Expression of Human Prostatic Acid Phosphatase Activity and the Growth of Prostate Carcinoma Cells

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ABSTRACT

Human prostatic acid phosphatase (PACP) is a tissue-specific differentiation antigen and is the major phosphotyrosyl (p-tyr) protein phosphatase in normal differentiated prostate epithelial cells. In prostate carcinomas, cellular PACP has a low expression. We examined the expression of cellular PACP activity and its correlation with cell growth that may lead us to understand the role of tyrosine phosphorylation in human prostate cells. LNCaP cells, which expressed the highest cellular PACP activity, had the slowest growth rate and the lowest p-tyr level among three human prostate carcinoma cell lines: LNCaP, DU145, and PC-3. This inverse correlation was further examined in LNCaP cells, since these cells remain hormone-sensitive. Androgen, a classical stimulator of prostate cells, stimulated the growth of LNCaP cells whereas cellular PACP activity decreased and p-tyr levels increased. This phenomenon was also observed when cells were treated with epidermal growth factor and fetal bovine serum. Both epidermal growth factor and fetal bovine serum stimulated the growth of LNCaP cells whereas cellular PACP activity decreased. Furthermore, when cell growth was arrested at low temperatures (23°C), cellular PACP activity was elevated. To establish the relationship of cellular PACP activity with cell growth rate, we transfected a complementary DNA encoding the full length PACP protein into another human prostate carcinoma line, PC-3, that lacks endogenous PACP. Two stable transfectants, designated PC-18 and PC-416 cells, were obtained and shown to express PACP mRNA transcribed from the transfected complementary DNA. The expression of PACP activity in PC-416 cells, but not PC-18 cells, was associated with a lower p-tyr level and a slower growth rate than control cells transfected with the expression vector alone. In conclusion, in LNCaP cells, the stimulated cell growth is associated with an increase in PACP activity and a decrease in cellular PACP activity. In PACP complementary DNA-transfected PC-416 cells, the low level of p-tyr corresponds to a slow growth rate.

INTRODUCTION

Protein tyrosine phosphorylation has been documented to play a crucial role in the control of cell growth mediated by several oncogene protein products and growth factor receptors (for review see Refs. 1-5). This, however, is a cyclical process of tyrosine phosphorylation and dephosphorylation, which can be regulated at either step. For example, many growth factor receptors have an endogenous tyrosine kinase activity that is activated in response to ligand binding (for review see Refs. 1, 3, and 5). The activation of receptor tyrosine kinases may function to stimulate various messenger production thereby increasing cell growth. However, this phosphorylation response is transient and the overall p-tyr level in cells returns to the basal level by a dephosphorylation mechanism within 2-5 h of hormonal stimulation (6, 7).

Since the phosphorylation level of a protein is apparently regulated by phosphorylation and dephosphorylation, an unbalanced expression of either activity may tip the level of phosphorylation toward one side or the other. For example, the treatment of the normal rat kidney 1 cells with o-vanadate, an inhibitor of several p-tyr protein phosphatases, causes an increase in intracellular p-tyr levels along with the induction of several parameters of the transformed phenotype (8). O-Vanadate also causes accumulation of p-tyr in Polyomavirus-transformed cells (9). The addition of o-vanadate to adipocytes has been reported to mimic insulin activation causing an increase in tyrosine phosphorylation (10).

Different p-tyr phosphatases are distinguished by their sizes, pH optima, specific inhibitors, and rates of dephosphorylation of and apparent affinities for various p-tyr-containing proteins and peptides (for review see Refs. 11-14). p-tyr protein phosphatases may play a role in the signal transduction pathway. The leukocyte common antigen CD45 has been shown to be a receptor-like p-tyr protein phosphatase (15). Results of further studies indicated that CD45 may play a role in the antigen activation signal transduction pathway by p-tyr dephosphorylation (16). One possible mechanism may be that CD45 directly, or indirectly, modulates the tyrosine kinase specific activity of p56lck (17). In addition, p-tyr dephosphorylation of P34cdc2 kinase has been suggested to play one of the essential roles in mitosis activation (18). The induction of granulocytic and monocytic maturation of HL-60 promyelocytic leukemia cells is associated with an increase in p-tyr phosphatase activity (19). Furthermore, receptor protein-tyrosine phosphatase γ has been proposed to be a candidate tumor suppressor and its functional loss may be involved in the pathogenesis of kidney and lung tumors (20).

In prostate epithelial cells, the cellular form of the major acid phosphatase, PACP, has been shown to have a low expression in carcinoma cells although its secretory form may be elevated and used as a parameter for tumor diagnosis in clinical laboratories (21-23). Recently, the major p-tyr protein phosphatase activity in prostate epithelial cells has been copurified with the cellular PACP protein (24). The purified PACP dephosphorylates p-tyr linkages in proteins at nm concentrations (24, 25). A neutral pH optimum was observed for PACP to dephosphorylate the auto-phosphorylated EGF receptor (26). Using two human prostate carcinoma cell lines, LNCaP and DU145, as the model system, an inverse correlation was observed between cellular PACP activity and tyrosine kinase specific activity (27, 28). It was therefore proposed that the cellular form of PACP may be involved in the regulation of p-tyr levels in cells.

In the present article, we studied the expression of phosphatase activities including PACP activity in prostate carcinoma...
cells to understand further the possible relationship of prostate cell growth and p-tyr dephosphorylation. We examined the correlation between the expression of PAcP activity and the cell growth in one prostate carcinoma cell line, LNCaP, since the inverse correlation between PAcP activity and tyrosine kinase activity was primarily observed in two different cell lines (27). We quantitated the expression of endogenous phosphatase activities in LNCaP cells when the cell growth rate was modulated. In addition, we established stable transfectants of the PAcP cDNA in PC-3 cells, a human prostate carcinoma cell line which lacks endogenous PAcP expression, and looked at the effect of PAcP expression on p-tyr levels and growth in these cells.

MATERIALS AND METHODS

Cells. Three prostate carcinoma cell lines, LNCaP (29), DU145 (30), and PC-3 (31), were derived from different metastatic lesions of human prostatic adenocarcinomas. They were all obtained from the American Type Culture Collection and maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 7% (v/v) FBS (Gibco) as described previously (26, 27). Of these three lines, LNCaP cells remain androgen sensitive, can express endogenous PAcP, and can be stimulated to grow by EGF (27, 29). PC-3 human prostate carcinoma cells are androgen insensitive (31) and lack endogenous PAcP expression (32). PC-3 cells were used for the PAcP cDNA transfection experiments. All other reagents used were described in previous publications (25–28).

Protein Determinations. The protein concentration in cell lysates was quantitated by the Bio-Rad dye protein assay. Bovine serum albumin was used as a standard (25).

Acid Phosphatase Activity Determinations. Since PAcP hydrolyzes PNPP at acidic pH (24, 25), the acid phosphatase activity assay was performed to quantify the specific activity of PAcP. The total acid phosphatase activity was measured using 50 μg of total cell lysate protein in 0.4 ml of 50 mM citrate, pH 5.6, with 3 mM PNPP as the substrate. The reaction was terminated by the addition of 2 ml of 0.1 N NaOH. The released p-nitrophenol was measured spectrophotometrically at 410 nm (25, 26). The reaction rate was linear for at least 15 min at 35°C (27).

1(+)·Tartrate has been demonstrated to be a relatively specific inhibitor of PAcP since it does not affect alkaline phosphatase and most other AcP activities (28, 33). In addition, over 90% of the tartrate-sensitive AcP activity in prostate epithelial cells including LNCaP cells was immunoprecipitated by specific polyclonal antibodies to PAcP (28, 29, 33). Therefore, 1(+)·tartrate sensitivity was used to distinguish cellular PAcP activity from other AcP activities unless it was specified.

Hormone Effects on Cellular Phosphatase Activity and Cell Growth. LNCaP cells were plated in RPMI 1640 containing 7% FBS and maintained in a 37°C incubator (7% CO2) for 72 h without disturbance since LNCaP cells do not attach to flasks very well (29). To test the effects of androgen on cell growth and cellular phosphatase activity, different concentrations of DHT were added to LNCaP cells that had been exposed to steroid-reduced medium for 72 h, in the medium containing 2% heat-inactivated, dialyzed FBS (molecular weight cutoff, 3500) (27). An aliquot of hormone-treated cells and control cells was counted to quantitate the cell growth. Another aliquot of cells was assayed for phosphatase activity.

Phosphotyrosine Level Analysis. To quantitate the relative p-tyr levels, hormone-treated LNCaP cells, control cells, and PC transfectants were maintained in the phosphate-free medium containing 2% dialyzed FBS and respective hormones and 5% dialyzed FBS, respectively, as specified in each experiment. Cells were then labeled with [32P]P for 16 h at 37°C. The [32P]-labeled proteins were extracted with phenol followed by chloroform-methanol (27, 34). The extracted proteins were subjected to partial acid hydrolysis for 90 and 60 min, respectively, and [32P]-amino acids were resolved by the high voltage paper electrophoresis in two dimensions as previously described (27).

[35S]Methionine Labeling. DHT (10−8 m)-treated and control cells after a 5-day treatment were maintained in methionine-free medium containing 2% heat-inactivated, dialyzed FBS and DHT/ethanol or ethanol alone, respectively, for 30 min at 37°C incubator (7% CO2). Tracer [35S]methionine/ml medium and incubated for an additional 2 h. Cells were collected for immunoprecipitation. It was found that an aliquot of 5 μl of rabbit antiserum (25) could precipitate 35S-P-AcP from up to 250 μg of total cell lysates.

Statistical Analyses. The data for the correlation between the cell growth and the PAcP specific activity were statistically analyzed by calculating the correlation coefficient (T) (35). The significance of difference between two groups of data was analyzed by paired two-tailed Student's t test (P) (35). P < 0.05 is considered significant.

Cloning of PAcP cDNA and Preparation of a cDNA Probe. The PAcP cDNA clone encoding the secretory form of PAcP was obtained by screening a normal human prostate λgt11 cDNA library (Clontech) with two oligonucleotides (17-mers) corresponding to the reported 5'- and 3'-end of PAcP coding sequence, respectively (36, 37), according to the protocol of Maniatis et al. (38). A positive clone that hybridized with both probes was obtained, the cDNA was digested with EcoRI restriction endonuclease, and three fragments of 0.22, 0.56, and 1.62 kilobases were recovered and subcloned separately into the pBluescript vector (Stratagene). The identity of these clones was confirmed by DNA sequencing using the Sequenase system (I. S. Biochemical) and specific synthetic oligonucleotides. The 2.4-kilobase PAcP cDNA contains a 1.1-kilobase coding region and a 1.3-kilobase 3'-noncoding region. A 294-base pair EcoRI/XbaI fragment from the 3'-end of the coding region was used as the probe for RNA blot analyses as described in a previous report (32).

Construction of PAcP Expression Vector. Since the PAcP coding region contains two endogenous EcoRI restriction sites (36, 37) and since the PAcP cDNA is inserted at the EcoRI site in λgt11 vector, PCR was utilized to amplify the full-length cDNA containing the signal sequence and the coding sequence of PAcP. The 2.4-kilobase cDNA in λgt11 vector was used as the template for PCR reactions. The primers used were the nucleotides with sequences corresponded to 5'-end and 3'-end of the target sequences, respectively, with a BamHI restriction site added at each end to create a unique site for subcloning. The specific sequences of the primers are 5' CCGGATCCGGATGGAG-AGCTTGACCCCTC 3' and 5' CCGGATCCGGATGTCAT-GTCTCAGT 3'. The resulting PCR product is about 1.2 kilobases. The PCR product was first subcloned in pBluescript and sequenced using Sequenase system to ensure that PCR product had an identical sequence to the template cDNA. The amplified PAcP cDNA was then ligated into the unique BamHI site of pCMV-Neo-Bam, an expression vector obtained from Dr. Bert Vogelstein at Johns Hopkins University (39). This vector contains approximately 0.6 kilobase polyadenylate downstream of the cDNA insert and a neomycin resistance gene for transfectant selection (39).

cDNA Transfection and Selection of Stable Transfectants. Human prostate carcinoma cells, PC-3, that lack the expression of PAcP were selected for our cDNA transfection experiments (32). Lipofectin reagent was used as the cDNA carrier and the protocol was performed essentially as recommended by the manufacturer (Gibco/BRL Laboratories) with minor modifications as follows. PC-3 cells were grown in RPMI 1640 supplemented with 7% FBS, 1% glutamine, and 0.5% gentamicin until they reached approximately 70% confluency. Cells were fed with OPTI-MEM I reduced serum medium and transfected with pCMV-Neo-Bam vector and pCMV-Neo-Bam containing a 1.2-kilobase cDNA-encoding PAcP protein (pCMV-PacP-CS) that were premixed with lipofectin, respectively. After 20 h incubation at 37°C in 7% CO2, cells received fresh RPMI 1640 containing 10% FBS and incubated for an additional 24 h. Stable transfectants were then selected and subcloned in the presence of 0.25 mg/ml G418 and maintained in G418 for approximately 2 months.

Northern Blot Analyses. Total RNA was prepared from LNCaP and PC-3 cells by the guanidine isothiocyanate method (38). Ten μg of each...
total RNA sample were electrophoresed on 1.2% agarose gels containing formaldehyde as a denaturing agent (40). After electrophoresis, the gel was stained with ethidium bromide, visualized to ensure approximately equal amounts of RNA per lane, and then blotted to nitrocellulose membranes by standard techniques (40). Filters were hybridized and washed under stringent conditions twice for 15 min in 0.5 × standard saline-citrate plus 0.1% sodium dodecyl sulfate at room temperature and once for 30 min in 0.5 × standard saline citrate (150 mM NaCl and 15 mM citrate) plus 0.1% sodium dodecyl sulfate at 52°C. cDNA probes were labeled with [α-32P]dCTP using random oligonucleotide primed synthesis (41) with a commercial system (Bethesda Research Laboratories). β-Actin cDNA probe was purchased from Clontech.

RESULTS

Cellular PAcP Activity and p-tyr Level in Prostate Carcinoma Cells. In order to understand the possible role of tyrosine phosphorylation in the growth regulation of prostate cells, we first conducted experiments to examine the correlation between the p-tyr level and the growth rate of currently available three prostate carcinoma cell lines, LNCaP, DU145, and PC-3. All cells were maintained in RPMI 1640 containing 7% FBS for three passages before conducting the experiments. Cellular PAcP specific activity was also quantitated.

As shown in Table 1, an inverse correlation between PAcP specific activity and p-tyr level as well as growth rate among the three cell lines was observed. The marginal PAcP activity in DU145 and PC-3 cells, respectively, is in part due to the co-precipitation of acid phosphatase-4, a minor species of L(+)-tartrate-sensitive acid phosphatase that is immunologically cross-reactive with PAcP antibodies, in prostate cells (33, 42). Nevertheless, LNCaP cells expressed the highest PAcP activity and had the lowest p-tyr levels and the longest generation time. These results indicated that the level of tyrosine phosphorylation is correlated with the growth rates of prostate carcinoma cells. This is compatible with the notion that cellular PAcP may be involved in regulating a fraction of p-tyr in proteins in prostate epithelial cells since large fluctuations of PAcP activity among different cell lines correlate with only some changes in total p-tyr level.

Androgen Effects on Cell Growth and Phosphatase Activity. We examined the expression of PAcP activity when cells were stimulated to grow in one cell line, LNCaP, by taking advantage of their androgen sensitivity (29). Androgen was reported to stimulate the growth of prostate carcinoma cells (29, 43, 44). This formulated the basis of the antiandrogen hormonal therapy used in the treatment of prostate cancer (43).

We examined the effects of androgen on the growth rate, p-tyr level, and cellular PAcP activity of LNCaP cells. As shown in Fig. 1, 10 nM DHT, an active form of endogenous androgens, stimulated the growth of LNCaP cells by approximately 60% over a 4-day treatment. This stimulation was comparable to results of the original report on LNCaP cells (29). The low stimulation by DHT could be due to the slow growth rate of LNCaP cells (Table 1). The increased cell growth was associated with a 35% increase in the p-tyr level (Fig. 1). Nevertheless, the activity of cellular PAcP was decreased by about 40% as measured by PPn hydrolase activity in tartrate sensitive assays (Fig. 1). The decrease in PAcP activity in cell lysates was further confirmed by immunoprecipitations with specific antiserum (data not shown). In contrast, there was no decrease in the tartrate-insensitive acid phosphatase activity (data not shown).

The specificity of DHT effects was further examined. LNCaP cells express low 5α-reductase activity and are therefore not significantly stimulated by testosterone (29). We then examined testosterone effects on cell growth and cellular PAcP activity. DHT and testosterone at concentrations of 10 nM each were used. After a 3-day treatment, testosterone stimulated cell growth by only about 10% with less than a 10% decrease in cellular PAcP activity (data not shown). However, DHT stimulated cell growth by 50% and caused a 30% decrease in cellular PAcP activity. Therefore, DHT stimulated the growth of prostate carcinoma cells with a corresponding decrease in cellular PAcP activity. In addition, all acid phosphatases showed a decrease in activity after DHT treatment.

Kinetics of Androgen Effect on Cellular PAcP Activity. The kinetics of the androgen effect on cellular PAcP activity and cell growth was further examined. Because LNCaP cells respond slowly to 10−8 M DHT, a high concentration of DHT (10−5 M) was used for these experiments. In addition, since the growth rate is too slow to be measured by cell counting, total cell mass was used to quantitate the stimulation of cell growth by DHT. As shown in Fig. 2, DHT caused a decrease in cellular PAcP activity after a 6-h treatment. The decrease of PAcP activity corresponds to an increase in total cell mass. Therefore, an
concentration) was added to cells that had been maintained in steroid-reduced medium for 72 h as described in "Materials and Methods." Cells in duplicate were harvested every 6 h for quantitations of PAcP-specific activity (O) and total cell mass (■). Bar, range of duplicate results. T = -0.85; P < 0.01.

Increase in cell growth corresponds kinetically with a decrease in PAcP activity. We observed that EGF (10 ng/ml) has approximately a 100% stimulation of LNCaP cell growth over a 3-day treatment. Therefore, we examined the effect of EGF on cellular PAcP activity and growth of LNCaP cells.

As shown in Table 2, EGF (10 ng/ml) treatment resulted in a decrease in more than 25% of cellular PAcP activity. The decrease in PAcP activity was inversely related to an increase in about 30% of LNCaP cell mass. This result was observed after a 16-h treatment with EGF, which corresponds to less than 30% of the generation time of LNCaP cells (Table 1). The effect of EGF on the activity of another group of phosphatases, the tartrate-insensitive acid phosphatases, was also measured as an internal control. EGF caused only about a 5% decrease in the activities of the tartrate-insensitive acid phosphatases (Table 2).

Other Factors Affecting PAcP Activity and Cell Growth. We further examined whether other growth modulators would alter intracellular PAcP activity. First, the effects of the FBS concentration on PAcP specific activity and growth of LNCaP cells were examined. Cell growth was not significantly affected by 0.1% FBS but was greatly stimulated by 7.5% FBS (Fig. 3). The increased growth of LNCaP cells was associated with a decrease in PAcP specific activity. However, there was no significant change in tartrate-insensitive acid phosphatase activity (Fig. 3).

Dialyzed FBS was prepared to examine whether FBS effects on cellular PAcP activity and cell growth were due to endogenous androgens and/or other growth factors present in FBS. FBS was dialyzed extensively against Hepes-saline, pH 7.2, using a M$\text{r}$ 12,000 cutoff membrane and then heat inactivated at 56°C for 30 min. Cells were plated in medium containing 7% FBS for 72 h, replaced with medium containing 1% dialyzed FBS for an additional 72 h, and then maintained in 1-10% dialyzed FBS for 11 days with couple changes of medium. Maximal LNCaP cell growth was obtained at concentrations of 10% dialyzed FBS with a more than 10-fold increase over cells in 1% dialyzed FBS ($P < 0.02$, $n = 4$; data not shown). Nevertheless, LNCaP cells expressed a low level of cellular PAcP activity with less than 40% of that in cells in 1% dialyzed FBS ($P < 0.02$, $n = 4$). In addition, tartrate-insensitive acid phosphatase was not significantly affected (data not shown). Therefore, the endogenous androgens and/or growth factors in undialyzed FBS were not the only causative agents affecting cell growth and cellular PAcP activity.

We examined further the association between cell growth and cellular PAcP activity because a decrease in cellular PAcP activity was associated with a stimulation of cell growth. Cells were plated and maintained in medium containing 7% FBS. Triplicate sets of cells were incubated at room temperature (23°C) for 24 h instead of at 37°C in order to diminish growth activity. Cells were harvested and quantitated for PAcP specific activity and total cell mass. Incubation at 23°C resulted in a 35% decrease in total cellular mass ($P < 0.001$, $n = 6$). This was associated with a 20% increase in cellular PAcP specific activity ($P < 0.001$, $n = 6$). Therefore, cellular PAcP specific activity was elevated when cell growth rate was decreased.

Table 2. Effect of EGF on cellular phosphatase activity

<table>
<thead>
<tr>
<th>EGF (10 ng/ml)</th>
<th>Normal FBS</th>
<th>Dialyzed FBS</th>
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<tr>
<td></td>
<td>TS*</td>
<td>TI</td>
</tr>
<tr>
<td>−</td>
<td>0.16$^b$</td>
<td>0.22</td>
</tr>
<tr>
<td>(100)$^d$</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>+</td>
<td>0.12$^b$</td>
<td>0.21</td>
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<tr>
<td>(75)</td>
<td>(95)</td>
<td>(72)</td>
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* TS, Tartrate-sensitive acid phosphatase activity; TI, tartrate-insensitive acid phosphatase activity.
$^b$ $P < 0.02$ ($n = 4$).
$^c$ $P < 0.005$ ($n = 7$).
$^d$ Numbers in parentheses, percentage.

Fig. 3. Effects of FBS concentrations on cell growth and phosphatase activity. Approximately 8 x 10$^4$ cells/cm$^2$ were plated in medium containing 7% FBS for 3 days. After incubation for an additional 3 days in medium containing 0.1% dialyzed FBS, LNCaP cells were switched to medium containing various concentrations of FBS as indicated. Cells in duplicate were harvested after 6 days for the determination of cell number (O), cellular PAcP activity (O), and tartrate-insensitive acid phosphatase activity (x). Bar, range of duplicate sets of cultures. T = −0.87; $P < 0.01$.

Table 3. Activities of tartrate-insensitive acid phosphatases in LNCaP cells

<table>
<thead>
<tr>
<th>FBS concentration (mg/ml)</th>
<th>Total activity (nmoles/mg protein/hr)</th>
<th>Specific activity (nmoles/mg protein/5 min)</th>
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<tbody>
<tr>
<td>−0.1</td>
<td>0.75</td>
<td>0.18</td>
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<tr>
<td>0.2</td>
<td>0.18</td>
<td>0.1</td>
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<tr>
<td>0.4</td>
<td>0.16</td>
<td>0.2</td>
</tr>
<tr>
<td>0.6</td>
<td>0.14</td>
<td>0.3</td>
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<tr>
<td>0.8</td>
<td>0.12</td>
<td>0.4</td>
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<tr>
<td>1.0</td>
<td>0.10</td>
<td>0.5</td>
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* M. F. Lin and G. M. Clinton, unpublished results.
Biosynthesis of PAcP in Androgen-treated Cells. We examined the possibility that the decrease in PAcP specific activity observed after stimulation of cell growth was due to androgen stimulation. The decrease in PAcP specific activity was observed to be accompanied by a decrease in PAcP protein level as demonstrated by Western blot analyses (data not shown). We sought to determine if the decrease in PAcP protein is due to androgen stimulation. DHT-treated cells and control cells were labeled with $[35S]$methionine and $[35S]$-PAcP was immunoprecipitated with specific polyclonal antiserum (25, 27). A ratio of 5 ml antiserum to 150 $\mu$g cellular protein was used to ensure the excess of antiserum (see “Materials and Methods”). As shown in Fig. 4A, the biosynthesis of PAcP was not significantly altered in DHT-treated cells. We further examined whether the decrease of cellular PAcP was due in part to an increase in PAcP secretion since DHT stimulates the secretion of PAcP (29). $[35S]$-PAcP in conditioned medium from DHT-treated and control cells was immunoprecipitated. As shown in Fig. 4B, the secretion of newly synthesized $[35S]$-PAcP was stimulated approximately 50% by DHT. The specificity of immunoprecipitation was demonstrated by competitive inhibition by the purified secretory form of PAcP (5 $\mug$). The reaction was not competitively inhibited by potato acid phosphatase, another $\alpha$-tyr phosphatase (Fig. 4A).

cDNA Transfection into PC-3 Cells. To more clearly establish the relationship between PAcP expression and cellular growth rate, we used PC-3 cells, a human prostate carcinoma line without endogenous PAcP (32), as the parent for the creation of stable transfectants of PAcP cDNA. The PAcP cDNA encoding a secretory form of PAcP protein was carried on a pCMV-Neo-Bam expression vector which also confers resistance to neomycin and the transfection was accomplished using the lipofectin technique. During transfection and subcloning processes, we obtained a considerably higher number of G418 sulfate-resistant transfectants that were transfected with the expression vector alone than transfectants that were transfected with the PAcP cDNA-containing expression vector. Furthermore, most slow-growing G418-resistant PAcP cDNA transfectants died before being established as subcloned cell lines. The difficulty in establishing subcloned cell lines may be due to the selection against the cell growth. These observations are very similar to the reports in cells that were transfected with cDNA encoding the wild-type p53 protein (39). In addition, the growth rate of some subcloned cells that were transfected with PAcP cDNA was apparently increased during the passage. After selection for neomycin resistance, two stable cDNA transfectants which we have designed, PC-18 and PC-416, were selected for further characterizations.

Northern blot analyses of PC-18 and PC-416 demonstrated expression of a 1.8-kilobase PAcP mRNA consistent with expression from the transfected cDNA (1.2 kilobases of PCR product, including both signal sequence and the coding region for the mature PAcP polypeptide, and a 0.6-kilobase polyadenylate sequence derived from the expression vector); endogenous PAcP mRNA from LNCaP cells has been shown to be 3.3 kilobases long (Fig. 5). In addition, the intensity of the 1.8-kilobase hybridization band in PC-18 cells is greater than 10-fold of that in PC-416 cells. There was no detectable level of hybridization with the PAcP-specific probe in either the PC-3...
parent cells or the vector-only transfectants (designated PC-CMV; Fig. 5). To ensure an equal quantity of each RNA was loaded, the electrophoresed gel was stained with ethidium bromide before being blotted (Fig. 5, bottom). After removal of the PAcP cDNA probe, the same blot was rehybridized with human β-actin cDNA as an internal control. A same intensity of hybridization band was observed in each sample (data not shown).

Biochemically, PC-18 cells and PC-416 cells expressed approximately 7-fold and 2-fold, respectively, greater L(+)-tartrate-sensitive acid phosphatase activity than the background level in PC-CMV cells (Table 3). The marginal level of L(+)-tartrate-sensitive acid phosphatase activity in PC-CMV cells is in part attributed to acid phosphatase-4 as discussed previously in PC-3 cells (Table 1). Unexpectedly, on the basis of per mg cellular protein, the secreted phosphatase activity by PC-18 cells is approximately 30-fold of that by PC-416 cells.

The p-tyr level in the PC transfectant was analyzed to examine its correlation with the expression of exogenous PAcP. For detecting possible minor differences in PC transfectants, we increased the p-tyr level by labeling cells with 32P in the presence of phosphate-free medium containing 5% dialyzed FBS and hydrolyzing 32P-proteins at 110°C for 1 h (34). As shown in Table 4, PC-416 cells had the lowest p-tyr level among three cells although PC-416 cells have only a low level of PAcP expression. Nevertheless, the expression of p-tyr level in PC-18 cells was apparently not affected by exogenous PAcP expression.

As described above, we observed that the stimulated cell growth is correlated with an increased p-tyr level and a diminished cellular PAcP activity in LNCaP cells. To investigate the possible correlation of p-tyr level with prostate cell growth, the growth rates of PC-18 and of PC-416 transfectants were compared with that of PC-CMV cells. The growth rate of PC-416 is approximately one-half that of PC-CMV cells (Fig. 6). Nevertheless, neither the growth rate nor the p-tyr level of PC-18 cells is affected by the expression of PAcP although PC-18 cells express a high level of PAcP. The generation time of PC-CMV control cells and PC-18 transfecant was about 28 and 30 h, respectively, while that of PC-416 cells was approximately 53 h. Therefore, the results demonstrated that the growth rate of PAcP cDNA-transfected PC cells is correlated with its p-tyr level.

We further examined the significance of the expression of exogenous PAcP activity in the slow growth rate of PC-416 cells since the slow growth rate may be attributed to a mutation in cells. Although PC-416 cells were obtained as a stable transfectant by G418 selection followed by subcloning, we observed an instability of PAcP expression in PC-416 cells. The expressed PAcP activity gradually decreased during the maintenance and passage (1 week per passage) in the absence of G418.

Increased cell growth rate, consistent with a putative role in growth regulation. By approximately 25 passages, cells regained the original rapid growth rate as that of PC-CMV control cells with the total loss of the transfected PAcP activity (data not shown). Therefore, despite what would appear to be a low level of PAcP expression, the growth regulation of PC-416 cells is apparently affected by the presence of PAcP expressed from the transfected cDNA.

**DISCUSSION**

The results of our experiments clearly demonstrated that there is a strong inverse correlation between cellular PAcP activity and cell growth of LNCaP cells that express endogenous PAcP. A stimulation in the growth of LNCaP cells was specifically associated with a concomitant decrease in cellular PAcP specific activity. For example, DHT stimulated the growth of prostate carcinoma cells along with an increase in p-tyr levels and a decrease in cellular PAcP activity (Fig. 1). Conversely, cellular PAcP specific activity was elevated when the growth of cells was arrested by low temperature.

It is possible that the decrease of cellular PAcP activity in the growth-stimulated cells is due to the fact that the phosphatase, PAcP, is a tissue-specific differentiation antigen because the inverse correlation between cell growth and differentiation has been established in a variety of systems. In addition, it was
observed that the p-tyr phosphatase activity is increased when HL-60 promyelocytic leukemia cells are induced to differentiate into granulocytes and monocytes (19). Nevertheless, the regulatory mechanism of this expression is not clear (46).

In normal prostate epithelial cells, differentiation is associated with an increase in cellular PAcP activity (21, 47, 48). Conversely, in prostate carcinoma cells, increased growth is associated with a decline of cellular PAcP activity (21–23, 47). It may therefore let us speculate that the stimulation of cell growth down-regulates the machinery for the biosynthesis of PAcP that results in a decline of cellular PAcP activity. However, in the presence of DHT, the biosynthesis of PAcP is not significantly affected as demonstrated by [35S]methionine labeling experiments (Fig. 4A). One possible explanation for the decrease of cellular PAcP activity in DHT-stimulated cells is that DHT stimulates the secretion of 35S-PAcP (Fig. 4B). This hypothesis is also supported by the observations that the secreted PAcP is a classical indicator for the growth of prostate carcinoma cells when prostate cancer patients are given androgen (43).

The significance of the observation that cellular PAcP activity is decreased when the growth of LNCaP cells is stimulated deserves thoughtful consideration because cellular PAcP has been proposed to be the major p-tyr phosphatase in differentiated prostate epithelial cells (24). In cells, including prostate cells, there are many species of tyrosine kinases (1, 3, 5). However, p-tyr in proteins in cells is present at extremely low levels and is the least abundant of the acid-stable phosphoamino acid (6, 34). It therefore would be expected that there is an equal number of p-tyr phosphatases or a high level and/or a highly efficient p-tyr phosphatase to play a role in regulating the level of p-tyr in cells (11–13, 28). In DHT-treated cells, the stimulated cell growth is associated with an increased p-tyr level and a declined cellular PAcP activity. The increased p-tyr level may be in part due to a direct androgen effect on tyrosine kinases since tyrosine kinase-specific activity is stimulated by DHT (27, 44). Nevertheless, the increased tyrosine kinase specific activity is partially inhibited by preincubating the kinase fractions with purified PAcP (27), while in control cells there is no effect on tyrosine kinase-specific activity by phosphatase treatment (27). These observations taken collectively may support the notion that PAcP may be involved in regulating the level of tyrosine phosphorylation in cells.

In order to clarify the effect of PAcP expression on cell growth, we transfected a PAcP cDNA encoding a secretory form of PAcP protein into prostate carcinoma cells that do not normally express the endogenous PAcP. The transfectants, PC-416 cells, express a low but significant amount of the exogenous PAcP and have an associated decrease in their p-tyr level and growth rate. Conversely, the loss of PAcP activity in PC-416 cells of high passage numbers is associated with an increased cellular growth rate. One possible explanation is that prostate carcinoma cells are able to exclude their negative growth regulator(s); consequently, the rapidly growing subclones that express low/no PAcP activity were selected during the passage process. The data may therefore indicate that the expression of exogenous PAcP activity is associated with a slow growth rate in PC-416 prostate carcinoma cells. However, in PC-18 cells, the expression of exogenous PAcP has no effect on p-tyr level or growth rate. It may be due to the fact that the expressed PAcP in PC-18 cells was merely for secretion. This hypothesis is supported by the observation that, on the basis of per mg cellular protein, the cellular PAcP in PC-18 cells is only approximately 5-fold of that in PC-416 cells, while the secreted PAcP by PC-18 cells is approximately 30-fold of that by PC-416 cells and corresponds to the ratio of mRNA label (Table 3; Fig. 5). Thus, the detected cellular PAcP activity in PC-18 cells may be the active secretory intermediates. Nevertheless, further experiments are required to delineate this mechanism.

The reason that there is only a 30–50% decrease in cellular PAcP activity when LNCaP cells are stimulated to grow is not known. One possible explanation is that with a 30–50% decrease the PAcP activity reaches the critical level in p-tyr regulation. It is also possible that the measured decrease of 30–50% in total PAcP activity in cells may actually represent a 100% turnover of one form of the enzyme since there are at least two forms of PAcP in cells (49, 50). Alternatively, the remaining PAcP activity could be due to cells that are not actively dividing and growing since these are not synchronized cultures. Since the specific antibody to either the secreted PAcP or the cellular PAcP is not available, and since the molecular structure of the cellular PAcP is not known (49, 50), the exact relationship between the cellular PAcP and the secreted PAcP is not clear at this moment.

In the past, cellular PAcP was thought to be a differentiation-specific antigen with unknown function in prostate epithelial cells, while its secretory form may be used as a marker for the diagnosis of prostate cancer (47, 48, 51). Recent reports that cellular PAcP is the major p-tyr phosphatase (24) have shed a new light on its possible function regarding prostate epithelial cell growth and regulation. In this report, we demonstrated that the endogenous PAcP expression in LNCaP cells is inversely correlated with cellular growth rate. Furthermore, the exogenous PAcP expression driven by cDNA transfection in PC-416 cells is also associated with a slow growth rate although it is not known why the exogenous PAcP expression in PC-18 cells has no effect on the cellular growth. Nevertheless, our data suggest that cellular PAcP may exhibit a putative function in the differentiated prostate epithelial cells.

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REFERENCES

7. Moria, A. O., and Wang, J. Y. J. Protein tyrosine phosphorylation in the cell
PROSTATIC ACID PHOSPHATASE AND HUMAN PROSTATE CARCINOMAS

Expression of Human Prostatic Acid Phosphatase Activity and the Growth of Prostate Carcinoma Cells

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