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Inhibition of Prostate Cancer Cell Growth by Human Secreted PDZ Domain 2 Protein, a Potential Autocrine Prostate Tumor Suppressor

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A possible role of the PDZ domain-containing protein 2 (PDZD2) in prostate tumorigenesis has been suggested. Besides, PDZD2 is posttranslationally cleaved by a caspase-dependent mechanism to form a secreted PDZ domain-containing protein 2 (sPDZD2) with unknown functions in humans. In this study, we demonstrate the endogenous expression of PDZD2 and secretion of sPDZD2 in cancerous DU145, PC-3, 22Rv1, LNCaP, and immortalized RWPE-1 prostate epithelial cells. Inhibition of endogenous sPDZD2 production and secretion by DU145, PC-3, 22Rv1, and RWPE-1 cells via the caspase-3 inhibitor Z-DEVD-FMK resulted in increased cell proliferation, which was abrogated by treatment with exogenous recombinant sPDZD2. Whereas sPDZD2-induced antiproliferation in DU145, PC-3, and 22Rv1 cells, it induced apoptosis in LNCaP cells. The data suggest that endogenous sPDZD2, produced by caspase-3-mediated cleavage from PDZD2, may function as a novel autocrine growth suppressor for human prostate cancer cells. The antiproliferative effect of sPDZD2 was apparently mediated through slowing the entry of DU145, PC-3, and 22Rv1 cells into the S phase of the cell cycle. In DU145 cells, this can be attributed to stimulated p53 and p21Cip1/WAF1 expression by sPDZD2. On the other hand, the apoptotic effect of sPDZD2 on LNCaP cells was apparently mediated via p53-independent Bad stimulation. Together our results indicate the presence of p53-dependent and p53-independent PDZD2/sPDZD2 autocrine growth suppressive signaling pathways in human prostate cancer cells and suggest a novel therapeutic approach of harnessing the latent tumor-suppressive potential of an endogenous autocrine signaling protein like sPDZD2 to inhibit prostate cancer growth. (Endocrinology 147: 0000–0000, 2006)
pathogenesis has recently been suggested by a significant increase in PDZD2 (AIPC) gene and protein expression in prostate cancer tissues (12). Besides, the protein has also been shown to be posttranslationally cleaved by a caspase-dependent mechanism to form a secreted PDZ domain-containing protein 2 (sPDZD2) (13). Taken together, it would be of interest to determine whether sPDZD2, a posttranslationally cleavage product of PDZD2 (AIPC), may act as a novel autocrine signal important in prostate cancer pathogenesis. In this report, we studied the expression of sPDZD2 in human prostate cancer cells as well as the actions and mechanisms of sPDZD2 on prostate cancer cell growth modulation.

Materials and Methods

Human cancerous-immortalized cell lines and recombinant sPDZD2 synthesis

Human prostate cancer cell lines LNCaP.FGC (CR/L-1740), DU145 (HTB-81), PC-3 (CRL-1435), and 22Rv1 (CRL-2505) as well as a human papillomavirus 18 transformed human prostate epithelial cell line RWPE-1 (CRL-11609) were obtained from American Type Culture Collection (Manassas, VA). LNCaP and 22Rv1 cells were propagated in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 1-glutamine and 10% fetal bovine serum (FBS) (Life Technologies), whereas DU145 and PC-3 cells were, respectively, propagated in Eagle’s MEM (Life Technologies) and F-12 (Ham) (Life Technologies) medium supplemented with 10% FBS. RWPE-1 cells were cultured in keratinocyte-serum free medium supplemented with 50 μg/ml bovine pituitary extract and 5 ng/ml human recombinant epidermal growth factor (Life Technologies). All cell lines were incubated at 37 C with 5% CO2 humidified atmosphere. Recombinant human sPDZD2 was synthesized and purified as previously described using the IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag)-CN system (New England Biolabs, Beverly, MA) (16).

Cell proliferation, viability, and apoptosis assays

DU145, PC-3, 22Rv1, and RWPE-1 cells (2 × 104/ml) were seeded in 96-well plates and incubated with or without 10−8, 10−7, and 10−6 M purified recombinant sPDZD2 or vehicle control for 24 and 48 h, respectively. Cells were washed 2× with PBS, and passive lysis buffer was added to each well before the cells were processed for cell proliferation studies. In separate experiments, DU145, PC-3, 22Rv1, and RWPE-1 cells were incubated with 10 μM specific caspase-3 peptide inhibitor, Z-DEVD-FMK (BD Biosciences, San Jose, CA), or 10 μM specific caspase-8 peptide inhibitor, Z-IETD-FMK (BD Biosciences), or 10 μM negative control peptide Z-FA-FMK (BD Biosciences), or vehicle for 24 and 48 h. In addition, DU145, PC-3, 22Rv1, and RWPE-1 cells were each coincubated with 10 μM Z-DEVD-FMK and 10 μM sPDZD2 for 48 h. Cell proliferation was measured by a tetrazolium-based Cell Titer 96 AQsens assay kit (Promega, Madison, WI). Absorbance at 490 nm was recorded 3 h after 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) addition. The proliferation of the cells was also monitored by a cell proliferation ELISA 5-bromo-2-deoxyuridine (BrdU) (colorimetric) kit (Roche, Stockholm, Sweden), according to the manufacturer’s protocol. Cell viabilities of LNCaP, DU145, PC-3, 22Rv1, and RWPE-1 cells, incubated with or without 10−7, 10−6, and 10−5 M purified recombinant sPDZD2 for 24 and 48 h, were measured by trypan blue dye exclusion assays. The number of viable cells was counted using hemocytometers. Any apoptotic effect was determined using Annexin V-FITC (BD Biosciences) and propidium iodide (PI) staining (BD Biosciences) and analyzed using the FACScan (Becton Dickinson). The percentage of viable cells was calculated using FACSDiva software (Becton Dickinson).

Immunoblot analyses

For studies on the expression of PDZD2 and sPDZD2, PBS-washed native cancerous or immortalized prostate epithelial cells were incubated in their respective culture media without any added FBS for 24 h before the cells and conditioned media were collected for immunoblotting with the rabbit anti-PDZD2 antibody, which has been described previously (13). To study the effect of caspase inhibitors, the cells were incubated with or without 10 μM caspase-3 inhibitor Z-DEVD-FMK, 10 μM caspase-8 inhibitor Z-IETD-FMK, or 10 μM negative control peptide Z-FA-FMK under serum-free conditions for 48 h before immunoblotting analysis. For studies on the effects of sPDZD2 on the expression of proteins involved in cell cycle and apoptosis control, prostate cancer and normal prostate cancer cells were harvested in lysis buffer (10 mM Tris-HCl (pH 7.4), 1% sodium dodecyl sulfate (SDS)) after they had been incubated with or without sPDZD2 for different time intervals. Lysates in sample buffer (0.2% SDS, 10% glycerol, 0.06 M Tris-HCl (pH 6.8), 100 mM dithiothreitol, and 0.01% bromophenol blue) were heated at 95 C for 5 min. Proteins in the conditioned media and recombinant sPDZD2 protein standards (10−12 to 10−7 M) were concentrated 50-fold using YM-10 Centricons (Millipore, Bedford, MA). Samples (10 μg) were resolved by SDS-PAGE and electroblotted to polyvinyl difluoride membranes (Millipore). The blots were blocked with 5% nonfat milk powder in TBS-T for 1 h at room temperature and then incubated with rabbit anti-PDZD2 antibody (1:10,000 dilution) overnight at 4 C. After washing with TBS-T, the blots were incubated with secondary antibodies against rabbit IgG (Zymed Madrid, San Francisco, CA) and the blots were visualized by enhanced chemiluminescence Western blotting system (Amersham Biosciences, Piscataway, NJ).

To study the expression of p21CIPI/WAF1, p27KIP1, cyclin B, cyclin D, Bad, polyclonal (polyhistidine) polymericase (PARP), and p53 as well as the phosphorylation status of p53, the blots were incubated with primary antibodies against p21CIPI/WAF1 (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), p27KIP1 (1:500 dilution; Santa Cruz Biotechnology); cyclin B (1:500 dilution; Santa Cruz Biotechnology); cyclin D (1:500 dilution; Santa Cruz Biotechnology); Bad (1:500 dilution; Santa Cruz Biotechnology); PARP (1:500 dilution; Santa Cruz Biotechnology); p53 (1:500 dilution; Santa Cruz Biotechnology); or primary phospho-p53 antibodies against Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392 phosphorylation sites (1:1000 dilution; Cell Signaling Technology, Beverly, MA) overnight at 4 C. After washing with TBS-T, the blots were incubated with secondary antibodies against mouse IgG (Amersham Biosciences) for p21CIPI/WAF1, p27KIP1, cyclin B, cyclin D, Bad, PARP, and p53 antibodies or secondary antibodies against rabbit IgG (Zymed Laboratories) for phosphory-p53 antibodies. Blots were stripped in 25 mm glycine buffer (pH 2.0) for 30 min for reprobing with α-tubulin (1:500 dilution; Santa Cruz Biotechnology). The signals were visualized by enhanced chemiluminescence Western blotting system (Amersham Biosciences), and densitometric analyses of the developed blot normalized against α-tubulin were performed.

Transient reporter assay

DU145 cells (5 × 103/ml) were seeded onto 24-well plates. After 24 h, the cells were transiently transfected with Fugene 6 reagent (Roche). To each well, a mixture of 0.6 μl Fugene 6, 0.2 μg p21CIPI/WAF1 reporter construct pGL3-21pro or p27KIP1 reporter construct pGL3-27pro, and 0.04 μg Renilla luciferase reporter pRL-tk (Promega) were added. sPDZD2 (10−9, 10−8, and 10−7 M) or vehicle was added directly to the medium at one tenth dilution 24 h after transfection. After 24 and 48 h of incubation, Dual-Luciferase reporter assay system (Promega) was used to measure both the firefly and Renilla luciferase activities, according to the manufacturer’s instructions. Briefly, the cells were washed thrice with PBS. Passive lysis buffer was added to each well and was shaken at room temperature for 15 min. Lysate (20 μl) from each sample was transferred to a 96-well plate for firefly and Renilla luciferase activity measurement by the microplate luminometer LB96V (EG&G Berthold, Bad Wildbad, Germany).

RNA preparation and semiquantitative RT-PCR analyses

Total RNA was extracted from DU145 cells treated with or without 10−7, 10−8, and 10−9 M sPDZD2 for 24 h using TRIzol (Invitrogen, Life Technologies). Total RNA (1 μg) was converted into cDNA (2 μl) using the Superscript II reverse transcriptase (Life Technologies) according to the manufacturer’s protocol. The cDNA (2 μl) was used as the template for the polymerase chain reaction (PCR) amplification on a thermocycler (Biometra). The PCR products were visualized on an agarose gel and the intensities of the bands were compared with relevant controls. The following primer pairs were used for amplification of the human DAX1 gene (20 bp): forward, GGAAGAGTGGCCGTTTCTAT; reverse, TCTGGCTGGTGGATAGTGTG.
Carlsbad, CA), and the RNA (500 ng) was then reversely transcribed into cDNA by the Superscript III first-strand synthesis system (Invitrogen) following the manufacturer’s instructions. $p_21^{CIP1/WAF1}$ cDNA was amplified by PCR using specific forward primer 5′-GGGCTTCCTCTTGGAGAA- GAT-3′ and reverse primer 5′-GGGCTTCCTCTTGGAGAA- GAT-3′ designed from published $p_21^{CIP1/WAF1}$ cDNA sequence as reported previously (18). Similarly, $p_27^{KIP1}$ cDNA was amplified by PCR using specific forward primer 5′-CCAGCGAGATTAACCCCGG-3′ and reverse primer 5′-GTCTGTCCACAGAAACCCGGG-3′ designed from published $p_27^{KIP1}$ cDNA sequence as described previously (18). Glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified by PCR using specific forward primer 5′-CGACGGGGGACCAAA- GGG-3′ and reverse primer 5′-GGCAGCCCCAGGCTGCAAAG-3′. cDNA amounts and cycle numbers were optimized to ensure that amplification was within the linear range for quantitative analysis. The PCR contained 2 μl cDNA, 1 pmol/μl primers, 1× deoxynucleotide triphosphate, 1× PCR buffer, 5 μM MgCl2, and 1 U Taq DNA polymerase. The 40 cycles of PCR amplification were preceded by denaturation at 94 °C for 2 min. Each PCR cycle consisted of denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 sec, and extension at 70 °C for 1 min. After the reaction, 10 μl of the PCR products were separated on an agarose gel [1.5% (wt/vol)]. The amplified $p_21^{CIP1/WAF1}$ and $p_27^{KIP1}$ cDNAs, normalized against glyceraldehyde-3-phosphate dehydrogenase cDNA in the same preparations, were quantitated.

Flow cytometry

Exponentially growing cells DU145, PC-3, and 22Rv1 were seeded in 25-cm² flasks at approximately 90% confluency. Cells were maintained after the reaction, 10 μl of the PCR products were separated on an agarose gel [1.5% (wt/vol)]. The amplified $p_21^{CIP1/WAF1}$ and $p_27^{KIP1}$ cDNAs, normalized against glyceraldehyde-3-phosphate dehydrogenase cDNA in the same preparations, were quantitated.

Statistical and data analysis

The data were analyzed with one-way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t-test. The level of significance for all statistical analyses was set at $P < 0.05$.

Results

Endogenous PDZD2 expression and sPDZD2 secretion in cancerous and immortalized prostate epithelial cells

Chaib et al. (12) reported up-regulation of the full-length PDZD2 protein in prostate cancer, but expression of the secreted form of PDZD2, sPDZD2, has not been similarly studied. Using a specific anti-PDZD2 antisemur that recognize both the full-length and secreted forms of PDZD2 (13), we analyzed the lysates and conditioned media from cultured LNCaP, DU145, PC-3, 22Rv1, and RWPE-1 cells by immunoblotting. Full-length PDZD2 (301 kDa) and sPDZD2 (37 kDa) were detected, respectively, in the cell lysates and conditioned media of all the prostate cell lines (Fig. 1A). The lack of sPDZD2 expression in the cell lysates suggests that sPDZD2 is predominantly secreted rather than stored intracellularly after its cleavage from PDZD2.

Effects of caspase inhibitors on endogenous PDZD2 expression and sPDZD2 secretion and cell growth

Full-length PDZD2 protein, which exhibits close sequence homology to pro-IL-16, is processed and cleaved by a caspase-dependent mechanism to generate sPDZD2 (13). In light of the fact that cleavage of pro-IL-16 to form the corresponding secretory IL-16 cytokine is mediated by caspase-3 (19), it would be of interest to determine whether the proteolytic cleavage of full-length PDZD2 to its secreted protein sPDZD2 is caspase-3 dependent. DU145, PC-3, 22Rv1, and RWPE-1 cells were incubated for 48 h with 10 μM Z-DEVD-FMK (a specific inhibitor of caspase-3), 10 μM Z-IETD-FMK (a specific inhibitor of caspase-8), or 10 μM Z-FA-FMK (a negative control peptide). Treatment of DU145, PC-3, 22Rv1, and RWPE-1 cells with Z-DEVD-FMK for 48 h resulted in 40, 28, 44, and 50% reduction of sPDZD2 secretion into the respective conditioned media, compared with cells treated with Z-FA-FMK (Fig. 1B). Concomitantly, PDZD2 expression in DU145, PC-3, 22Rv1, and RWPE-1 cells showed respective 3-, 2-, 1.5-, and 4-fold increases, compared with Z-FA-FMK-treated cells (Fig. 1C). However, there were no changes in sPDZD2 secretion and PDZD2 expression after the cells were treated with the specific caspase-8 inhibitor Z-IETD-FMK (10 μM) for 48 h (data not shown). These data indicated that the proteolytic cleavage of PDZD2 is mediated by caspase-3.

To determine whether alterations in PDZD2 and sPDZD2 levels in inhibitor-treated cells are associated with any changes in the cell growth rate, we also monitored DU145, PC-3, 22Rv1, and RWPE-1 cell proliferation by MTS-based assays. Interestingly, there were significant ($P < 0.01$) increases in DU145 (20.1–22.7%), PC-3 (22.9–24.8%), 22Rv1 (23.4–26%), and RWPE-1 (20.9–24.2%) cell proliferation after the cells were treated with the caspase-3 inhibitor Z-DEVD-FMK (10 μM) for 24 and 48 h (Fig. 1D–G). No changes in the proportion of cells in different cell-cycle phases were detected after treatment with the caspase-3 inhibitor for 24 and 48 h (data not shown). There were also no changes in the proliferation of the cells after 24 and 48 h treatment of 10 μM caspase-8 inhibitor Z-IETD-FMK (data not shown). To further examine whether the observed increases in cell proliferation were due to inhibition of sPDZD2 secretion, we tested the ability of exogenously applied recombinant sPDZD2 (16) to counteract the growth-promoting effects of the caspase-3 inhibitor. As shown in Fig. 1H, the increases in DU145, PC-3, 22Rv1, and RWPE-1 cell proliferation induced by caspase-3 inhibitor treatment were abolished by coincubating with 10−8 M sPDZD2. These results suggest that the antiproliferative effects were predominantly mediated by sPDZD2 instead of PDZD2.

Effects of recombinant sPDZD2 on cancerous and immortalized prostate epithelial cell growth

Recombinant sPDZD2 induced a significant ($P < 0.001$) concentration-dependent inhibition of cell proliferation in DU145, PC-3, and 22Rv1 cells (Fig. 2). No significant changes in RWPE-1 cell proliferation in response to recombinant sPDZD2 were observed (data not shown). Treatment of DU145 cells with 10−6 and 10−7 M sPDZD2 for 24 h resulted...
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in, respectively, 25.8 and 29.9% decreases in cell proliferation ($P < 0.001$). Treatment of PC-3 cells with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 24 h resulted in, respectively, 13.5 and 22.6% decreases in cell proliferation ($P < 0.01$). Treatment of 22Rv1 cells with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 24 h resulted in, respectively, 12.7 and 15.4% decreases in cell proliferation ($P < 0.001$). On the other hand, DU145 cell proliferation exhibited 38.6 and 43.5% decreases ($P < 0.001$) after the cells were treated with $10^{-8}$ and $10^{-7}$ m sPDZD2, respectively, for 48 h. PC-3 cell proliferation exhibited 23.8 and 41.6% decreases ($P < 0.001$) after the cells were treated with $10^{-8}$ and $10^{-7}$ m sPDZD2, respectively, for 48 h. 22Rv1 cell proliferation exhibited 21.7 and 27.7% decreases ($P < 0.001$) after the cells were treated with $10^{-8}$ and $10^{-7}$ m sPDZD2, respectively, for 48 h (Fig. 2, A–C). Similar antiproliferative effects of sPDZD2 on DU145, PC-3, and 22Rv1 cells were also demonstrated using the Cell Proliferation ELISA BrdU (colorimetric) kit (Roche) (Fig. 2, D–F). No changes in DU145, PC-3, 22Rv1, and RWPE-1 viabilities were detected (data not shown).

**Effects of sPDZD2 on regulation of proteins involved in cell cycle control**

Given that sPDZD2 induced the strongest antiproliferative effects on DU145 cells, compared with PC-3 and 22Rv1 cells (Fig. 2), we selected DU145 as the principal prostate cancer cell model to explore the intracellular mechanisms that may mediate the growth-inhibitory effects of sPDZD2. DU145 cells were treated with $10^{-8}$, $10^{-7}$, and $10^{-6}$ m sPDZD2 for 48 h, and any sPDZD2-induced changes in the expression levels of p21CIP1/WAF1, p27KIP1, p53, cyclin B, and cyclin D were monitored. As shown in Fig. 3A, treatment of DU145 cells with $10^{-9}$, $10^{-8}$, and $10^{-7}$ m sPDZD2 for 48 h did not result in any changes in the level of p27KIP1. No changes in the expression of cyclin B and cyclin D were also observed (data not shown). However, sPDZD2-induced changes in p21CIP1/WAF1 and p53 in growth-inhibited DU145 cells (Fig. 3A). Treatment of DU145 cells with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 48 h resulted in 4.5- and 4.4-fold increases in p21CIP1/WAF1, respectively. In correlation, DU145 cells treated with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 48 h, respectively, exhibited 3.9- and 3.8-fold increases in p53 expression. Of note, increased p53 expression (1.4-fold) was detected in DU145 cells after the cells had been incubated with $10^{-8}$ m sPDZD2 for 12 h. Relative to p53, the rise (2.6-fold) of p21CIP1/WAF1 expression was delayed by 12 h in DU145 cells treated with $10^{-8}$ m sPDZD2 (Fig. 3B). To further elucidate the signaling mechanisms of sPDZD2 in DU145 cells, the phosphorylation status of p53 in response to sPDZD2 treatment was investigated. No significant changes in the phosphorylation status of p53 at Ser6, Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392 phosphorylation sites were detected in DU145 treated with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 1, 4, and 8 h (data not shown). On the other hand, no significant changes in p21CIP1/WAF1, p27KIP1, cyclin B, and cyclin D expression were observed in PC-3 cells, which showed no p53 expression (data not shown). Similarly, no significant changes in p21CIP1/WAF1, p27KIP1, p53, cyclin B, and cyclin D expression were observed in 22Rv1 cells (data not shown).

**Apoptotic effects of sPDZD2 on LNCaP cells**

Recombinant sPDZD2 did not induce any changes in the viabilities of DU145, PC-3, 22Rv1, and RWPE-1 cells. However, incubation of LNCaP cells with $10^{-7}$ m sPDZD2 for 24 h resulted in 26.2% decrease in cell viability ($P < 0.001$), whereas LNCaP cell viability exhibited 14.7 and 18.6% decreases ($P < 0.001$) after treatment with $10^{-8}$ and $10^{-7}$ m sPDZD2, respectively, for 48 h (Fig. 4A). The observed decreases in LNCaP cell viability in response to sPDZD2 were found to be due to apoptosis induction (Fig. 4B), as measured by cell death detection ELISA (Roche). Significant ($P < 0.001$) increases in absorbance at 405 nm, which reflects an increase in mono- and oligonucleosomes in the cell cytoplasm, were observed in LNCaP cells treated with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 24 h. Because p53 is also a key regulator of apoptosis besides cell cycle progression (20), we also monitored the expression of p53 and the phosphorylation status of p53 at Ser15, Ser20, Ser37, and Ser46 phosphorylation sites, which are important in apoptosis control (21), in LNCaP cells. Treatment of the cells with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 1, 2, and 4 h did not induce any changes in p53 levels and in its phosphorylation status at Ser15, Ser20, Ser37, and Ser46 (data not shown). Interestingly, treatment of LNCaP cells with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 24 h resulted in respective 3.7- and 3.1-fold up-regulation in Bad expression. In addition, treatment of LNCaP cells with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 48 h increased the expression of Bad by 2.6- and 2.1-fold, respectively (Fig. 4C). The cleavage of PARP, which serves as an early specific marker of apoptosis, was also observed after treatment of LNCaP cells with $10^{-8}$ and $10^{-7}$ m sPDZD2 for 24 and 48 h (Fig. 4C). In the presence of $10^{-9}$ m dihydrotestosterone, the apoptotic effects induced by $10^{-8}$ and $10^{-7}$ m sPDZD2 were significantly reduced (data not shown), indicating that androgen may confer protection against sPDZD2-induced apoptosis in LNCaP cells.

**Effects of sPDZD2 on p21CIP1/WAF1 and p27KIP1 transcription**

To determine whether the elevated p21CIP1/WAF1 protein expression was caused by an increase in p21CIP1/WAF1 gene tran-
We monitored p21CIP1/WAF1 promoter activity in DU145 cells in response to sPDZD2 treatment using luciferase reporter assays. Treatment of DU145 cells with 10−8 and 10−7 M sPDZD2 for 24 h resulted in, respectively, 1.4- and 1.8-fold increases (P < 0.01) in p21CIP1/WAF1 promoter activity. Similarly, treatment of DU145 cells with 10−8 and 10−7 M
significant changes in the promoter activity of p21CIP1/WAF1, p27KIP1, and p53. Treatment of DU145 cells with 10⁻⁸ M recombinant sPDZD2 for 48 h resulted in 1.4- and 2.2-fold increases in p21CIP1/WAF1 and p27KIP1 mRNA levels, respectively (Fig. 5A). No significant changes in the promoter activity of p21CIP1/WAF1 could be detected (Fig. 5B). The p21CIP1/WAF1 and p27KIP1 promoter activity changes induced by sPDZD2 were in good correlation with sPDZD2-induced changes in p21CIP1/WAF1 and p27KIP1 mRNA levels as quantitated by RT-PCR. Treatment of DU145 cells with 10⁻⁸ and 10⁻⁷ M sPDZD2 for 24 h resulted in 2.6- and 2.9-fold increases in p21CIP1/WAF1 mRNA levels, whereas no changes in p27KIP1 mRNA could be detected (Fig. 5C).

Effects of sPDZD2 on cell cycle progression

The growth suppressive effects of sPDZD2 on DU145 cells were monitored by flow cytometry. Treatment of synchronized DU145 cells with 10⁻⁸ M sPDZD2 delayed the entry of the cells to the S phase of cell cycle. Without sPDZD2 treatment, the synchronized DU145 cells reenter into S phase by 12 h after replating, as reflected by an increase in the percentage of S phase cells from 22.1 to 38.9% (Fig. 6A). In contrast, most of the sPDZD2-treated cells remained in the G₀/G₁ phase, and no significant increase in the percentage of S phase cells was observed until 48 h after replating (Fig. 6B). Similar to DU145 cells, untreated synchronized PC-3 and 22Rv1 cells reenter into S phase by 12 h after replating, as reflected by an increase in the percentage of S phase cells from 25.4 to 34.8% and from 25.4 to 33.7%, respectively, whereas most of the sPDZD2-treated PC-3 and 22Rv1 cells remained in the G₀/G₁ phase until 48 h after replating (data not shown).

Discussion

In this communication, we demonstrated the endogenous intracellular expression of sPDZD2 and extracellular secretion of its cleavage product sPDZD2 in human prostate cancer LNCaP, DU145, PC-3, and 22Rv1 cell lines as well as the immortalized RWPE-1 cells (Fig. 1A). Furthermore, the cleavage of sPDZD2 from its precursor PDZD2 in prostate cancer cells is shown to be mediated by a caspase-3-dependent mechanism, similar to that reported for the proteolytic processing of IL-16 from pro-IL-16 (19). Importantly, inhibition of endogenous sPDZD2 production and secretion by DU145, PC-3, 22Rv1, and RWPE-1 cells via the specific caspase-3 inhibitor Z-DEVD-FMK resulted in increased cell proliferation, which can be abrogated by supplementation with exogenous recombinant sPDZD2 (Figs. 1, D–H). The results suggest that endogenous sPDZD2 may exert an autocrine physiological antiproliferative action on human prostate cancer cells. Although we estimated the concentration of endogenous sPDZD2 in the conditioned cell culture media to be in the range of 10⁻¹⁰ to 10⁻¹¹ M, it remains to be determined whether this reflects the actual concentration of sPDZD2 in human interstitial fluid or plasma. Notwithstanding this limitation of the yet-unknown physiological concentration of sPDZD2, it is noteworthy that the predicted antiproliferative actions can indeed be induced in the cancerous prostate epithelial cells by treating them with recombinant sPDZD2 (Fig. 2), which provide support for our postulation that sPDZD2 may function as an autocrine growth suppressor for human prostate cancer cells. It is also evident from our studies that sPDZD2 can suppress prostate cancer cell growth by not only antiproliferation as shown in DU145, PC-3, and 22Rv1 cells but also apoptosis induction in LNCaP cells (Fig. 4). Together with the reported up-regulation of gene and protein expression of PDZD2, also known as AIPC, in human prostate cancer tissues (12), our data indicate that sPDZD2, a proteolytic secretory product of PDZD2, may indeed play an important role in growth dysregulation in prostate cancer tumorigenesis and progression.

Flow cytometric data have indicated that antiproliferative effects of sPDZD2 on DU145, PC-3, and 22Rv1 cells are largely mediated via slowing the entry of these cancer cells into the S phase of the cell cycle (Fig. 6). In DU145 cells, we found an increase in p53 protein expression, followed by correlative increases in the gene and protein expression of p21CIP1/WAF1, which is an important p53 transcriptionsal target (22) (Figs. 3 and 5). The observed slowing of DU145 cell cycle progression from G₁ to S in response to sPDZD2 can thus be adequately explained by sPDZD2-induced up-regulated gene and protein expression of p21CIP1/WAF1, which is a well-known cyclin-dependent kinase inhibitor crucial for G₁ mitotic checkpoint regulation (23). Interestingly, the DU145 cell line, in which more than 90% of the cells show positive immunoreactivity for p53 (24), carries two mutant p53 alleles producing p53²²³Leu and p53²⁷⁴Phe, whose transactivation abilities on p53-responsive genes differ from each other (25). It has been shown that p53²²³Leu was as active as wild-type p53 in transactivation of p53-responsive genes such as p21CIP1/WAF1, whereas p53²⁷⁴Phe has no detectable transactivation activity on those genes in a direct transactivation assay (25). Apparently sPDZD2 has the ability to activate p53²²³Leu-p21CIP1/WAF1 signaling pathway in DU145 cells to mediate its cytostatic actions observed in the present study, although the remote possibility of p21CIP1/WAF1 activation by p53-independent mechanisms may still need to be
FIG. 4. Effects of sPDZD2 on LNCaP cell growth. A, LNCaP cells were treated with 10^{-9}, 10^{-8}, and 10^{-7} M recombinant sPDZD2 for 24 and 48 h. The effects of sPDZD2 on cell viability were monitored by trypan blue dye exclusion assays. B, Cell death detection ELISA®-PLUS assay kit (Roche) was used to determine the apoptotic effects of sPDZD2 on LNCaP cells. After treatment of the cells with 10^{-8} and 10^{-7} M recombinant sPDZD2 for 24 h, the cells were lysed and the presence of nucleosomes in the cytoplasm was detected by measuring absorbance values at 405 nm. Data are shown as mean ± SE. **, *P < 0.001, compared with control. C, LNCaP cells were treated with 10^{-9}, 10^{-8}, and 10^{-7} M recombinant sPDZD2 for 24 and 48 h. The level of Bad and the processing of PARP were monitored by immunoblotting.
Considered in future studies. In an attempt to further define the mechanisms involved in p53 up-regulation and activation in DU145 cells by sPDZD2, we examined any sPDZD2-induced changes in phosphorylation at specific serine residues of p53 in DU145 cells, using a panel of antibodies against phospho-p53 (Ser6, Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392). Intriguingly, we were unable to detect any significant sPDZD2-induced changes in p53 phosphorylation at the aforementioned serine residues in DU145 cells. Because active p53 is subject to a diverse array of covalent posttranslational modifications, any sPDZD2-induced changes in protein phosphorylation at sites outside those examined and in
other modifications such as acetylation, ribosylation, O-glycosylation, ubiquitination, and sumoylation (21) will need to be examined in future studies.

Given that p53 is an important node that integrates diverse oncogenic and DNA damage signals to mediate cytostasis and apoptosis (26), we also examined whether p53 activation is likely to be a common mechanism through which sPDZD2 induces its antiproliferative or apoptotic effects on other prostate cancer PC-3, 22Rv1, and LNCaP cells. Of note, we could not detect any expression of p53 in the PC-3 cell line, which has been shown to harbor a frameshift deletion mutation in its single p53 allele, causing a stop signal at codon 169 of the protein (27). Moreover, sPDZD2 induced no significant changes of p21CIP1/WAF1 and p27KIP1 in PC-3 cells. The above findings suggested that, distinct from DU145 cells, the mechanism mediating sPDZD2-induced antiproliferation in PC-3 cells is p53 independent. Similarly, no significant changes of p21CIP1/WAF1 and p27KIP1 were observed in 22Rv1 cells, which probably expresses a wild-type p53 in addition to a mutated p53331Arg, when cultured under standard conditions (24). Clearly further studies were needed to determine the importance of p53 in the antitumor mechanisms induced by sPDZD2 on 22Rv1 cells. Whereas sPDZD2 induced LNCaP cells to undergo apoptosis as shown by increases in Bad expression and PARP cleavage (Fig. 4C), no significant changes in total cellular p53 were observed, despite the fact that the LNCaP cell line is known to express wild-type p53 (24, 28) because its expression can be up-regulated by DNA damage signal induced by irradiation (28). Moreover, sPDZD2 did not induce, in LNCaP cells, any changes in p53 phosphorylation at Ser15, Ser20, Ser37, and Ser46, which are phosphorylation sites that are important in apoptosis control by p53 (21). These results suggest that the apoptotic effect of sPDZD2 on LNCaP cells is probably mediated through p53-independent Bad up-regulation (29).

In summary, we have obtained evidence to support the existence of PDZD2/sPDZD2-mediated autocrine growth suppressive signaling pathways in human prostate cancer cells. The p53-dependent and p53-independent mechanisms of sPDZD2 in growth inhibition of DU145, PC-3, 22Rv1, and LNCaP prostate cancer cell lines observed in our studies indicate that PDZD2/sPDZD2 signaling may use different and, perhaps, new signal transduction pathways of the intrinsic tumor suppressive network in prostate cancer in different developmental or progression stages to mediate its growth suppressor actions. Detailed characterization of these PDZD2/sPDZD2 signaling pathways and identification of the membrane receptor(s) involved may yield not only new therapeutic targets for antiprostate cancer drug discovery and development but also provide novel ways, such as harnessing therapeutically the latent tumor-suppressive potential of an endogenous autocrine signaling protein like sPDZD2, to inhibit prostate cancer growth. Besides the potential therapeutic implications mentioned above, unraveling the autocrine signaling mechanisms of PDZD2/sPDZD2 in prostate cancer growth suppression will help to increase our understanding of the physiological growth-inhibitory
networks contributed by vitamin D₃/vitamin D receptor (30) and melatonin/MT₁ receptor (31–34) signaling at the endocrine level and fatty acid metabolites/peroxisome proliferator-activated receptor-γ (35, 36) signaling at the paracrine level.

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