

Expression of Human Prostatic Acid Phosphatase Correlates with Androgen-stimulated Cell Proliferation in Prostate Cancer Cell Lines*

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Androgen plays a critical role in regulating the growth and differentiation of normal prostate epithelia, as well as the initial growth of prostate cancer cells. Nevertheless, prostate carcinomas eventually become androgen-unresponsive, and the cancer is refractory to hormonal therapy. To gain insight into the mechanism involved in this hormone-refractory phenomenon, we have examined the potential role of the androgen receptor (AR) in that process. We have investigated the expression of AR and two prostate-specific androgen-responsive antigens, prostatic acid phosphatase (PacP) and prostate-specific antigen (PSA), for the functional activity of AR in LNCaP and PC-3 human prostate carcinoma cells. Our results are as follows. (i) Clone 33 LNCaP cells express AR, PacP, and PSA, and cell growth is stimulated by 5 α -dihydrotestosterone (DHT). Stimulation of cell growth correlates with decreased cellular PacP activity. (ii) In clone 81 LNCaP cells, the expression of PacP decreases with a concurrent decrease in the degree of androgen stimulation of cell growth, whereas the expression of PSA mRNA level is up-regulated by DHT, as in clone 33 cells. Conversely, in PacP cDNA-transfected clone 81 cells, an additional expression of cellular PacP correlates with an increased stimulation by androgen, higher than the corresponding control cells. (iii) PC-3 cells express a low level of functional AR with no detectable PacP or PSA, and the growth of PC-3 cells is not affected by DHT treatment. Nevertheless, in two PacP cDNA-transfected PC-3 sublines, the expression of exogenous cellular PacP correlates with androgen stimulation. This androgen stimulation of cell growth concurs with an increased tyrosine phosphorylation of a phosphoprotein of 185 kDa. In summary, the data indicate that the expression of AR alone is not sufficient for androgen stimulation of cell growth. Furthermore, in AR-expressing prostate cancer cells, the expression of cellular PacP correlates with androgen stimulation of cell proliferation.

Androgen plays an important role in male physiology and pathology by regulating the expression of various genes in different cells (for a review, see Ref. 1). One of the target organs of androgen is the prostate. Development and maintenance of differentiated function of the normal prostate gland require androgen (2). Androgen has also been implicated in the carcinogenesis of prostate epithelium (3, 4). This is evidenced by observations that, at least in the early phase of prostate carcinogenesis, the growth of carcinoma cells can be stimulated by androgen and arrested by androgen withdrawal (5). Thus, hormonal manipulation including anti-androgen and androgen deprivation therapy is the predominant treatment of advanced cancer, being approximately 70% effective (4, 6, 7). Hormone therapy, however, is not curative, and disease relapse will inevitably occur, usually within 24 months (4). The molecular mechanism(s) underlying this transition from androgen-responsive to androgen-unresponsive prostate cancer is not understood.

Since steroid action on target cells is mediated by an intracellular receptor protein, it has been inferred that loss of steroid response is caused by a loss of corresponding receptors. Results from several studies in different cancer cell lines demonstrate a lower level of ligand binding in steroid-insensitive cells than in steroid-sensitive cells (8–11). It has therefore been proposed that steroid depletion of initially steroid-responsive cancers may result in the selective outgrowth of tumor cells lacking steroid receptors.

For androgen action, the correlation of AR¹ expression with androgen sensitivity of prostate cancer cells has been characterized in a Dunning rat model system. In those rat prostate cell lines, the loss of androgen binding and responsiveness correlates with the lack of AR mRNA and protein (9–11). However, results from studies with human prostate carcinomas contradict these findings. Carcinomas from endocrine therapy-resistant patients following prolonged treatment with androgen deprivation do not have a significant change in the expression level of AR or the percentage of AR-positive cells (12, 13). Additionally, a high percentage of those hormonally manipulated tumor cells express PSA, an androgen-regulated antigen, implying the functional activity of AR in carcinomas (13).

Similarly, in several experimental systems, data have been

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¹ The abbreviations used are: AR, androgen receptor; ER, estrogen receptor; PSA, prostate-specific antigen; PacP, prostatic acid phosphatase; PTPase, protein-tyrosine phosphatase; FBS, fetal bovine serum; DHT, 5 α -dihydrotestosterone; AcP, acid phosphatase; Ab, antibody; PP-1 and PP-2A, protein phosphatase-1 and -2A, respectively; RT, reverse transcriptase; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; pp185, a phosphoprotein with a molecular mass of 185 kDa; kb, kilobase pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

accumulated to show that steroid-insensitive tumor cells may still express an unchanged level of corresponding receptors. In human breast cancer, for example, a considerable percentage of ER-positive breast cancers do not respond to hormonal therapy, and several estrogen-unresponsive human breast cancer cell lines do express ER (14–16). Mouse mammary tumor cells may retain functional steroid receptors, while they lose their sensitivity to steroids (17, 18). Additionally, lymphoma cells express glucocorticoid receptor but are insensitive to glucocorticoid treatments (19, 20). Thus, it is imperative to investigate further the role of steroid receptor in growth regulation.

In this study, we utilized human prostate carcinoma cell lines including LNCaP and PC-3 cells as model systems to investigate the role of AR expression in the androgen-stimulatory effect on cell growth. We also analyzed the expression of AR in different LNCaP cells that show different androgen responsiveness. To test the functional activity of AR, we examined the expression of PSA, since the expression of this antigen is regulated by androgens (13, 21). Additionally, we examined PACP expression because it is also an androgen-responsive enzyme (22) and because the cellular PACP has been implied to be involved in androgen regulation of cell proliferation by participating in the tyrosine phosphorylation signal transduction pathway (22–24). Preliminary results demonstrated that, among different LNCaP cells, the expression of cellular PACP correlated with androgen responsiveness of cell growth. To explore this correlation further, we examined the effect of androgen on the growth of PACP cDNA-transfected LNCaP and PC-3 cells.

EXPERIMENTAL PROCEDURES

Materials—FBS and RPMI 1640 medium were purchased from Life Technologies, Inc. The heat-inactivated dialyzed FBS that was a certified grade FBS containing less than 74 pM testosterone, was prepared as described previously (25). The steroid-reduced medium consisted of RPMI 1640 medium supplemented with 2 or 5% (v/v) heat-inactivated dialyzed FBS. Thus, the final concentration of testosterone was less than 4 pM (25). Hepes, bovine serum albumin, Tris, *p*-nitrophenyl phosphate, DHT, L-(+)-tartaric acid, citric acid, formaldehyde, and EtBr were all purchased from Sigma. [α - 32 P]dCTP was from NEN Life Science Products. All other reagents were obtained as described in previous publications (22, 24–26).

Cell Cultures—Human prostate carcinoma cell lines, LNCaP-FGC (LNCaP) (27), DU145 (28), and PC-3 (29), were originally obtained from the American Type Culture Collection (Rockville, MD) and routinely maintained in RPMI 1640 medium supplemented with 7% FBS, 1% glutamine, and 0.5% gentamicin, as described previously (22, 30). Cultured cells were fed twice per week and trypsinized once per week; one passage equals one trypsinization. The doubling time of LNCaP (clone 33), DU145, and PC-3 cells in 7% FBS was approximately 60, 29, and 35 h, respectively (22). For experiments, LNCaP cells that had passage numbers less than 33 were designated as clone 33, passage numbers over 80 as clone 81, and passage numbers between 34 and 80 as clone 51. To investigate basal growth rates of different subcloned cells, cells were seeded and maintained in steroid-reduced medium containing 5% heat-inactivated dialyzed FBS. To quantify cell growth, attached cells were trypsinized and neutralized with medium-FBS. The total cell number including the suspended and attached cells were counted using a hemocytometer via trypan blue exclusion or a Coulter Counter Z1 model (22, 24).

PC-3 and Clone 81 LNCaP Cell Transfection with a PACP cDNA Expression Vector—PC-411 and PC-416 cells were subclones of PC-3 parent cells transfected with a full-length human PACP cDNA, as described in our previous publications (22, 24). The expression of PACP cDNA was driven by a pCMV-neo expression vector (22, 24). PC-CMV cells were a subcloned cell line of PC-3 that was transfected with the expression vector alone (22). PC-411 and PC-416 cells have been characterized and shown to express a putative cellular form of exogenous PACP (22, 24).²

Clone 81 LNCaP cells were also transfected with the same expression

vector containing a full-length human PACP cDNA (22). Two subcloned cell lines, designated as LNCaP-23 and LNCaP-34, were established and characterized in this study. LNCaP-CMV cells were a subline of clone 81 LNCaP that was transfected with the expression vector alone.

Androgen Effect on Cell Growth, Protein Tyrosine Phosphorylation, PSA, and PACP Expression—Cells were plated at a density of approximately 1×10^4 cells/cm² in RPMI 1640 medium containing 7% FBS and maintained in a 37 °C incubator (5% CO₂) for 3 days (22, 25). To investigate androgen effect on cell growth and protein tyrosine phosphorylation, cells that had been maintained in the steroid-reduced medium containing 5% heat-inactivated dialyzed FBS for an additional 48 h were grown in fresh steroid-reduced medium in the presence or absence of different concentrations of DHT. After a time period specified in each set of experiments, cells were harvested and then counted using a hemocytometer or Coulter Counter (22, 24). To analyze androgen effects on PSA and PACP expression, cells that had been grown in the steroid-reduced medium containing 2% heat-inactivated dialyzed FBS for 48 h were maintained in fresh steroid-reduced medium in the presence or absence of 10 nM DHT for an additional 3 days (25). Cells were harvested for preparation of total RNA and total cell lysate proteins as described below.

Protein Determination—For biochemical experiments, cells were harvested by scraping, rinsing, and pelleting in 20 mM Hepes, 0.9% NaCl, pH 7.0. Cell pellets were lysed in 20 mM Hepes, pH 7.0, containing 0.5% Nonidet P-40, 0.5 mM dithiothreitol, and various protease inhibitors (30, 31). The protein concentration in cell lysates was quantified by the Bio-Rad dye protein assay using bovine serum albumin as a standard.

Acid Phosphatase Activity Determination—*p*-Nitrophenyl phosphate was used as the substrate to quantify the AcP activity at pH 5.5 by measuring the absorbance of released *p*-nitrophenol at 410 nm (26, 30). L-(+)-tartrate is a conventional inhibitor of PACP (23). In LNCaP cells, greater than 90% of L-(+)-tartrate-sensitive AcP activity is precipitated by anti-PACP Ab (30). Thus, the L-(+)-tartrate-sensitive AcP activity is taken to represent PACP activity (23, 30).

Protein Phosphatase Activity Assay—The activity of serine/threonine protein phosphatase was determined as described previously (32). Briefly, cells were homogenized in 10 mM Tris (pH 7.4) containing a mixture of various protease inhibitors, and centrifuged at $15,000 \times g$ for 15 min at 4 °C. The protein concentration of each supernatant was determined and adjusted to 1 mg/ml. Serial dilutions of the cellular lysates were incubated in buffer containing 32 P-phosphorylase *a* as the substrate (1.0 μ g/ μ l in a 30- μ l total reaction volume) for 15 min at room temperature. The reaction was stopped by the addition of 100 μ l of trichloroacetic acid (10% solution), and the released radioactivity was determined by scintillation counting. The ratio of PP-1 to PP-2A activity was determined by the inclusion of okadaic acid (5 nM to inhibit only PP-2A) and calyculin-A (1 μ M to inhibit both PP-1 and PP-2A) in the *in vitro* dephosphorylation reaction. PP-2A is the activity that is inhibited by okadaic acid, whereas PP-1 is the activity that is sensitive to calyculin A subtracted from the activity that is blocked by okadaic acid (although PACP could dephosphorylate Ser(P)/Thr(P) in proteins, PACP activity is not affected by 20 nM okadaic acid (33)). Each reaction was performed in triplicate from three sets of cell cultures.

Western Blot Analysis—Subconfluent cells were trypsinized, pelleted, and rinsed with Hepes-buffered saline, pH 7.0, and then lysed in a hypotonic cell lysis buffer containing various protease inhibitors as described above. An aliquot of total cellular lysates (200 μ g/lane) was electrophoresed on a 7.5% SDS gel and blotted onto a NitroPlus filter membrane (Micron Separations Inc.) (24, 33). The filter was incubated with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 at 24 °C for 60 min, followed by rabbit polyclonal anti-human AR Ab (PharMingen, San Diego, CA) or rabbit polyclonal anti-human PACP Ab (24, 33) for 2 h. After rinsing, the filter was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG Ab (Life Technologies, Inc.) at 24 °C for 1 h (33) or incubated with 125 I-labeled protein A (24). The intensity of 125 I was detected by autoradiography. The peroxidase activity was detected by utilizing an ECL reagent kit from Amersham Corp. (33). The relative levels of AR and PACP protein were semiquantified by densitometric analyses of autoradiograms with different exposure time periods utilizing Molecular Dynamics equipment and software program.

Northern Blot Analysis—Total RNA was prepared from cells by a single step guanidine isothiocyanate-phenol-chloroform method (34, 35). An aliquot of each total RNA sample was electrophoresed in a 1.2% agarose gel containing formaldehyde as a denaturing agent (22, 35). After electrophoresis, the gel was stained with EtBr, visualized to ensure the quality of RNA and approximately equal amounts of RNA per lane, and then blotted to Zeta-Probe GT membranes (Bio-Rad) by

² M.-F. Lin, T.-C. Meng, and F.-F. Lin, unpublished results.

standard techniques (35, 36). Filters were hybridized and washed under stringent conditions as described previously (22, 25). cDNA probes were labeled with [α - 32 P]dCTP using random oligonucleotide-primed synthesis (37) with a commercial system from Life Technologies. Both PACp (0.29 kb) and GAPDH (0.78 kb) cDNA probes were prepared as described previously (25, 35). PSA cDNA probe (0.214 kb) was a RT-PCR product (35) and is described under "Polymerase Chain Reaction." The PACp and PSA mRNA bands were visualized by autoradiography followed by densitometric scanning for quantitation.

Synthesis of cDNA by Reverse Transcriptase Reaction—The cDNA was synthesized in an RT reaction mixture with a total volume of 20 μ l including PCR buffer (10 mM Tris, pH 8.3, containing 50 mM KCl), 5 mM MgCl₂, 1 mM each of deoxynucleotides (dCTP, dGTP, dTTP, and dATP from Perkin-Elmer), 1 unit/ μ l RNase inhibitor (Boehringer Mannheim), 2.5 units/ μ l Moloney murine leukemia virus RT (Life Technologies), 2.5 μ M random primers (Life Technologies), and 1 μ g of total RNA. The reaction was performed by incubating the mixture at room temperature for 10 min, 42 °C for 15 min, 99 °C for 5 min, and 5 °C for 5 min, as described previously (35).

Polymerase Chain Reaction—For RT-PCR, the PCR reaction mixture contained 2 mM MgCl₂, a 0.4 mM concentration each of dCTP, dGTP, dTTP, and dATP, 2.5 units of *Taq* polymerase (Perkin Elmer), and a 1 μ M concentration each of specific primers in PCR buffer along with the cDNA synthesis reaction mixture (20 μ l). The total reaction volume was 100 μ l. The PCR reaction was carried out in a Perkin-Elmer apparatus by denaturation at 94 °C for 30 s, annealing at 54 °C for 1 min, and extension at 72 °C for 2 min for 30 cycles and subsequently at 72 °C for 10 min and then soaking at 4 °C (35). For the semiquantitative analysis, a 28-cycle amplification was performed, since preliminary results demonstrated that the PCR, under described conditions with a 28-cycle amplification, followed a linear relationship (data not shown).

The primers used for AR, PACp, PSA, and actin in PCR reactions were synthesized and prepared as described previously (22, 35). The sequences of primers A and B for AR cDNA that were specific to the ligand-binding domain (approximately 0.76 kb) were as in a previous report (35, 38): 5'-GTGGAAATAGATGGGCTTGA-3' and 5'-TCACACATTGAAGGCTATTGA-3'. The sequence of primers for PACp cDNA coding sequence (1.1 kb) was described in a previous report (22): 5'-CGGGATCCCGATGAGAGCTGCACCCCTC-3' and 5'-CGGGATCCCGCTAATCTGTACTGTCTCCTCAGT-3'. The sequences of primers for a specific region of PSA cDNA (214 base pairs) were obtained from a previous report (39): 5'-GAGGTCCACACACTGAAGTT-3' and 5'-CCTCCTGAA-GAATCGATTCTCCT-3'. The primer sequence for a portion of actin cDNA (154 base pairs) was 5'-CACTGTGTTGGCGTACAGGT-3' and 5'-TCA-TCACCATTGGCAATGAG-3' (40).

Chloramphenicol Acetyltransferase Assay—Cells that had been grown in medium containing 5% charcoal-stripped FBS were transfected with the pMSG-CAT reporter plasmid, pSG5 vector DNA, or pSG5-AR plasmid by the liposome-mediated transfection method (22, 41). After a 24-h incubation period, cells were maintained in medium supplemented with 5% steroid-reduced FBS in the presence or absence of 10 nM R1881, a synthetic androgen, in ethanol (0.01%, v/v). After an additional 24-h incubation, CAT assays were performed as described (35, 38).

Statistical Analyses—The significance of difference between two groups of data was analyzed by paired two-tailed Student's *t* test (*p* value) (22). *p* < 0.05 was considered significant.

RESULTS

AR Expression and Androgen Sensitivity to Growth Stimulation—To explore the potential role of AR expression in androgen stimulation of prostate cell growth, we examined the cellular growth of three commonly used human prostate carcinoma cell lines, LNCaP, DU145, and PC-3, after exposure to androgen. 10 nM DHT stimulated the growth of clone 33 LNCaP cells by approximately 1.5–2-fold (Fig. 1A), similar to the original report (27). Nevertheless, DHT did not have a significant effect on the growth of PC-3 and DU145 cells (Fig. 1A). Thus, DHT could stimulate the growth of LNCaP cells (clone 33), but not DU 145 or PC-3 cells.

Androgen insensitivity of PC-3 and DU 145 cellular growth was investigated by examining the expression of AR in these two cell lines. RT-PCR analyses demonstrated that clone 33 LNCaP cells expressed AR mRNA, as indicated by the presence of the specific androgen-binding domain (Fig. 1B). DU145 cells

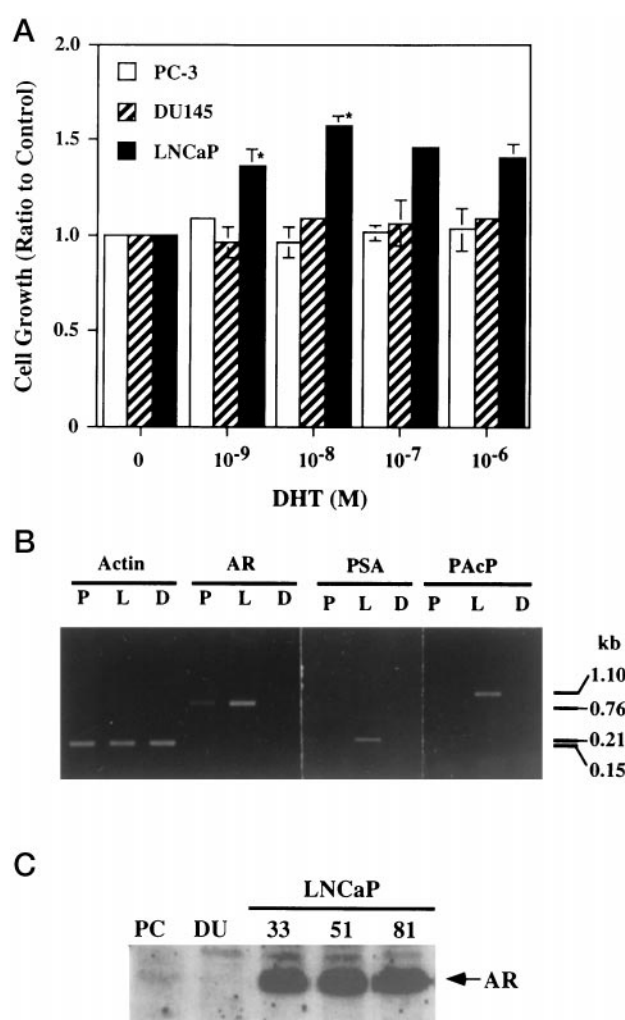


Fig. 1. Growth stimulation by androgen and expression of endogenous AR, PSA, and PACp in prostate carcinoma cells. A, DHT effect on cellular growth. LNCaP (clone 33), PC-3, and DU145 cells were seeded at a density of approximately 1×10^4 cells/cm² in medium containing 7% FBS in six-well plates for 3 days. After they were grown in a steroid-reduced medium for 2 days, cells were then exposed to different concentrations of DHT as indicated in the figure. The total cell number was counted at day 3 for PC-3 and DU 145 cells and at day 4 for LNCaP cells. Cell number in each control well was defined as ratio 1.0. The data shown were the average of duplicate wells, and similar results were observed in two sets of independent experiments. Bar, the range of results from duplicate flasks. *, *p* < 0.05 versus control cells (*n* = 4). B, RT-PCR analyses. AR, PSA, PACp, and actin mRNAs in 1 μ g each of total RNA from PC-3, LNCaP (clone 33) and DU 145 cells were amplified by RT-PCR with specific primers as described under "Experimental Procedures." P, PC-3 cells; L, LNCaP (clone 33) cells; D, DU 145 cells. C, Western blots. An aliquot of total cell lysates (200 μ g) was analyzed for AR expression after electrophoresis in SDS-polyacrylamide gel followed by hybridizing with anti-AR antibody. PC, PC-3 cells; DU, DU 145 cells; 33, 51, and 81 in LNCaP cells indicated clone 33, 51, and 81 cells, respectively.

did not express a detectable level of AR message (Fig. 1B). Unexpectedly, PC-3 cells also expressed AR mRNA, although the level was lower than LNCaP cells (Fig. 1B). Western blot analyses confirmed the expression of low level of AR protein in PC-3 cells (Fig. 1C). RT-PCR analyses also demonstrated that clone 33 LNCaP cells expressed specific mRNAs of PACp and PSA, while PC-3 and DU 145 cells did not have a detectable level of mRNAs of these two antigens (Fig. 1B). Thus, PC-3 cells expressed a low level of AR but not PACp or PSA, while its growth rate was not responsive to androgen stimulation.

Androgen Sensitivity of Different LNCaP Cells—We further examined the effect of androgen on the growth of different

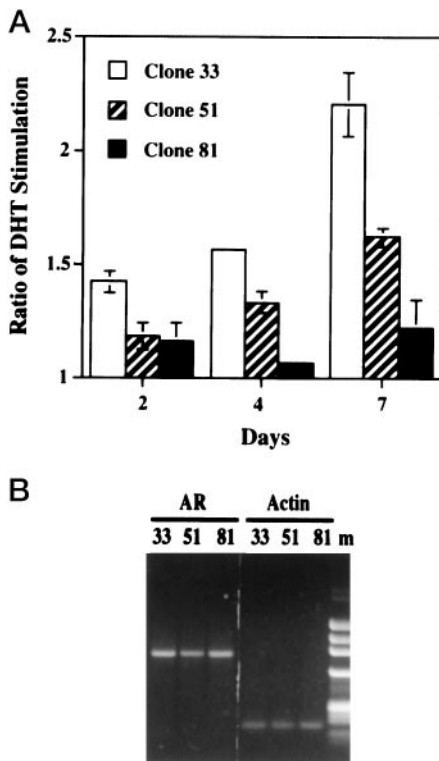


FIG. 2. Androgen effect and AR expression in different LNCaP cells. *A*, androgen effect on cell growth. Different LNCaP cells were seeded in six-well plates at a density of 5×10^4 cells/well as described under "Experimental Procedures" and subsequently maintained in steroid-reduced medium containing 5% dialyzed FBS. Cells were then fed with steroid-reduced medium in the presence or absence of 10 nM DHT. Total cell numbers were counted at days 2, 4, and 7, while 3 ml/well fresh medium with or without DHT was added to the remaining cultures at days 2 and 4. Ratios of DHT stimulation were calculated from cell numbers in wells with DHT divided by wells without DHT. The data shown are the average of duplicate wells. Similar results were observed in three sets of independent experiments. *Bar*, the range of results from duplicate wells. *B*, RT-PCR analyses on AR expression. The mRNAs of AR and actin in 1 μ g each of total RNA from different LNCaP cells were amplified by RT-PCR with specific primers. 33, 51, and 81, clone 33, 51, and 81 LNCaP cells. *m*, λ HindIII and ϕ x174 HaeIII digest DNA markers. Sizes from top to bottom are 2.3, 2.0, 1.3, 1.0, 0.87, 0.6, 0.56, 0.31, 0.28, 0.27, 0.19, 0.18, 0.12, and 0.11 kb.

LNCaP cells. A 7-day treatment with 10 nM DHT had approximately a 20% stimulation on the growth of clone 81 LNCaP cells, while it exhibited up to a 2.5-fold stimulatory effect on clone 33 cells in the same set of experiments (Fig. 2A). To clarify the relationship between the expression of AR and the degree of androgen-stimulated cell growth, we investigated the AR expression. By using RT-PCR with a linear reaction rate (see "Experimental Procedures"), the level of AR mRNA in clone 81 LNCaP cells remained approximately the same as that in clone 33 cells (Fig. 2B). The similar expression level of AR protein in different LNCaP cells was further demonstrated by Western blot analyses (Fig. 1C). By semiquantitation with a densitometer, the AR protein level in clone 81 cells was more than 90% of that in clone 33 cells (data not shown). Therefore, in different LNCaP cells, despite a similar level of AR expression, the degree of androgen stimulation was altered.

Expression of Prostate-specific Differentiation Antigens in Different LNCaP Cells—Since PSA mRNA level and cellular PAcP activity were responsive to androgen treatment (21, 22), we investigated their expression in clone 33 LNCaP cells after they were exposed to DHT. Androgen stimulation of clone 33 LNCaP cells correlated with an up-regulation of PSA mRNA level (Fig. 3A) and a diminished level of cellular PAcP (Fig. 3B).

The expression of these two antigens in different LNCaP

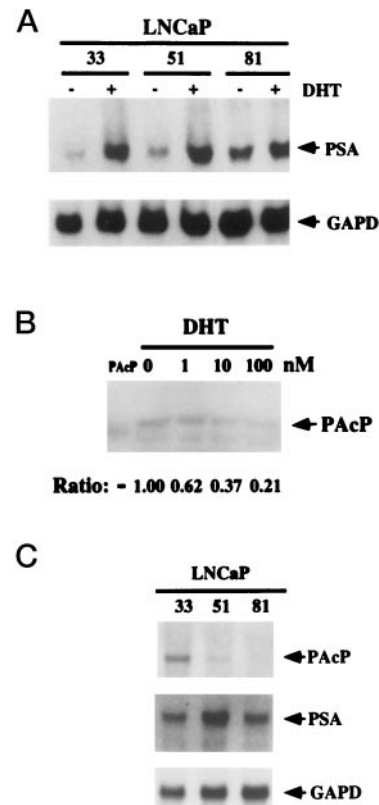


FIG. 3. Expression of PSA and PAcP in different LNCaP cells. *A*, DHT effects on PSA mRNA expression. Different LNCaP cells were plated as described above and treated with 10 nM DHT for 3 days. Total RNA was prepared from experimental cells or control cells receiving solvent alone. After electrophoresis and transfer, the same blot was hybridized first with PSA followed by GAPDH (*GAPD*) cDNA probes. 33, 51, and 81, clone 33, 51, and 81 of LNCaP cells, respectively. *B*, DHT effect on cellular PAcP protein level. LNCaP cells (clone 33) were treated with different concentrations of DHT as indicated in the figure for 3 days. The PAcP level was demonstrated by Western blotting using anti-PAcP antiserum. Signals were detected by autoradiography following 125 I-protein A hybridization. The first lane shows the position of PAcP purified from seminal fluid. *C*, Northern blot analyses. Total RNA with 15 μ g each from different LNCaP cells grown in steroid-reduced medium containing 5% dialyzed FBS was electrophoresed in denaturing agarose gels. After blotting, the same membrane was hybridized with PAcP, PSA, and GAPDH (*GAPD*) cDNA probes, sequentially. 33, 51, and 81, clone 33, 51, and 81 cells, respectively.

cells was further examined. In all three clones, LNCaP cells expressed PSA mRNA (Fig. 3, A and C), and its level was up-regulated by DHT as indicated by RT-PCR (data not shown) and Northern blot analyses (Fig. 3A). To semiquantify the androgen induction, autoradiograms were densitometrically analyzed, and the intensity of the PSA band was then normalized to that of GAPDH band. The results demonstrated that, in each clone of LNCaP cells, there was a 2–3-fold induction by DHT (data not shown). However, in clone 81 cells, the expression of PAcP diminished as shown by RT-PCR (data not shown), Northern blot analyses (Fig. 3C), and PAcP activity assays (Fig. 4A). Thus, in clone 81 LNCaP cells that express a functional AR protein, the degree of androgen stimulation of cell growth diminished with a parallel decrease in PAcP.

Growth Rate and Protein Phosphatase Activity of Different LNCaP Cells—To investigate the biological significance of PAcP expression in different LNCaP cells, we quantified the serine/threonine PP activity and compared that with cellular PAcP activity. As shown in Fig. 4A, the total serine/threonine PP activity, the PP-1 activity, and the PP-2A activity were elevated in clone 81 cells, higher than that in clone 33 and clone 51 cells. Thus, in different LNCaP cells, the expression of PAcP

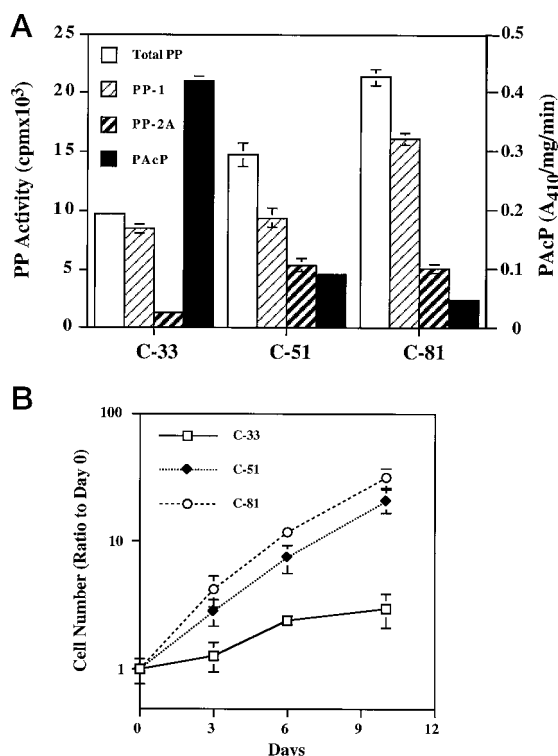


FIG. 4. Protein phosphatase activity and growth rates of different LNCaP cells. A, PACp and serine/threonine protein phosphatase activities. PACp and protein phosphatase activities in different LNCaP cells were analyzed. The PACp activity was the L-(+)-tartrate-sensitive AcP activity in 1 mg of total cell lysate proteins, while the Ser/Thr protein phosphatase activity was obtained using a 1:500-fold dilution of cellular extract (see "Experimental Procedures" for details). PACp activity represented the average of triplicate results, and Ser/Thr protein phosphatase activity was the mean cpm \pm S.D. ($n = 9$) of $^{32}\text{P}_i$ that was released from the substrate during the incubation period. B, growth rates of different LNCaP cells. LNCaP cells were plated in steroid-reduced medium containing 5% dialyzed FBS to avoid serum androgen effect. One set of attached cells was harvested and counted as day 0. The remaining cells were fed with fresh medium containing dialyzed FBS. The total cell number was then counted on days 3, 6, and 10, while fresh steroid-reduced medium (3 ml/well) was added to the remaining cultures on days 3 and 6. The data shown are the average of duplicate wells after normalization to day 0, indicating cell growth rates. Similar results were observed in three sets of independent experiments. Bar, the range of results from duplicate wells.

activity decreased, while the Ser/Thr protein phosphatase activity increased.

Since cellular PACp has been implicated to be involved in growth regulation (22, 24), we examined the growth rate of different LNCaP cells in steroid-reduced medium to avoid serum androgen effect. As shown in Fig. 4B, the growth rates of clone 81 LNCaP cells increased, higher than clone 33 cells. Under described conditions using medium containing 5% steroid-reduced FBS, the doubling time of clone 81, 51, and 33 cells was approximately 29, 48, and 110 h, respectively. Therefore, a decreased expression of PACp correlated with an increased growth rate. Using PC-3 cells as controls, there was no significant effect on the growth rate with increasing passage levels (Refs. 22 and 24, and data not shown).

Androgen Effect on PACp cDNA-transfected Clone 81 LNCaP Cells—To further delineate the relationship of cellular PACp expression with androgen responsiveness of cell proliferation, we transfected clone 81 LNCaP cells with a PACp cDNA expression vector driven by a cytomegalovirus promoter. After G418 selection and subcloning, two independent transfected cells, LNCaP-23 and LNCaP-34, were established as sublines. Biochemically, in these two transfectants, the cellular PACp

protein level and activity were increased (Fig. 5A) with a concomitant decrease in growth rate, lower than that of the corresponding control clone 81 parent cells (Fig. 5B). Since cellular PACp exhibits the PTPase activity, the Tyr(P) level in cellular proteins of LNCaP-23 and -34 cells was investigated. As shown in Fig. 5C, an increased expression of PACp correlated with a decreased Tyr(P) in cellular proteins from both transfected cells. It was noted that the Tyr(P) level of two phosphoproteins with a respective molecular size of approximately 185 and 150 kDa decreased significantly, although some other proteins also had decreased Tyr(P) levels (Fig. 5C). Thus, the expression of cellular PACp correlated with decreased Tyr(P) levels in cellular proteins and a diminished growth rate.

To examine the effect of additional PACp expression on the androgen responsiveness, cells were exposed to different concentrations of DHT. As shown in Fig. 5D, the degree of androgen stimulation of these two transfectants was enhanced, the stimulant effect being higher than that of the corresponding clone 81 parent cells (Fig. 5D) as well as LNCaP-CMV control cells transfected with the vector alone (data not shown). Thus, an increased expression of cellular PACp restored the androgen sensitivity of clone 81 LNCaP cells.

Androgen Effect on PACp cDNA-transfected PC-3 Cells—One of the major differences between LNCaP and PC-3 cells with respect to androgen sensitivity is that only a marginal level of AR is expressed in PC-3 cells (Fig. 1, B and C). Using a transient expression assay, we first investigated whether AR in PC-3 cells could have an androgen action. As shown in Fig. 6A, R1881, a synthetic androgen, reproducibly had approximately a 2-fold stimulation of the CAT activity in PC-3 cell lysate proteins. Additionally, R1881 had approximately a 9-fold stimulation of the CAT activity in human AR cDNA-transfected PC-3 cells, as in LNCaP cells (Fig. 6A). As a control, R1881 did not have an effect on the CAT activity in DU 145 cells, since no AR expression was detected in those cells (data not shown). Thus, PC-3 cells expressed a functional AR, although the level was lower. The degree of androgen stimulation on the CAT activity apparently correlated with the expression level of AR.

Since the expression of cellular PACp in LNCaP cells correlated with the growth stimulation by androgen, we investigated the androgen effect on the growth of two sublines of PACp cDNA-transfected PC-3 cells, *i.e.* PC-411 and PC-416 cells that express an exogenous, cellular form of PACp (22, 24). By RT-PCR analyses, PC-416 and PC-411 cells expressed low levels of AR mRNA (data not shown), as in PC-3 parent cells (Fig. 1B). Biochemically, PC-416 and PC-411 cells expressed an exogenous cellular PACp and had a slow growth rate (Fig. 6B) as well as a decreased Tyr(P) level in cellular proteins including pp185 and pp150 (Fig. 6C). Nevertheless, the Tyr(P) level of two other phosphoproteins with a molecular size of approximately 70 and 55 kDa, respectively, was also changed notably (Fig. 6C). Furthermore, the growth of PC-411 and PC-416 cells was stimulated significantly by DHT ($p < 0.05$) (Fig. 6D). Thus, in PC-416 and PC-411 cells that express an endogenous AR, the expression of an exogenous, cellular PACp correlated with androgen stimulation of cellular growth.

Androgen Effect on Protein Tyrosine Phosphorylation—To elucidate a possible mechanism by which androgen stimulates cell growth, we analyzed protein tyrosine phosphorylation in cellular proteins from different androgen-responsive and -unresponsive cells including LNCaP clone 33, clone 81, and LNCaP-34 cells and PC-3 as well as PC-416 cells after exposure to androgen. As shown in Fig. 7, upon androgen stimulation, the Tyr(P) level of pp185 in androgen-responsive cells including LNCaP clone 33, LNCaP-34, and PC-416 cells was consistently increased, higher than that in corresponding control cells in the

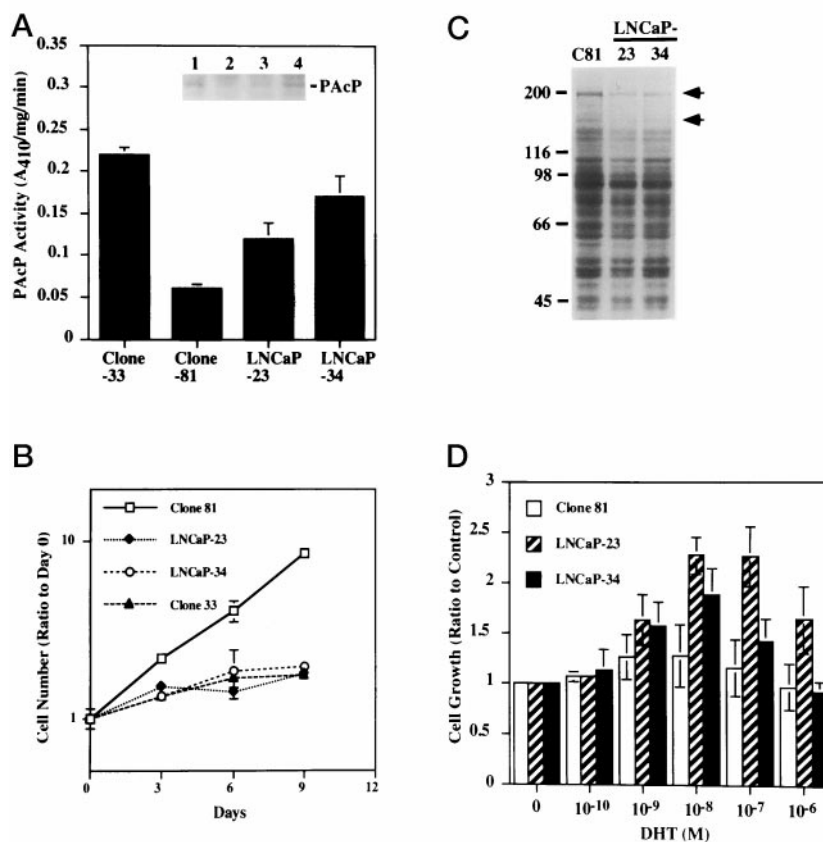


FIG. 5. Androgen effect on the growth of PAcP cDNA-transfected clone 81 LNCaP cells. *A*, PAcP activity. Total cell lysate proteins were harvested from clone 33, clone 81 LNCaP cells, and PAcP cDNA-transfected LNCaP-23 (passage 8) and LNCaP-34 (passage 6) sublines. The A_{410} represented the PAcP activity in 1 mg of total cell lysate proteins. *Inset*, Western blot analyses. An aliquot of 50 μ g of total cell lysate proteins/lane was electrophoresed, blotted to membrane filters, and hybridized with an anti-PAcP antiserum. *Lane 1*, clone 33; *lane 2*, clone 81; *lane 3*, LNCaP-23; *lane 4*, LNCaP-34 cells. *B*, effect of additional PAcP expression on the basal cell growth rate. PAcP cDNA transfectants, LNCaP-23 (passage 8), LNCaP-34 (passage 6) cells, and parent clone 81 cells were seeded in steroid-reduced medium containing 5% dialyzed FBS. For comparison, clone 33 cells were also plated. 3 days after seeding, one set of attached cells in duplicate wells was harvested and counted as day 0. The remaining cells were fed with fresh steroid-reduced medium containing 5% dialyzed FBS. Cells were harvested at days 3, 6, and 9 for counting. Fresh medium was added on days 3 and 6 to the remaining cells. Similar results were observed in two sets of independent experiments in duplicate wells. *C*, the Tyr(P) level in total cellular proteins. An aliquot of 50 μ g of total cellular lysate proteins from each cell line was electrophoresed and blotted into nitrocellulose membranes. After the membrane was blocked with 5% skim milk, the membrane filter was reacted with anti-Tyr(P) Ab. Clone 81 parent cells (*C81*) and LNCaP-23 (passage 8) and -34 (passage 10) PAcP cDNA-transfected LNCaP cells are shown. *Arrows* indicate the respective positions of 185 and 150 kDa. *D*, androgen effect on the cell growth rate. Cells, including clone 81, LNCaP-23 (passage 8), and LNCaP-34 (passage 10), were plated, harvested at day 7, and counted as described in the Fig. 2 legend for different LNCaP cells. The growth stimulation by DHT was normalized to the control cells (as 100%) that were maintained in the absence of androgen. The data shown are the average of duplicate wells. Similar results were obtained in three sets of independent experiments with duplicate wells.

absence of androgen. Furthermore, androgen had little or no inhibitory effect on tyrosine phosphorylation of pp185 in androgen-unresponsive LNCaP clone 81 and PC-3 cells (Fig. 7). Thus, androgen stimulation of cell growth correlated with an increased Tyr(P) level of pp185.

DISCUSSION

We examined the role of AR in the progression of human prostate carcinoma cells from androgen-sensitive to androgen-insensitive carcinomas. The transition from androgen-responsive to androgen-insensitive growth could be due to the preferential outgrowth of AR-negative tumor cells. For example, in the R-3227 Dunning rat prostate carcinoma model system, the androgen-resistant AT-1 and MAT-Lu sublines lacked immunostaining for the AR protein, and their AR mRNA levels were less than 10% of that in the androgen-sensitive H subline cells (11). Similarly, among human cancer lines, DU 145 cells did not express a detectable AR and were androgen-unresponsive for growth stimulation (Fig. 1).

Nevertheless, our results indicate that the expression of a functional AR is apparently required, but not sufficient, for the androgen stimulation of human prostate carcinoma cell

growth. This is because androgen has no significant stimulation on the growth of clone 81 LNCaP and PC-3 cells, although both these cells express a functional AR protein (Figs. 1C, 3A, and 6A), similar to clone 33 cells. For example, in androgen-unresponsive clone 81 LNCaP cells, the expression of PSA mRNA level is up-regulated by DHT, similar to that in clone 33 cells (Fig. 3A), indicating factors in addition to AR, are involved in androgen responsiveness of cell proliferation.

If the androgen unresponsiveness of PC-3 cells is merely due to the low level of AR expression (Fig. 1, *B* and *C*; Refs. 35 and 42), which is below the threshold of androgen effect, an increased expression of functional AR should result in androgen stimulation. However, an additional expression of functional AR driven by a cDNA expression vector in AR cDNA-transfected PC-3 subcloned cells does not have any stimulatory effect on cell growth (35, 43). Instead, the growth rate of those cells is decreased by exposure to androgen (35, 43), similar to that observed in rat prostate cancer cells (44). This androgen inhibition of the AR cDNA-transfected prostate carcinoma cells is also similar to estrogen inhibition of estrogen receptor cDNA transfected breast carcinoma cells (45). Although the molecular

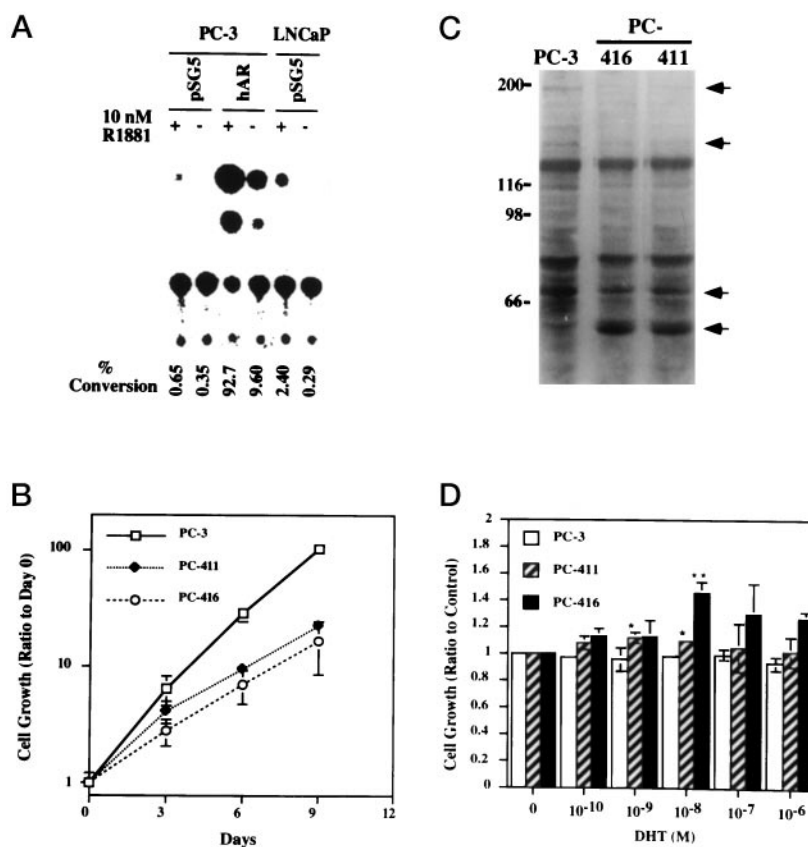


FIG. 6. Androgen effect on the growth of PACp cDNA-transfected PC-3 cells. A, expression of functional AR in PC-3 cells. PC-3 cells were transfected with 10 μ g of pSG5 vector and 10 μ g of pMSG-CAT plasmid (lanes 1 and 2) or 10 μ g of pSG5-AR and 10 μ g of pMSG-CAT plasmids (lanes 3 and 4). As controls, LNCaP cells (clone 33) were also transfected with 10 μ g of pSG5 vector and 10 μ g of pMSG-CAT plasmid (lanes 5 and 6). After a 24-h culture, cells were treated with 10 nM R1881 (lanes 1, 3, and 5) or solvent ethanol alone (lanes 2, 4, and 6). Following a 48-h incubation, cells were harvested, and 50 μ g of protein from each cytosol was used in the CAT assay. The percentage of conversion of [¹⁴C]chloramphenicol to monoacetylated product was indicated. B, expression of PACp and the basal cell growth rate. PC-411 (passage 5), PC-416 cells (passage 7), and PC-3 parent cells were plated as described above. 24 h after seeding, the attached cells in one set of duplicate wells were harvested and counted as day 0. The remaining cells were fed with fresh steroid-reduced medium containing 5% dialyzed FBS at days 0, 3, and 6. Total cells were harvested on days 3, 6, and 9 for cell counting. The data shown are the average results of duplicate wells, and similar results were obtained from three independent experiments. Bar, the range of results from duplicate wells. C, the Tyr(P) level in cellular proteins from PC-transfectants. An aliquot of 150 μ g total cellular proteins was electrophoresed and blotted. The membrane was reacted with anti-Tyr(P) Ab as described above. Arrows, 185, 150, 70, and 55 kDa, respectively. D, androgen effect on the growth of PC-411 and PC-416 cells. PC-411 (passage 5), PC-416 (passage 6), and PC-3 parent cells were treated with different concentrations of DHT. Total cells were harvested and counted on day 5. The cell number in each control well was defined as ratio 1. The data shown are the average of duplicate wells from one of three sets of independent experiments. Bar, the range of results from duplicate wells. *, $p < 0.05$ versus control ($n = 6$); **, $p < 0.01$ versus control ($n = 6$).

mechanism of these steroid inhibitions is not known, the data taken collectively indicate that the expression of functional AR has little direct linkage to androgen stimulation of the growth of prostate carcinoma cells.

Unexpectedly, we observed that, in AR-expressing LNCaP and PC-3 prostate carcinoma cells, the expression of PACp inversely correlates with the androgen responsiveness of cell growth as well as the basal growth rate. It is possible that the increased basal growth rate of clone 81 LNCaP cells is merely due to the decreased expression of PACp protein, which results in a reduced protein mass effect and/or a reduced usage of the translational machinery. If this is the case, we would expect to obtain an even higher stimulation by DHT of clone 81 cells than of clone 33 cells, since more machinery is available for cellular growth in clone 81 cells. However, the degree of DHT-stimulatory activity of cell growth is diminished in those cells (Fig. 2A). Conversely, an additional expression of cellular PACp by cDNA transfection in clone 81 cells correlates with an androgen-stimulated cell growth (Fig. 5D). Thus, the biochemical characteristics of clone 81 cells resemble that observed in endocrine therapy-resistant human prostate adenocarcinomas in their diminished androgen responsiveness with an unchanged level of functional AR (12, 13, 46). In our studies with human pros-

tate carcinoma cells, to the best of our knowledge, this is the first report that a decreased expression of a differentiation-associated PTPase, PACp, correlates with a reduced stimulation of cellular growth by a steroid, androgen. Conversely, exogenous expression of PACp by cDNA transfection in androgen-insensitive cells restores the androgen responsiveness of growth stimulation.

These findings imply that cellular PACp plays a role in androgen stimulation of cell growth. This may, therefore, provide an explanation for clinical observations in the higher grades/stages of prostate carcinomas, which correlate with lower levels of cellular PACp expression (3, 47–50). These high grade tumors will eventually escape from anti-androgen or androgen deprivation therapy, despite unaltered expression of functional AR in these carcinomas (12, 13, 46). Nevertheless, it is also possible that the decreased expression of PACp, a differentiation antigen, in high passage carcinoma cells is due to the dedifferentiation of carcinoma cells upon passage. Further experiments are required to delineate the mechanism of decreased PACp expression in clone 81 cells.

Since cellular PACp expression correlates with androgen responsiveness of cellular growth, it is imperative to understand the possible molecular mechanism by which cellular PACp is

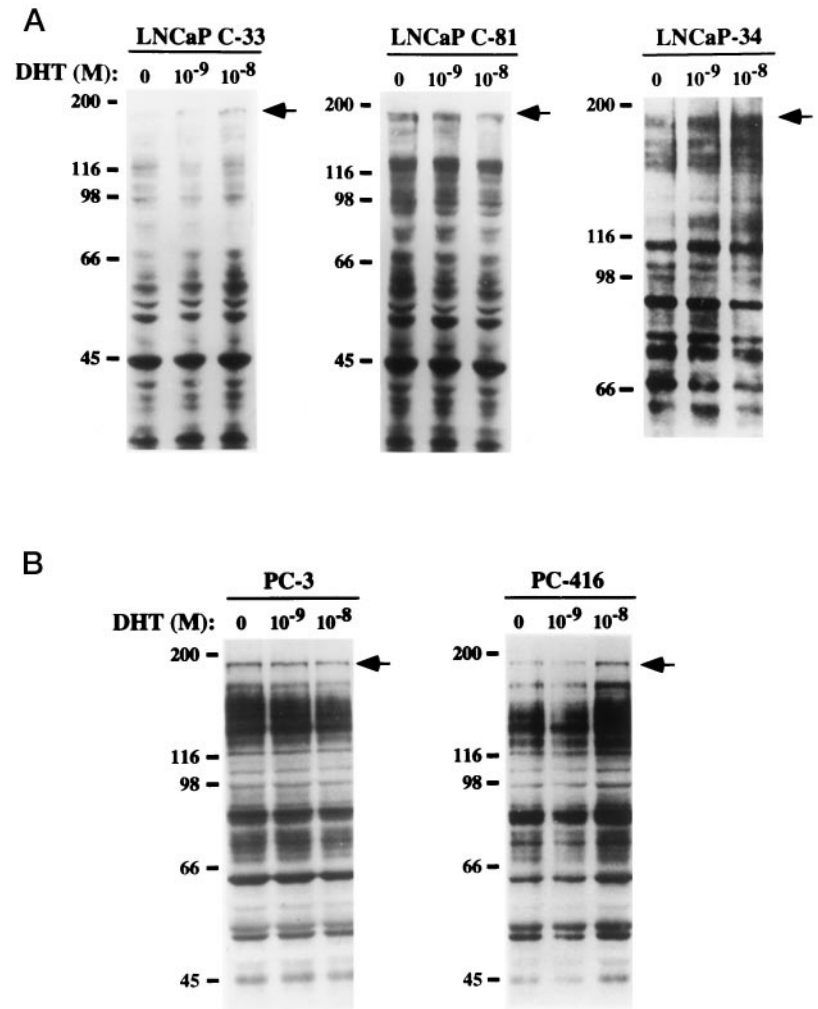


FIG. 7. **Androgen effect on protein tyrosine phosphorylation.** To analyze androgen effect on protein tyrosine phosphorylation, cells were grown in steroid-reduced medium in the presence or absence of different concentrations of DHT for 3 days. An aliquot of total cell lysate proteins was electrophoresed and analyzed by Western blotting with anti-Tyr(P) Ab. A, LNCaP clone 33, clone 81, and LNCaP-34 cells; B, PC-3 and PC-416 cells. Arrow, 185 kDa.

involved. Utilizing phosphomonoesters as substrates, PACP has classically been classified as a "histidine" acid phosphatase without known function (23). Recently, several lines of evidence support the notion that the cellular form of PACP could indeed function as a neutral "cysteine" PTPase in prostate epithelial cells (22, 31, 33, 51, 52). Thus, the involvement of cellular PACP in androgen regulation of cell growth is apparently by participation in the tyrosine phosphorylation signal transduction pathway (Figs. 5C and 6C), resulting in down-regulating cellular growth as in PACP cDNA-transfected LNCaP and PC-3 cells (Figs. 5B and 6B). For example, the tyrosine phosphorylation of pp185 is decreased in those PACP-expressing cells (Fig. 5C and 6C). Subsequently, that diminished growth rate would result in a distinct growth stimulation by androgen, which causes a decrease in cellular PACP activity (Fig. 3B; Refs. 22 and 30) and an increased Tyr(P) level of pp185 (Fig. 7). Conversely, clone 81 LNCaP and PC-3 cells lacking PACP expression have an increased Tyr(P) level of pp185 (Figs. 5C and 6C; Refs. 22, 30, and 33), a rapid growth rate (Figs. 5B and 6B), and a diminished androgen responsiveness (Figs. 1A, 2A, 5D, and 6D). Thus, cellular PACP may be involved in androgen stimulation of cell growth by regulating tyrosine phosphorylation of pp185. This notion is further supported by observations in PACP cDNA-transfected PC-3 cells. For example, PC-411 cells express a lower level of PACP than PC-416 cells; consequently, the Tyr(P) level of pp185 is higher, and the degree of androgen stimulation is lower than that in PC-416 cells (Fig. 6, C and D). Our preliminary results indicate that pp185 co-migrates with

ErbB-2/neu protein in SDS gels.³ Interestingly, in several independent systems, an induction of cellular differentiation by various differentiation agents is associated with a decreased cell growth and an increased expression of cellular PTPase activity (53–57). Thus, the study of PACP, a differentiation-associated PTPase, for its possible functional role in growth regulation of prostate epithelium may provide us with useful information in understanding the putative role of other differentiation-associated PTPases in their respective cell growth regulation.

In summary, our data clearly demonstrate that the expression of AR is apparently required, but not sufficient, for androgen stimulation of the growth of human prostate carcinoma cells. Furthermore, a decreased expression of cellular PACP, an androgen-responsive putative PTPase, correlates with a diminished androgen-stimulatory activity of cell growth. Thus, the expression of cellular PACP may serve as a useful marker for androgen responsiveness of growth regulation. Further experiments are required to clarify the role of cellular PACP, if any, in cell growth regulation signal transduction by androgen and its molecular mechanism and clarify the identity of pp185.

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³ T. C. Meng and M. F. Lin, manuscript in preparation.

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