PC-1/PrLZ Contributes to Malignant Progression in Prostate Cancer

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Abstract

PC-1/PrLZ gene overexpression has been identified to be associated with prostate cancer progression. Previous studies have revealed that PC-1 possesses transforming activity and confers malignant phenotypes to mouse NIH3T3 cells. However, the functional relevance of PC-1 expression changes during prostate cancer development and progression remains to be evaluated. In this study, gain-of-function and loss-of-function analyses in LNCaP and C4-2 cells, respectively, were implemented. Experimental data showed that PC-1 expression was in positive correlation with prostate cancer cell growth and anchor-independent colony formation in vitro, as well as tumorigenicity in athymic BALB/c mice. Moreover, PC-1 expression was also found to promote androgen-independent progression and androgen antagonist Casodex resistance in prostate cancer cells. These results indicate that PC-1 contributes to androgen-independent progression and malignant phenotypes in prostate cancer cells. Furthermore, molecular evidence revealed that PC-1 expression stimulated Akt/protein kinase B signaling pathway, which has been implicated to play important roles in promoting androgen refractory progression in prostate cancer. Increased PC-1 levels in C4-2 cells may represent an adaptive response in prostate cancer, mediating androgen-independent growth and malignant progression. Inhibiting PC-1 expression may represent a novel therapeutic strategy to delay prostate cancer progression.

Introduction

Prostate cancer is one of the most commonly diagnosed and the second leading cause of death in the men in the western countries (1). More than 40,000 men die of prostate cancer in United States every year (2). Prostate cancer is a hormone-associated cancer and is regulated by androgen receptor (AR) signaling pathway triggered by dihydrotestosterone. Because of this, besides prostatectomy and radiotherapy for local prostate cancer, androgen ablation through surgery or chemical methods is the most effective therapeutic approach for advanced prostate cancer. Unfortunately, despite being effective in the initial period, prostate cancer eventually relapses to androgen-independent stage and resists additional androgen withdrawal (3). The mechanism mediating prostate cancer progression from androgen dependence to androgen independence remains unclear. Understanding the molecular mechanism and associated gene expression change occurring in the transition process should be the first step for understanding the molecular biology of prostate cancer, as well as for designing novel therapeutic targets for prostate cancer development and progression (4, 5).

The LNCaP/C4-2 human prostate cancer progression model was established to investigate the mechanism mediating prostate cancer progression. LNCaP is an androgen-responsive, nonmetastatic, and marginally tumorigenic prostate cancer cell (6), which is most frequently used in prostate cancer research. The C4-2 subline was derived from LNCaP through interaction with stromal cells under androgen-depleted condition in vivo and acquired the phenotypes of androgen independence and osseous metastases (7, 8). Unlike other prostate cancer cell model, LNCaP/C4-2 progression model has the unique advantage of remarkably mimicking the phenotypic and genotypic changes often observed in clinical human prostate cancer (9). Gene expression–differentiated analysis between the LNCaP and lineage-related C4-2 subline may provide clues to the molecular mechanism mediating the transition from androgen dependence to androgen independence, from nonmetastases to bone metastasis.

PC-1 gene, also named PrLZ, was identified to be up-regulated in C4-2, in comparison with LNCaP parent cell line through cDNA microarray analysis (10). PC-1 belongs to TPDS2 family. Many members of this family are associated with cellular proliferation and a series of tumors, including breast, lung, and prostate adenomas and adenocarcinomas (11–14). Besides its distinctive NH2-terminal domain, PC-1 contains conserved domain of TPDS2 family, including a coiled coil leucine zipper in the central region, through which DS2 proteins form homomeric and heteromeric complex (15, 16). PC-1 and TPDS2 transcripts may derive from the same gene by selective splicing. DS2 proteins have been associated with multiple biological processes through interacting with a variety of partners. A series of proteins have been identified to be associated with DS2 proteins, including MAL2, Annexin VI, and SNARE complex, indicating DS2 proteins may be involved in vesicle transport, membrane trafficking, and exocytotic secretion (17–19).

14-3-3 proteins, crucial players in Ras signaling, protein kinase B (PKB) signaling, and intracellular protein localization, have also been isolated to be interacting partners of hD53, another member of TPDS2 family (20). Furthermore, human DS3L1 physically interacts with ASK1 and functions as a positive regulator of ASK1-induced apoptosis (21). Although the C-terminal domain of PC-1 is homologous with TPDS2, PC-1 and TPDS2 are functionally different. Being different from TPDS2 and the other members of this family, PC-1 expression is primarily specific in prostate tissue and can be induced by androgen. A series of genes, including insulin-like growth factor-I receptor, fibroblast growth factor 8, ErbB1, and ezrin, which are up-regulated by androgen stimulation, are associated with malignant phenotypes of prostate cancer and contribute to prostate cancer progression (22–26). Most importantly, PC-1 expression is associated with prostate cancer...
development, whereas TPD52 expression is uniform in LNCaP, C4-2, and other prostate cancer cell lines and is not found to be correlated with prostate cancer development. PC-1 gene is localized at chromosome 8q21.1, a locus most frequently amplified in human prostate cancer specimens (27). Fluorescence in situ hybridization using PC-1–specific probe has revealed that PC-1 gene is amplified in a number of prostate cancer cases. Evidence of clinical specimens has also shown that PC-1 is overexpressed in prostate epithelial cells in prostate intraepithelial neoplasia and prostate cancer while remaining at low level in normal prostate tissue (10). Moreover, our previous studies showed that immortalized mouse NIH3T3 fibroblasts overexpressing PC-1 was transformed and acquired malignant characteristics of anchor-independent growth and tumorigenicity (28). Through in vitro transwell invasion assay, migration ability of C4-2 cell line was found to be suppressed as the result of inhibition of PC-1 expression (29). These results indicate that overexpression of PC-1 is associated with prostate cancer progression and that PC-1 possesses the oncogenic property and ability to initiate transformation and malignancy. However, the functional importance of changes in PC-1 expression during prostate cancer progression and the molecular mechanism mediating PC-1 function remain to be elucidated. In this study, gain-of-function and lose-of-function analyses were done to investigate the functional significance of PC-1 in prostate cancer progression. The effect of PC-1 expression on Akt signaling cascade was also examined to determine the molecular regulation PC-1 may be involved in after overexpression in LNCaP cell line.

Materials and Methods

Cell culture and reagents. LNCaP and C4-2 cell lines were cultured in RPMI 1640 (Invitrogen) with 8% fetal bovine serum (FBS; Hyclone), 10 mM/L HEPES, and 1.0 mM/L sodium bicarbonate. All cells were cultured at 37°C with 5% CO₂ in a humidified incubator. To study the cell growth in androgen-depleted condition, LNCaP and C4-2 cells were cultured in fresh phenol red–free RPMI 1640 with 5% to 10% dextran/charcoal absorbed FBS (cFBS; Hyclone). G418 was obtained from Life Technologies, and Casodex was supplied by Sigma.

Antisense and overexpressing PC-1 cDNA vector constructs and stable transfection. PC-1 overexpression vector was prepared by ligating the coding region of human PC-1 cDNA into the BamHI (sense orientation) sites of pcDNA3.1(−)/−Myc-His B vector (Invitrogen) in frame with His-Myc tag. The 599-bp PC-1–specific cDNA fragment was cloned into EcoRV (antisense orientation) site of pcDNA3.1(−)/−Myc-His B to decrease PC-1 expression. Plasmid DNA corresponding to each construct was transfected into LNCaP and C4-2 cells with LipofectAMINE 2000 according to the manufacturer’s protocol, with empty vector used as control. Transfected LNCaP cells and C4-2 cells were first selected for their ability to grow with G418 (LNCaP 1000 μg/mL, C4-2 800 μg/mL). Stable clones were selected after 5 weeks. Antisense and overexpressing clones were analyzed for either inhibition of PC-1 expression or increase in PC-1 expression by Western blot analyses. In the case of overexpressing clones, all of the selected clones (total of eight clones) similarly overexpressed PC-1. Studies presented here were done using representative clones. Similar data were obtained with other randomly picked clones.

Western blot assay. Cells were treated with lysis buffer containing 150 mM/L Tris base (pH 7.5), 50 mM/L NaCl, 1 mM/L EDTA (pH 8.0), 1% NP40, 1 mg/mL leupetin, and 1 mM/L phenylmethylsulfonyl fluoride. Protein concentrations were determined by bicinchoninic acid protein assay (Pierce Chemical Co.). Equal amounts of protein denatured in 2× SDS sample buffer [100 mM/L Tris base (pH 6.8), 200 mM/L DTT, 4% SDS, 20% glycerol, and 0.005% bromphenol blue] were loaded into 15% SDS–PAGE gels and gels were transferred onto nitrocellulose membranes (Amersham Biosciences). The membranes were blocked overnight in TBS containing 5% (w/v) skimmed milk powder and were then stained by dilution (1:250) of primary antibodies against PC-1 N-terminal 46 amino acids residues (made by our laboratory), β-actin (Santa Cruz Biotechnology, Inc.), Phosphorylated Akt pathway starter kit (Cell Signaling Technology) was used to examine the phosphorylated Akt(Ser473), phosphorylated Akt(Thr308), total Akt, phosphorylated glycogen synthase kinase (GSK)-3β(GluSer), and phosphorylated Raf(SerTH20) after a series of washes, the blots were further incubated with goat anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Zhongshan Golden Bridge Biotechnology Co., Ltd.) and detected using the enhanced chemiluminescence kit (Pierce).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell growth rate was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay. Briefly, 2,000 to 3,000 cells were seeded in 96-well plates. Cell growth was examined at indicated time points. Before testing, 20 μl of MTT reagent (2.5 mg/mL MTI in PBS, Amresco, Inc.) was added, and the cells were incubated for a further 4 h at 37°C. Then 150 μl of dissolving reagent DMSO (Amresco) was added to dissolve the formazan crystals. The absorbance (A) was measured at wavelength of 490 nm on a microplate reader. Pilot experiments were conducted to determine optimal cell concentration for the experiments.

Colony formation in soft agar. A total of 400 to 10,000 cells were suspended in 0.07% low melting agarose (Difco Laboratories) dissolved in 0.2 ml of RPMI 1640 with 10% FBS or with 10% androgen-free FBS medium and plated on top of 2 ml of medium underlayer of 0.5% agarose in the same medium in six-well culture plates. After 3 weeks of incubation at 37°C in a 5% CO₂ incubator with humidified atmosphere, the colonies with >15 cells were counted.

In vivo tumorigenicity study. Male nude athymic BALB/c mice, 6 to 8 weeks old (V ctor Experimental Animals Technique Ltd.) were used for examine the tumorigenicity of PC-1 overexpressing LNCaP and the control. A total of 5 × 10⁶ cells at the exponential growth phase were suspended in 200 μl serum-free RPMI 1640 containing Matrigel (1:1, vol/vol; BD Biosciences) and injected into two sites per mouse on opposite sides of the abdomen of the mice s.c. via 27-gauge needle. Mice bearing tumors were castrated 4 to 5 weeks postinjection. Tumor volume measurements were done once weekly. Tumor volumes were calculated by the formula \( V = \frac{L \times W^2}{2} \times 0.5236 \). Tumors derived from LNCaP stable transfectants were removed at necropsy, fixed in 4% neutral buffered formalin for at least 20 h, and embedded in paraffin. Sections cut at 4 μm were placed on slides and were stained with H&E.

Figure 1. PC-1 levels in stably transfected cells. A, LNCaP cells and LNCaP-pc-1 clone transfected with PC-1 expressing vector. B, C4-2, C4-2-po-1, and C4-2-anti clones transfected with sense and antisense PC-1 vector, respectively.
Statistical analysis. Analyses were done using the statistical software SAS/STAT. Data analyses over time were undertaken by repeated measures analysis using SAS/STAT. \( P < 0.01 \) was considered the threshold value for statistical significance.

Results

Isolation of sense and antisense PC-1 transfected human prostate cancer cells. The sense and antisense PC-1 cDNA expression construct was transfected into prostate cancer of different stages: LNCaP and C4-2 cells, respectively, as described in Materials and Methods. Western blot analyses were done to determine the protein levels of PC-1 using polyclonal antibody against PC-1. The LNCaP and C4-2 clones that showed increased level of PC-1 expression by immunoblotting were designated as LNCaP-pc-1 and C4-2-pc-1, respectively (Fig. 1A and B). C4-2 clones that showed decreased level of PC-1 expression were defined as C4-2-anti, with PC-1 expression decreased by 40% to 50% (Fig. 1B).

Effects of PC-1 expression on malignant phenotypes of prostate cancer cells in vitro and in vivo. Growth ability of transfected cells were measured in normal serum-supplemented medium using MTT assay. Growth curve showed that PC-1 overexpression stimulated the growth rate of LNCaP cells in monolayer culture. After 5 days in culture, the value of \( A_{490} \) for LNCaP-pc-1 cells was increased by 4.6-fold compared with the empty vector control (\( P < 0.01; \) Fig. 2C). As expected, the colony formation rate of C4-2 antisense PC-1 transfectants was reduced by \( >40\% \) compared with C4-2-pc-1 cells (\( P < 0.01; \) Fig. 2B). These results show that PC-1 expression is in positive correlation with the growth ability of prostate cancer cells of different stages.

Based on the results described above, we next examined the effects of PC-1 expression on the anchorage-independent growth ability of prostate cancer cells to form colonies in soft agar, a property believed to be an in vitro variable reflective of malignancy. As shown in Fig. 2C and D, both LNCaP-pc-1 and C4-2-pc-1 showed enhanced clonogenicity in semisolid agar compared with mock-transfected control, regardless of the number of cells seeded in a six-well plate. The number of colonies formed by LNCaP-pc-1 transfectants was increased by 4.6-fold compared with the empty vector-transfected control, when 1 \( \times \) \( 10^4 \) cells per well were inoculated in a 6-well plate (\( P < 0.01; \) Fig. 2C). The rate of colony formation for C4-2-pc-1 transfectants was increased by 1.5-fold compared with the control when seeded as \( 8 \times 10^3 \) cells per well (\( P < 0.01; \) Fig. 2D). As expected, the colony formation rate of C4-2 antisense PC-1 transfectants was reduced by \( >40\% \) compared with empty vector control and reduced by 60% compared with C4-2 PC-1 transfectants (\( P < 0.01; \) Fig. 2D). PC-1 expression has a positive effect on the anchorage-independent growth of prostate cancer cell lines representing different status of prostate cancer development.
Intrinsic anchorage-independent growth (soft agar colony formation) activity in vitro closely reflects the tumorigenicity of target epithelial cells (30). Because of the experimental evidence from colony formation in soft agar, we further investigated whether PC-1 overexpression affects the tumorigenic property of LNCaP cells. LNCaP-pc-1 transfectants and the control cells (5 × 10^6) were s.c. injected into male athymic mice to determine whether PC-1 overexpression in LNCaP would have an effect on tumor formation in nude mice. As the result, all mice injected with LNCaP PC-1 transfectants developed tumors with 100% (7 of 7) incidence. Tumors were visible as early as 7 days and grew rapidly after injection. In contrast, mice carrying control cells showed a reduced tumor incidence of 71% (5 of 7). Tumor developed as late as 21 days in the injection. Further, histologic analysis revealed that LNCaP-pc-1 producing tumors comprised of more malignant cells with prominent multiple nuclei and ~2-fold nuclear/cytoplasmic ratio increase as well as frequent pathologic karyokinesis compared with the control (Fig. 3C). These histologic analyses provide further evidence for PC-1 function in increasing LNCaP malignancy.

Effects of PC-1 expression on androgen-independent progression of prostate cancer. To characterize the role of PC-1 in androgen-independent progression, we investigated the effects of PC-1 expression on growth in monolayer culture, anchorage-independent growth ability, and tumorigenicity of prostate cancer cells in androgen-depleted condition.

Figure 3. Effect of PC-1 expression on tumorigenicity of LNCaP in vivo. A, tumor growth assay in vivo. Fourteen male nude mice were randomly divided into two groups and received s.c. injections with LNCaP-neo and LNCaP-pc-1 cells (5 × 10^6), respectively, at two sites per mouse on opposite sides of the abdomen. Tumor volumes were monitored weekly once tumors were palpable, and the mean LNCaP-pc-1 tumor volume was found to be significantly greater than the LNCaP empty vector control tumor volume. Mice bearing tumors were castrated 4 to 5 wks postinjection. Tumor volumes were monitored weekly, and the mean LNCaP-pc-1 tumor volume was found to be significantly greater than the LNCaP empty vector control tumor volume in the castrated mice. Points, mean; bars, SD. The differences in tumorigenicity between PC-1 sense and the control achieved statistical significance (P < 0.01); B, comparison of mice injected with LNCaP-pc-1 and the control transfected cells. The nude mice were photographed several weeks after s.c. injection. C, H&E staining of tumor derived from LNCaP transfectants. a and c, captured digitally using microscopic magnification of ×200. b and d, captured digitally using microscopic magnification of ×400. LNCaP-pc-1-producing tumors (a and b) and tumors derived from LNCaP-pc-1 cells (c and d) with increased nuclear/cytoplasmic ratio and frequent pathologic karyokinesis (arrow). Inset, high power view.

Growth rate of transfected cells were measured in androgen-depleted medium, phenol red–free RPMI 1640 supplemented with 5% charcoal-treated FBS. The LNCaP PC-1 transfectants and the control were cultured for 10 days with androgen withdrawal. MITT assay was carried out by measuring the absorbency of each well on days 0, 2, 4, 6, 8, and 10. As shown in Fig. 4A, LNCaP cells overexpressing PC-1 were able to proliferate in androgen ablation condition with a doubling time of ~3 days, whereas the vector-only control exhibits very slow proliferation with a doubling time of ~8 days (P < 0.01). In addition, the growth rate of C4-2 antisense PC-1 transfectants reduced by 42% compared with the vector-only control cells in androgen-depleted condition (P < 0.01; Fig. 4B).

Similarly, LNCaP PC-1 transfectants showed enhanced clonogenicity in semisolid agar with androgen ablation over the control cells. The colony-forming rate of empty control cells remained only 2.6% in androgen-depleted condition supplied with 10% charcoal-treated FBS, whereas the rate of LNCaP-pc-1 increased to 12.4% in the same environment (P < 0.01; Fig. 4C).

Based on the results above discovered through androgen ablation in vitro, we next evaluate the effect of PC-1 expression on androgen-independent tumor growth in vivo. Male nude mice bearing LNCaP transfectants producing tumors were castrated, and the tumor volume measurements were done on a weekly basis. We found that, at 2 weeks after castration, LNCaP-pc-1 tumors exhibited a rapid recovery in tumor growth over mock-transfected control and that, at 4 weeks postcastration, the average volumes of
experiments, each done in triplicate. Colonies with at least 15 cells were counted. LNCaP PC-1 transfectants and the control achieved statistical significance (Figs. 3A and B). These results suggest that PC-1 expression can overcome the Casodex inhibition partially and that increased PC-1 expression in LNCaP cells leads to Casodex (androgen antagonist) resistance.

Positive effect of PC-1 expression on Akt signaling pathway. Perturbations in Akt signaling axes are significantly associated with the progression of prostate cancer. To investigate the mechanism mediating PC-1 function in prostate cancer progression, we further examined the effect of PC-1 overexpression on Akt signaling pathway in LNCaP and C4-2 cells. As the result, PC-1 overexpression was observed to increase Akt kinase activity. As shown in Fig. 6A, the stable transfection of PC-1 expressing plasmid into LNCaP cells obviously enhanced phosphorylation of Akt/PKB at Thr308 and Ser473 compared with the control. Consistently, the phosphorylation of Akt at Thr308 was also shown to be decreased in C4-2-anti transfectant in comparison with C4-2 empty vector control (Fig. 6B). Moreover, the kinase activities of two major targets of phosphoinositide-3-kinase/Akt cascade including GSK-3β and Raf were also examined. As expected, the phosphorylation levels of GSK-3β and Raf were also increased in LNCaP PC-1 transfectants in comparison with the empty control (Fig. 6C). These results reveal that expression of PC-1 leads to higher activity level of Akt signaling pathway, which may provide molecular evidence to explain PC-1 function in prostate cancer development.

Discussion
In the present study, we set out to characterize the functional significance of PC-1 expression during prostate cancer development and progression using overexpression and antisense inhibition approaches in the LNCaP progression prostate cancer model. The growth curve revealed that PC-1 overexpression mediated stimulatory effect on the growth of LNCaP and C4-2 cells in monolayer culture, whereas C4-2 with reduced PC-1 expression displayed decreased growth rate. These results suggest that the growth rate of prostate cancer cells is proportional to the PC-1 expression level. Moreover, LNCaP is an androgen-sensitive, non-metastasis human prostate cancer (6). C4-2 subline was established from LNCaP parent line and acquired the characteristics of androgen independence and metastasis to lymph node and bone (8). The fact that PC-1 stimulates growth rate of prostate cancer cells representing different status indicates that PC-1 may play an important role in different stages of prostate cancer progression from androgen-dependent to androgen-independent stage.

To determine whether PC-1 expression in prostate cancer cells contributes to their tumorigenic properties, we next examined the effect of PC-1 overexpression on the anchorage-independent growth in vitro and tumor growth in vivo. The results showed that PC-1 overexpression mediated dramatically increased clonogenicity in LNCaP, whereas PC-1 reduction in C4-2 cells consequently exhibits decreased colony formation. Experimental data have shown that the signaling pathways that stimulate growth in monolayer culture and confer anchorage independence are not identical (31). Thus, it is significant that PC-1 overexpression augments both growth pattern of anchorage dependence and independence.

Insert diagram here
studies indicate that PC-1 up-regulation in LNCaP cells results in a significant stimulation of tumorigenicity. Histologic observation also showed that PC-1 expression augmented malignant phenotype of LNCaP-producing tumors. The experiments in vitro and in vivo presented here show that overexpression of PC-1 may play an important function in carcinogenesis and tumorigenesis. Therefore, the PC-1 gene product fulfills several of the traditional criteria relating a gene product to tumorigenesis, being highly expressed in many transformed epithelial cell lines, mitogenic in vitro, supporting colony formation in soft agar, as well as promoting tumor growth in vivo.

Previous studies revealed that PC-1 was a downstream target of AR, as PC-1 expression was transcriptionally regulated by AR activity (10). Evidence described above reveals that PC-1 expression is in positive correlation with growth in culture and tumorigenicity in vitro and in vivo in serum supplement condition. These facts suggest that PC-1 expression is required for androgen-dependent growth and may function in the early stage of prostate cancer development.

Antiandrogen therapy is most generally given to the advanced prostate cancer in the androgen-dependent stage. However, a negative outcome of this treatment is the appearance of androgen refractory tumors with hormone therapy resistance and eventual fatal prognosis. Clinically, progression to androgen independence and acquisition of potential to form metastasis are characterized as the lethal phenotypes of human prostate cancer. Cellular and molecular events that mediate androgen-independent progression consist of a complex process involving both colony selection and adaptive mechanism occurring in heterogeneous tumors. Changes in expression of various genes, including bcl-2 (32) and AKT (33, 34), or recruitment of alternative stimulatory pathway that replace androgen requirement for growth and survival are key elements of androgen-independent progression (35, 36). Elucidation of the pathogenic role of candidate genes implicated in tumor progression is a rapidly progressing field of prostate cancer research and has provided a stably growing list of candidates. Targeting the cancer-relevant genes may offer the possibility to design novel anticancer agents for prostate cancer therapy.

To investigate the role of PC-1 expression in androgen refractory development of prostate cancer, we examined the effect of PC-1 expression in AR-positive prostate cancer cells on their androgen-independent growth and the androgen antagonist Casodex resistance. Increased expression of PC-1 in LNCaP led to cells being able to proliferate robustly in the absence of androgen and in resistance to the androgen antagonist Casodex. PC-1 overexpression also promoted the tumorigenic phenotype in vitro and in vivo in the androgen-depleted condition. LNCaP cell is not nearly able to form colonies in androgen-depleted soft agar, whereas LNCaP overexpressing PC-1 acquires the ability. LNCaP-pc-1 producing tumor in athymic mice displayed a more rapid recovery and growth rate postcastration in comparison with the control. Furthermore, decreased expression of PC-1 in C4-2 cells mediated inhibition of cell proliferation in the absence of androgen. The present studies point to the possible role of increased PC-1 expression in the transition from androgen dependence to androgen-independence.

Figure 5. PC-1 overexpression decreased sensitivity to androgen inhibitor Casodex in LNCaP cell. A, LNCaP-neo and LNCaP-pc-1 were seeded in 96-well plate at confluence of 2,000 cells per well. The cells were exposed to different concentrations of the androgen inhibitor Casodex (10, 20 μmol/L) or equal amount of ethanol as indicated. Cell viability was examined at 24, 48, and 72 h postexposure time points. Cells were observed through the microscope 48 h posttreatment. B, cell viability was measured using MTT assay at indicated time points. The result was consistent with (A), n = 2 independent experiments, each done in triplicate. Points, mean; bars, SD. The differences in Casodex resistance between PC-1 sense versus the control acquired statistical significance (P < 0.01).
Figure 6. PC-1 plays a role as a positive regulator in the Akt signaling pathway. A-C, the results showed that the expression of PC-1 functioned as a positive regulator in phosphorylation of Akt, GSK-3β, and Raf, with total Akt and β-actin used as controls.

and androgen antagonist treatment resistance, which is a critical stage for the development of prostate cancer with poor prognosis. PC-1 up-regulation contributes to androgen-independent growth of androgen-dependent cells and is required for the growth of androgen-independent cells in androgen-depleted condition. PC-1 expression may play a potential role in the development and sustenance of androgen-refractory prostate cancer.

Enhancement of AR signaling has been proposed as a mechanism contributing to androgen-independent progression (5, 36). To assess whether PC-1 bypasses androgen dependency through an increase in AR signaling, we analyzed expression of prostate-specific antigen (PSA), a well-defined AR-induced gene. As the result, Western blot assay showed that PSA expression was not elevated with the increased PC-1 expression. Additionally, transactivation assays showed that PC-1 could not up-regulate PSA and ARE promoter, thus supporting the previous observation (data not shown). These preliminary experimental results indicate that PC-1 induces androgen-independent growth, independent of AR signaling pathway.

PC-1 overexpression is associated with prostate cancer development and contributes to the malignant phenotypes in prostate adenocarcinoma. However, comparatively little is known about the mechanism mediating the function of PC-1 in prostate tumorigenesis other than that directly attributable to its role as a member of the TPD52 family. The PKB/Akt signaling pathway plays an important role in prostate cancer development and progression. Increased Akt kinase activity is associated with poor prognosis in human prostate cancer and correlates with a hormone therapy-resistant phenotype (37–39). Transgenic mice expressing activated Akt1, specifically in the prostate tissue, display murine prostatic intraepithelial neoplasia (40). The experimental results presented here have shown that PC-1 expression plays a role as a positive regulator in the Akt signaling pathway. Activation of Akt/PKB requires phosphorylation of two key regulatory sites. Thr308 phosphorylation is necessary for Akt activation, and Ser473 phosphorylation is required for its maximal activation (41). In this article, we found that PC-1 expression in LNCaP cells led to increase in Akt/PKB activity, as measured by the specific antibodies against phosphorylation of Akt/PKB on Ser473 and Thr308, respectively, and decrease in PC-1 expression in C4-2 correspondingly reduced phosphorylation of Akt on Thr308. The activation of Akt leads to the phosphorylation and regulation of a wide spectrum of its substrates involved in multiple cellular processes, including cell proliferation, cell differentiation, and cell metabolism. Our data presented in this article further showed that PC-1 overexpression led to increased phosphorylation of multiple Akt downstream targets, including GSK-3β and Raf. These proteins affect cell survival through diverse mechanisms. Previous studies have indicated that GSK-3β is not only a kinase for glycogen synthesis but also a regulator for several signaling pathways through its kinase activities for a variety of downstream targets, including c-Myc, β-catenin, CAMP-responsive element binding protein, and nuclear factor-κB, which are frequently found to be up-regulated in multiple types of cancers (42). Moreover, Akt interacts with Raf and phosphorylates this protein at a highly conserved Ser259 residue, which leads to inhibited activation of the Raf/MEK/extracellular signal-regulated kinase signaling pathway and shifts the cellular response from cell cycle arrest to proliferation in a human breast cancer cell line (43). As we have known, PKB/Akt is constitutively active in LNCaP cells (44), which would provide significant advantages for survival and malignant progression of prostate cancer cells. However, PC-1 overexpression may represent additional protection. We speculate that overexpression of PC-1 may specially provide prostate cancer cells selection advantages after androgen withdrawal therapy and may provide advantageous condition for prostate cancer development to androgen refractory stage. The association of PC-1 expression with Akt signaling pathway and its targets provides molecular evidence to show that PC-1 expression plays an important role in prostate cancer development and provides new insight into molecular mechanism mediating prostate cancer progression.

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