry into S phase by the ectopic expression of PTOV1 contain significantly higher levels of the cyclin D1

protein, as shown by Western blotting of lysates from cells transfected with PTOV1 (Figure 5E). These results show that PTOV1 induces progression through the cell cycle by enhancing entry into the S phase.

Discussion

We report here the cell-cycle-dependent shifts in subcellular localization of PTOV1, a novel protein of unknown function, and that its overexpression drives cells into a proliferative state. The distinguishing structural feature of PTOV1 is its duplicated domain, of which a single copy is present also in a second protein that we have called PTOV2.[20] Very recently deposited sequence entries (GenBank accession AAM20739) indicate that the latter protein could be identical to the transcriptional co-regulator ARC92.[31] Nevertheless, the PTOV domain does not resemble any other protein motif,[20] and thus examination of its sequence, per se, does not provide useful functional clues. Despite this lack of *a priori* functional knowledge, the fact that PTOV1 is overexpressed in a significant proportion of prostate cancers[17,20] led us to suggest that this gene and protein could have a role in the biological behavior of these tumors.[20] This notion is now reinforced by our observations, that successively associate 1) *in vivo*, high levels of PTOV1 with proliferative status and nuclear localization in carcinomatous cells; 2) *in vitro*, nuclear exclusion of PTOV1 with proliferative quiescence and nuclear localization with the S and M phases of the cell cycle; and 3) overexpression of transfected PTOV1 with induction of proliferation and entry into the S phase of the cell cycle.

The correlation found in tumor samples between high levels and nuclear localization of PTOV1 with elevated proliferative indexes, suggests a direct link of PTOV1 and the proliferative status of the tumor cells. This relationship was strongest in samples in which PTOV1 was localized in the nucleus. The statistically significant correlation found between tumors with PTOV1 expression in the nucleus and higher PSA serum levels might give additional support to a role for PTOV1 in the aggressive behavior of prostate tumors, because higher PSA levels are associated with more locally advanced disease.[32] On the other hand, the lack of correlation between PTOV1 expression and p27 levels, or the Gleason score, could be related to the fact that the cases analyzed did not include advanced stage tumors with highly undifferentiated morphologies. Loss of expression of p27 has been associated with advanced prostate tumors and high Gleason scores,[22,25,26] and therefore it would be interesting to study the levels of expression of PTOV1 in a set of samples corresponding to more advanced disease.

The correlation observed in tumor samples between Ki67 index and PTOV1 levels and subcellular localization prompted us to investigate in more detail the relationship between the cell-cycle status of the cells, the subcellular localization of PTOV1, and its overexpression. In our time-course experiments with PC-3 prostate cells, the nuclear translocation of PTOV1 occurred 9 hours after serum stimulation of quiescent cells, coincident with the exit of
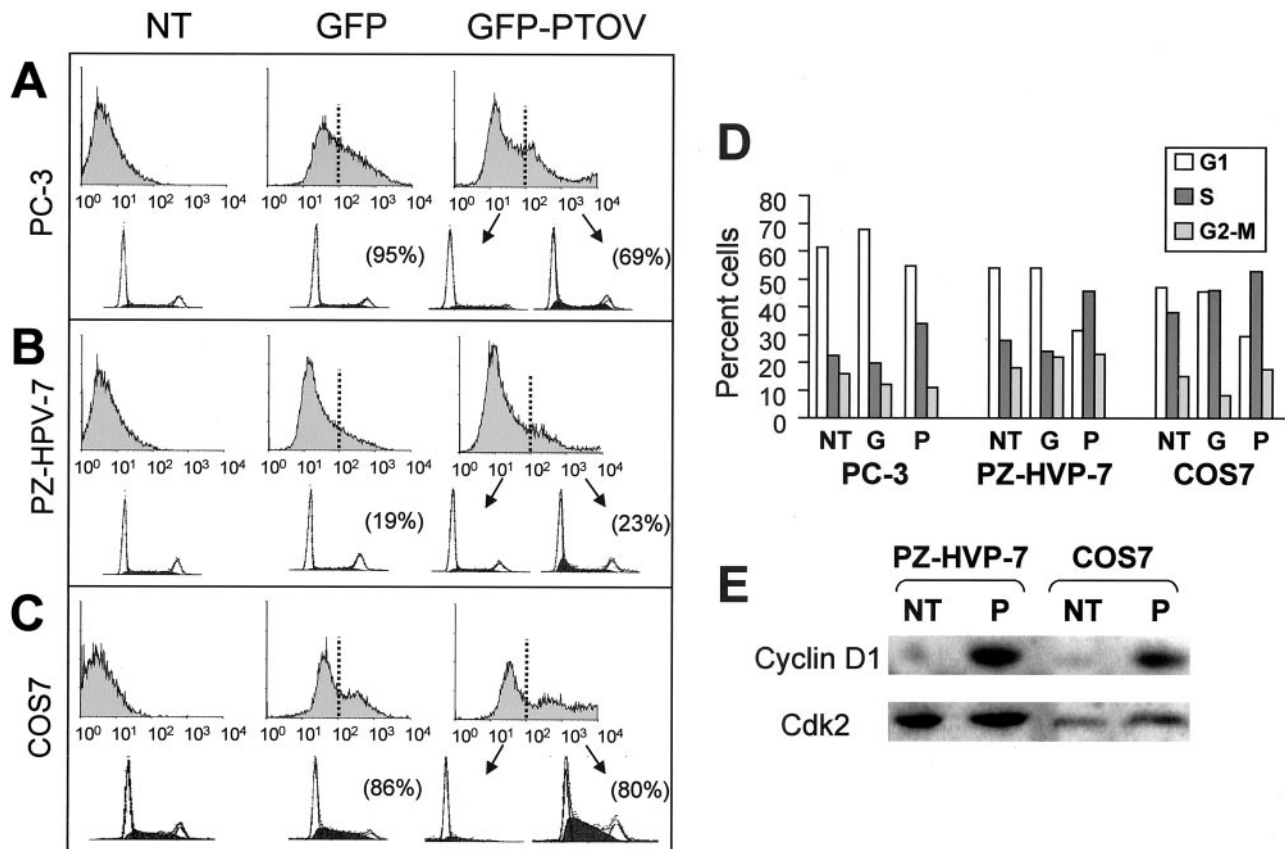
Figure 5. Overexpression of GFP-PTOV1 forces entry into the S phase of the cell cycle. **A—C** Flow cytometry analysis of untransfected cells (NT), cells transfected with vector alone (GFP), or with GFP-PTOV1. The threshold for positivity for GFP was established as the levels corresponding to untransfected cells. Cells with green fluorescence below (not transfected) or above (transfected) this threshold were analyzed separately for DNA content. The histograms at the **top** of each panel correspond to GFP levels, and the histograms at the **bottom** of each panel correspond to analysis of DNA content and cell-cycle assignments. For each experiment, transfection efficiency is shown in **brackets**. **D:** Histograms of distribution of cell populations to the G_1, S, or G_2 to M phases of the cell cycle, corresponding to the flow cytometry analysis on the **left** panels. One representative experiment of four performed with each cell line is shown. **E:** Levels of cyclin D1 are elevated in cells transfected with GFP-PTOV1. PZ-HPV-7 or COS7 cells were either not transfected (NT) or transfected (P) with GFP-PTOV1, lysed 24 hours after transfection, and analyzed by Western blotting with antibodies to cyclin D1 or CDK2.

cyclin D1 from the nucleus, which marks the beginning of the S phase.[28] PTOV1 remained in the nucleus until the end of mitosis, suggesting a role for this protein not only in S, but also throughout G_2 and M. The fact that entry of PTOV1 into the nucleus did not occur early after serum stimulation, but only after a 9-hour lag, suggests a functional link between nuclear translocation of PTOV1 and the cell-cycle regulatory machinery, and also that this translocation is not a direct or immediate consequence of the stimulus, as is the case for the nuclear import of certain proteins that are part of signaling cascades.[33,34] The enhanced entry of cells in S as a result of the forced expression of GFP-PTOV1 points to an active role of this protein in this stage of the cell cycle. A number of cell-cycle regulatory proteins undergo nuclear-cytoplasmic shifts in association with specific stages of the cell division cycle[28,30,35] and their localization to given subcellular compartments can determine the rate of progression through one or more cell-cycle transition points.[36] As discussed above, cyclin D1 localizes in the nucleus during G_1, and is actively exported to the cytoplasm during the S phase,[28] in a process dependent on phosphorylation by GSK-3β[29] and on p21^{Cip1} and p27^{Kip1}.[37] Mutant forms of cyclin D1 that fail to translocate in this way and

do not form functional cyclin-CDK complexes cause the accumulation of cells in G_1.[38] This could be relevant to our present findings, because the facts that PTOV1 drives the G_1 to S transition and that the levels of cyclin D1 greatly increase after overexpression of GFP-PTOV1 point to the CDK4-cyclin D1 complex as a potential target for positive regulation by PTOV1, either by increasing the stability of the cyclin D1 subunit or by inducing its expression. Alternatively, PTOV1 could negatively regulate one or more inhibitors of the G_1 cyclin-dependent kinases.

Taken together, our observations suggest that the overexpression of PTOV1 can play an active role in the proliferative status of neoplastic prostate epithelial cells, and thus in their biological behavior. The characterization of the molecular mechanisms by which PTOV1 exerts its mitogenic effects will require further exploration.

Acknowledgments

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