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# Tyrosine Phosphorylation of c-ErbB-2 Is Regulated by the Cellular Form of Prostatic Acid Phosphatase in Human Prostate Cancer Cells*

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**Human prostatic acid phosphatase (PAcP) is a prostate epithelium-specific differentiation antigen. In prostate carcinomas, the cellular PAcP is decreased. We investigated its functional role in these cells. Several lines of evidence support the hypothesis that cellular PAcP functions as a neutral protein-tyrosine phosphatase and is involved in regulating prostate cell growth. In this study, we identify its *in vivo* substrate. Our results demonstrated that, in different human prostate cancer cell lines, the phosphotyrosine (Tyr(P)) level of a 185-kDa phosphoprotein (pp185) inversely correlates with the cellular activity of PAcP. On SDS-PAGE, this pp185 comigrates with the c-ErbB-2 oncoprotein. Immunodepletion experiments revealed that c-ErbB-2 protein is the major pp185 in cells. Results from subclones of LNCaP cells indicated the lower the cellular PAcP activity, the higher the Tyr(P) levels of c-ErbB-2. This inverse correlation was further observed in PAcP cDNA-transfected cells. In clone 33 LNCaP cells, L-(+)-tartrate suppresses the cellular PAcP activity and causes an elevated Tyr(P) level of c-ErbB-2 protein. Epidermal growth factor stimulates the proliferation of LNCaP cells, which concurs with a decreased cellular PAcP activity as well as an increased Tyr(P) level of c-ErbB-2. Biochemically, PAcP dephosphorylates c-ErbB-2 at pH 7.0. The results thus suggest that cellular PAcP down-regulates prostate cell growth by dephosphorylating Tyr(P) on c-ErbB-2 oncoprotein in those cells.**

Protein tyrosine phosphorylation and dephosphorylation play a key role in regulating the proliferation and differentiation of normal and tumor cells (1, 2). In cells, the tyrosine phosphorylation status is apparently regulated by a dynamic equilibrium of protein-tyrosine kinases and PTPases[1] (3, 4). In human cancers, an increased level of tyrosine phosphorylation is implicated in a high cellular proliferation rate and an eventual development of tumors (5). This effect of increased tyrosine phosphorylation could be due to activation of protein-tyrosine kinases, inactivation of PTPases, or both.

The biological function of PTPases in antagonizing protein-tyrosine kinase activity and cellular transformation has been demonstrated in cell culture models (for a review, see Ref. 6). For example, protein-tyrosine phosphatase 1B and LAR PTPase dephosphorylated the IR on tyrosine residues, resulting in a decreased tyrosine kinase specific activity of IR (7, 8). Additionally, SHP-1, an Src homology 2 domain-containing PTPase, could be recruited by an erythropoietin receptor and subsequently down-regulated JAK2 through a dephosphorylation mechanism (9, 10). Furthermore, by cDNA transfection, the expression of an exogenous protein-tyrosine phosphatase 1B suppressed the malignant transformation of NIH3T3 cells induced by the c-*erbB-2* oncogene (11). Similarly, an induced expression of DEP-1, a PTPase, inhibited cell growth by 5–10-fold in human breast cancer cell line MCF-7, which is c-*erbB-2*-amplified (12). In a xenograft animal model, c-*erbB-2* gene-transformed 18-Hn1 human breast carcinoma cells developed tumors in athymic nude mice. Following LAR transfection, the tumor growth was significantly inhibited (13). Thus, PTPases could counteract the growth-stimulating and oncogenic activity of protein-tyrosine kinases.

Human PAcP is a prostate epithelium-specific differentiation antigen. Two forms of PAcP have been identified: one stays intracellular, while the other is secreted (14). Its cellular enzyme activity and mRNA level is decreased in prostate carcinomas, compared with normal or benign prostate hypertrophy cells (15, 16). Results from many studies have demonstrated that the cellular form of PAcP could function as a PTPase in cells. Briefly, PAcP exhibits endogenous PTPase activity and dephosphorylates tyrosine-phosphorylated proteins (17, 18). Biochemically, the tyrosine phosphorylation level and the tyrosine kinase-specific activity of EGFR could be down-regulated by PAcP at a neutral pH (19). Expression of an exogenous PAcP via either cDNA transfection (20, 21) or protein incorporation (22, 23) was associated with decreased Tyr(P) levels of cellular proteins. Additionally, crystallographic analyses and titration experiments revealed that PAcP could indeed function as a "cysteine"-mediated PTPase (24, 25). Taken collectively, these data indicate that the cellular form of PAcP is involved in regulating tyrosine phosphorylation in prostate epithelia.

Although previous investigations suggested that PAcP acts as a negative regulator of tyrosine phosphorylation signals, the *in vivo* substrate of PAcP was not identified yet. In this study, we analyzed protein tyrosine phosphorylation in several pros-

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[1] The abbreviations used are: PTPase, protein tyrosine phosphatase; PAcP, prostatic acid phosphatase; IR, insulin receptor; LAR, leukocyte common antigen-related; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; Ab, antibody; Tyr(P), phosphotyrosine; PAGE, polyacrylamide gel electrophoresis; CMV, cytomegalovirus; pp185, a phosphoprotein with a molecular mass of 185 kDa.


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tate cancer cells that express different levels of cellular PAcP. Our results demonstrated that the Tyr(P) level of pp185, a 185-kDa phosphoprotein, negatively correlates with the cellular activity of PAcP. We clarified the identity of pp185 to be the c-ErbB-2 oncoprotein. Furthermore, using PAcP cDNA transfection, we elucidated the relationship between PAcP expression and the Tyr(P) level of c-ErbB-2 in prostate cancer cells. Thus, our data suggest that c-ErbB-2 protein is an *in vivo* substrate of cellular PAcP.

## EXPERIMENTAL PROCEDURES

*Materials*—FBS, RPMI 1640 culture medium, gentamicin, EGF, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgGs were purchased from Life Technologies, Inc. Charcoal/dextran-treated, certified FBS (lot AGD6463, testosterone ≤ 3.0 ng/dl) was from Hyclone (Logan, UT). Protein molecular weight standard marker, acrylamide, and a protein assay kit were obtained from Bio-Rad. Polyclonal anti-ErbB-2 Abs (C-18 and K-15) for immunoblotting, monoclonal anti-ErbB-2 Ab (9G6) for immunoprecipitation, and normal mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-PAcP antiserum was obtained as described previously (26). Monoclonal anti-Tyr(P) antibody (4G10) was from Upstate Biotechnology Inc. (Lake Placid, NY). Purified PAcP was isolated from human seminal fluid as described (27). An enhanced chemiluminescence (ECL) detection system was purchased from Amersham Pharmacia Biotech. All other reagents were from Sigma.

*Cell Culture*—Human prostate carcinoma cell lines LNCaP, DU 145, and PC-3 were obtained from the American Type Culture Collection (Rockville, MD) and routinely maintained in RPMI 1640 medium supplemented with 5% FBS, 1% glutamine, and 0.5% gentamicin. Cells were split once per week, defined as one passage. LNCaP cells that had passage numbers less than 33 were designated as clone 33, greater than 80 as clone 81, and between 34 and 80 as clone 51 (28). Since cellular PAcP activity is regulated by androgen in prostate cells (20, 29), a steroid-reduced medium, *i.e.* RPMI 1640 medium containing 5% charcoal-treated, heat-inactivated FBS, was used in this study (26, 28). For doubling time determination, after 72 h of culture, one set of attached cells was harvested and counted, defined as cell number on day 0. The remaining cells were fed with fresh medium and then counted on days 3, 5, and 7. Fresh medium (3 ml/well in six-well plates) was added to the remaining cultures on days 3 and 5. To quantify cell growth, attached cells were trypsinized and combined with the suspended cells, and the total cell number was counted using a Coulter Counter Z1 model.

*PAcP cDNA Transfectants*—PC-411, -412, and -416 cells were subclones of PC-3 parental cells transfected with a full-length human PAcP cDNA driven by a pCMV-neo expression vector, as described previously (20). Clone 81 LNCaP cells were also transfected with the same expression vector containing human PAcP cDNA. Two subclones designated as LNCaP-28 and -40 were obtained as the stable transfectants after neomycin selection. LNCaP-CMV-13 is a subline of clone 81 LNCaP cells transfected with the pCMV-neo vector alone.

*Acid Phosphatase Activity Assay*—*p*-Nitrophenol phosphate was utilized as the substrate to quantify the acid phosphatase activity at pH 5.5 by measuring the absorbency of released PNP at 410 nm (30). Since L-(+)-tartrate is a classic inhibitor of PAcP and since greater than 90% of L-(+)-tartrate-sensitive acid phosphatase in LNCaP cells can be precipitated by anti-PAcP antiserum, the L-(+)-tartrate-sensitive acid phosphatase activity was used to represent the PAcP activity (30).

*Immunoprecipitation and Immunoblotting*—Subconfluent cells were harvested by scraping, pelleted, and rinsed with ice-cold 20 mM Hepes-buffered saline, pH 7.0, and then lysed in ice-cold cell lysis buffer containing protease and phosphatase inhibitors (*i.e.* 20 mM Hepes, pH 7.0, 0.5% deoxycholic acid, 0.15 M NaCl, 0.1% SDS, 1% Nonidet P-40, 4 mM EDTA, 10 mM NaF, 0.1 mM $ZnCl_2$, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl flouride, 1 trypsin-inhibitory unit of aprotinin, 4 $\mu$M leupeptin, and 1 $\mu$M pepstatin A). The lysates were spun at 1,600 × *g* for 10 min at 4 °C. The supernatants were transferred, and protein concentration was determined using a Bio-Rad protein assay kit. For immunoprecipitation, 1 mg of supernatant protein was incubated with 9G6 anti-ErbB-2 Ab-conjugated protein A-Sepharose complex for 2 h at 4 °C. The immunocomplexes were spun at 700 × *g* for 5 min, washed four times with ice-cold lysis buffer, and suspended in SDS-PAGE sample buffer. For immunoblotting, an aliquot of total lysate protein (50 $\mu$g) or the supernatant of immunoprecipitated complexes in SDS-PAGE sample buffer was electrophoresed and transferred to a nitrocellulose filter (Micron Separation Inc., MA). Filters were incubated with appropriate antibodies, and the protein bands were visualized by the ECL detection system (28). For rehybridization, the filters were stripped by a stripping buffer, *i.e.* 62.5 mM Tris-HCl, pH 6.7, containing 100 $\mu$M 2-mercaptoethanol and 2% SDS, for 30 min at 50 °C. After two washes with TBST buffer (*i.e.* 20 mM Tris-buffered saline, pH 7.5, containing 0.1% Tween 20), the filters were reacted with specific Abs, and the signal was detected by the ECL method.

## RESULTS

*PAcP Expression and Tyrosine Phosphorylation of pp185 in Different Prostate Cancer Cells*—Previous studies demonstrated that cellular PAcP could function as a neutral PTPase in prostate cancer cells (19, 22, 23). To understand its biological role, we investigated its *in vivo* substrate. We initially examined PAcP expression in different subclones of LNCaP cells as well as PC-3 and DU 145 cells. As shown in Fig. 1*A*, PAcP protein level in clone 33 LNCaP cells was higher than that in clone 51 and 81 cells, while PC-3 and DU 145 cells did not express a detectable PAcP protein. To examine the relationship between PAcP expression and protein tyrosine phosphorylation, the same membrane was rehybridized with anti-Tyr(P) Ab. As shown in Fig. 1*B*, among different subclones of LNCaP cells, the tyrosine phosphorylation level of a protein with a molecular mass of approximately 185 kDa (pp185) inversely correlates with the cellular level of PAcP. Significantly, levels of pp185 tyrosine phosphorylation were high in PC-3 and DU 145 cells (Fig. 1*B*), which lack PAcP expression (Fig. 1*A*).

To further explore the correlation between the Tyr(P) level of pp185 and the cellular PAcP activity, we performed PAcP cDNA transfection experiments utilizing clone 81 LNCaP cells, which express a low level of PAcP. Two stable transfectants were established and designated as LNCaP-28 and LNCaP-40. Immunoblotting analyses demonstrated that PAcP expression was increased in these two transfectants, higher than that in clone 81 parental cells (Fig. 2*A*, *top*). The effect of exogenous PAcP expression on protein tyrosine phosphorylation was examined with anti-Tyr(P) Ab via Western blotting. As shown in Fig. 2*A* (*bottom*), in both LNCaP-28 and -40 cells, the Tyr(P) level of pp185 conspicuously decreased, as compared with that in control clone 81 parental cells. We also investigated if an inverse correlation exists between PAcP expression and the Tyr(P) level of pp185 in PAcP cDNA-transfected PC-3 cells. Immunoblotting results showed that three transfectants (*i.e.* PC-411, PC-412, and PC-416) expressed an exogenous PAcP (Fig. 2*B*, *top panel*) with a decreased Tyr(P) level of pp185, lower than that in the control parental cells (Fig. 2*B*, *bottom*). These data indicated that although there are other phosphoproteins with altered Tyr(P) levels, the pp185 is the only one that exhibits a diminished Tyr(P) level in all PAcP-expressing cells.

Since c-ErbB-2 has a molecular mass of 185 kDa and is one of the major tyrosine kinases in prostate epithelial cells (31), we examined whether pp185 might be the c-ErbB-2 protein. Hybridization of the same membranes shown in Fig. 1*B*, and Fig. 2, *A* and *B*, with anti-ErbB-2 Ab revealed that c-ErbB-2 protein comigrates with pp185 on SDS-PAGE (data not shown). Furthermore, in all of these cell systems analyzed, although the tyrosine phosphorylation status was altered, there was no significant change in the protein expression level of c-ErbB-2, independent of its tyrosine phosphorylation status (data not shown). Apparently the differences in Tyr(P) levels of c-ErbB-2 in different subcloned cells are not due to differences in its protein expression.

*Immunodepletion of pp185 by Anti-ErbB-2 Ab*—To examine if c-ErbB-2 is the major phosphoprotein at 185 kDa, c-ErbB-2 was immunodepleted from total lysates of clone 81 LNCaP cells. As shown in Fig. 3*A*, the immunoprecipitated amounts of

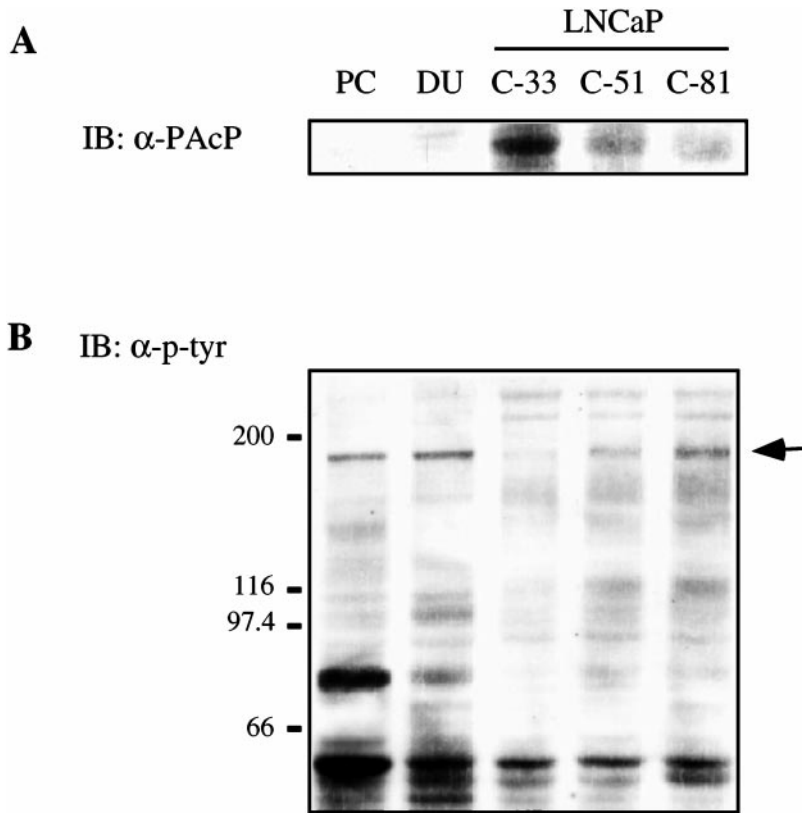


FIG. 1. **Endogenous PAcP expression and tyrosine phosphorylation of pp185.** Subconfluent PC-3, DU 145, and different subclones of LNCaP cells were harvested, lysed, and aliquoted for immunoblotting experiments. Total cell lysates (50 $\mu$g) were electrophoresed on a 7.5% SDS-PAGE and transferred to a nitrocellulose filter. *A*, immunoblotting with rabbit anti-PAcP antiserum. *B*, tyrosine phosphorylation level of total cell proteins. The filter shown in *A* was stripped and then rehybridized with anti-Tyr(P) (*α-p-tyr*) Ab. The *arrow* indicates the pp185. *PC*, PC-3 cells; *DU*, DU 145 cells; *LNCaP C-33*, *C-51*, and *C-81*, LNCaP clone 33, clone 51, and clone 81 cells, respectively. *IB*, immunoblotting.

c-ErbB-2 protein increased following the dosage of Ab. The immunodepleted cell lysates were then immunoblotted with anti-Tyr(P) Ab. In these depleted lysates, the tyrosine phosphorylation level of pp185 decreased, proportionally to the increase of Ab dosage (Fig. 3*B*). Greater than 80% of the Tyr(P) level of the pp185 was depleted from 1 mg of cell lysate by 10 $\mu$g of Ab (Fig. 3*B*). However, normal mouse IgG had no effect on immunoprecipitating c-ErbB-2 protein (Fig. 3*A*) and did not affect the Tyr(P) level of pp185 (Fig. 3*B*). The same membrane was subsequently reacted with anti-ErbB-2 Ab. A diminished amount of c-ErbB-2 protein in immunodepleted lysates corresponded to the decreased tyrosine phosphorylation level of pp185 (Fig. 3, *C versus B*). Thus, c-ErbB-2 protein is the major pp185 whose Tyr(P) level inversely correlates with the cellular PAcP activity in prostate cancer cells.

*Tyrosine Phosphorylation of c-ErbB-2 in Different Prostate Cancer Cells*—To investigate whether c-ErbB-2 could be a substrate of cellular PAcP, we first examined the Tyr(P) level of c-ErbB-2 in subclones of LNCaP cells. The c-ErbB-2 protein was immunoprecipitated, and its tyrosine phosphorylation level was analyzed by immunoblotting with anti-Tyr(P) Ab. The results demonstrated the lower the cellular PAcP activity, the higher the Tyr(P) level of c-ErbB-2 protein in LNCaP cells (*top* of Fig. 4*A versus* Fig. 1*A*). The same membrane was reacted with anti-ErbB-2 Ab after stripping. As shown in the *bottom* of Fig. 4*A*, there was no significant change in c-ErbB-2 protein levels among clone 33, 51, and 81 LNCaP cells. The relative Tyr(P) level of c-ErbB-2 protein was semiquantified by normalizing to its protein level after densitometric analyses. In clone 81 cells, the tyrosine phosphorylation of c-ErbB-2 was approximately 5-fold higher than that in clone 33 cells, while the PAcP activity was 5-fold lower (Table I).

To evaluate the effect of exogenous PAcP on the tyrosine phosphorylation of c-ErbB-2, we performed similar analyses using PAcP cDNA-transfected LNCaP-28 and -40 cells. As shown in Fig. 4*B* (*top*), in both LNCaP-28 and LNCaP-40 cells, the tyrosine phosphorylation of c-ErbB-2 was lower than that in clone 81 LNCaP parental cells as well as CMV vector control cells. The same membrane was subsequently incubated with anti-ErbB-2 Ab. The immunoblotting result revealed that c-ErbB-2 protein levels were essentially the same among all cells examined (*bottom* of Fig. 4*B*). Densitometric analyses indicated that an elevated expression of PAcP by cDNA transfection in clone 81 LNCaP cells correlated with a decrease of the Tyr(P) level of c-ErbB-2 by up to 65% (Table I).

We further examined tyrosine phosphorylation levels on c-ErbB-2 in PC-3 transfectants. Similarly, this exogenous expression of PAcP resulted in a diminished Tyr(P) level of c-ErbB-2 in all three transfected clones (Fig. 4*C*, *top*), while the protein levels of c-ErbB-2 were apparently the same in all cells (Fig. 4*C*, *bottom*). Densitometric analyses demonstrated an up to 70% decrease in tyrosine phosphorylation of c-ErbB-2 in PAcP cDNA transfectants, as compared with that in PC-3 parental cells (Table I).

*L-(+)-Tartrate Effect on Tyrosine Phosphorylation of c-ErbB-2*—Since results from PAcP cDNA transfection studies revealed that an additional PAcP expression correlates with a decreased Tyr(P) level on c-ErbB-2 protein in prostate cancer cells, we investigated this relationship further by treating clone 33 LNCaP cells with L-(+)-tartrate, a classic inhibitor of PAcP (23, 30). Cells were exposed to different doses of L-(+)-tartrate, and c-ErbB-2 protein was immunoprecipitated and followed by immunoblotted for analyzing tyrosine phosphorylation status. As shown in Fig. 5*A*, the tyrosine phosphorylation level of c-ErbB-2 increased following an increase of inhibitor concentrations. However, the c-ErbB-2 protein expression levels were very similar among the cells exposed to different doses of L-(+)-tartrate (Fig. 5*B*). The relative Tyr(P) level of c-ErbB-2 in cells exposed to 5 mM L-(+)-tartrate was approximately 4.5-fold of that in control cells (Fig. 5*B*). Conversely, the cellular PAcP activity was decreased by around 20% (data not shown), as we reported previously (22, 23). Thus, an inhibition of PAcP activ-

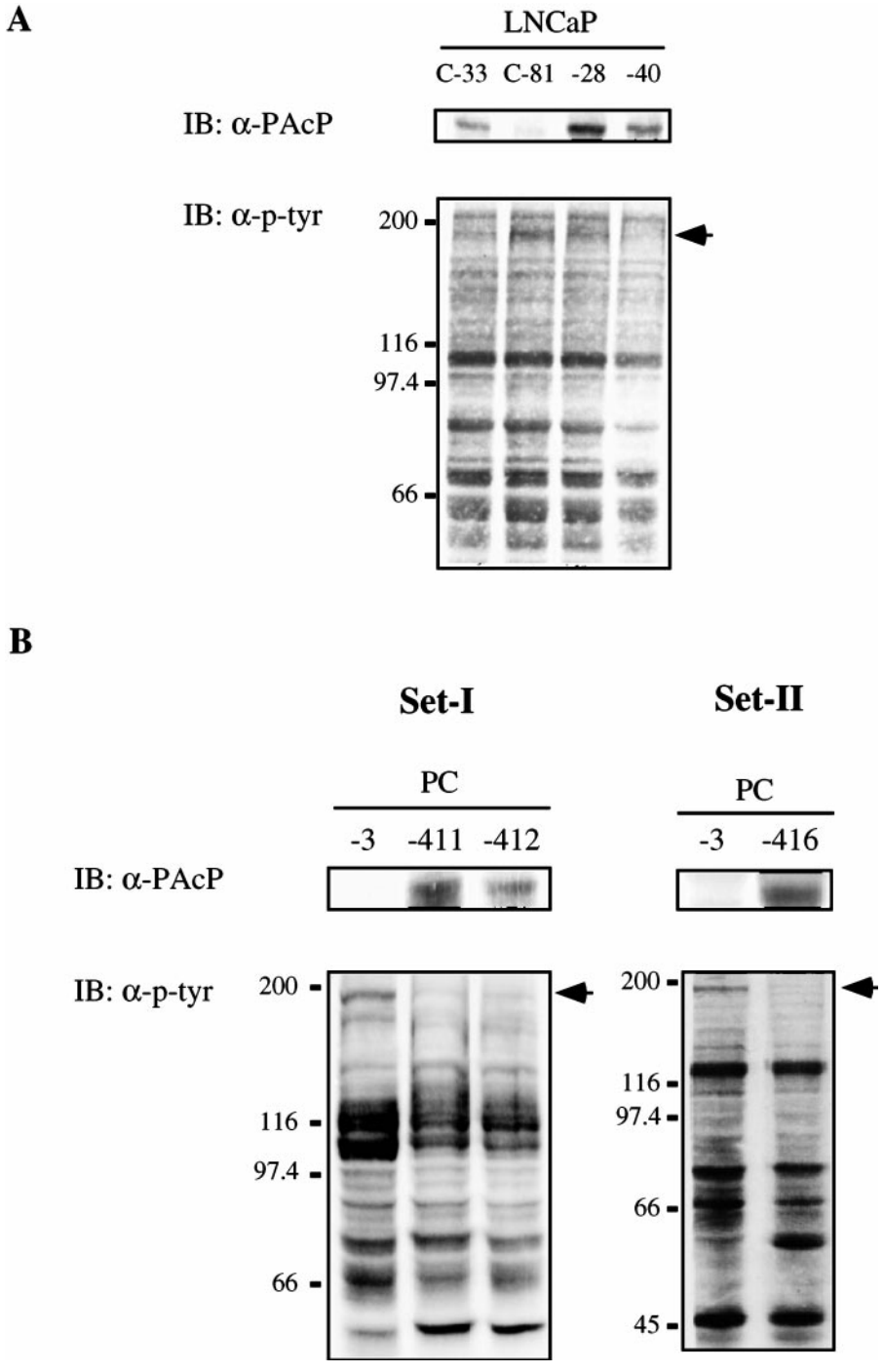


FIG. 2. **Exogenous PAcP expression and tyrosine phosphorylation of pp185.** Subconfluent cells were harvested for immunoblotting experiments. *A*, PAcP expression and Tyr(P) (*p-tyr*) level of pp185 in PAcP cDNA-transfected clone 81 LNCaP cells. *Top*, PAcP level in 50 $\mu$g of total cell lysates. Immunoblotting was performed with rabbit anti-PAcP antiserum. *Bottom*, tyrosine phosphorylation of total cell proteins. The filter shown at the *top* was stripped and rehybridized with anti-Tyr(P) Ab. *B*, PAcP expression and Tyr(P) level of pp185 in PAcP cDNA transfected PC-3 cells. *Top,* PAcP level in 50 $\mu$g of total cell lysates. *Bottom*, tyrosine phosphorylation of total cell proteins. *Set-I* and *Set-II*, two sets of independent experiments. *Arrows*, the positions of pp185. *IB*, immunoblotting.

ity concurs with an increase of tyrosine phosphorylation of c-ErbB-2 in prostate cancer cells.

*EGF Effect on Cell Growth, PAcP Activity, and Tyrosine Phosphorylation of c-ErbB-2*—EGF stimulates the growth of LNCaP cells (20) and prostate primary cells (32). Since cellular PAcP is possibly involved in down-regulating the growth of prostate cells, we also analyzed whether a stimulation of cell growth by EGF correlates with a decrease of PAcP activity in clone 33 LNCaP cells. EGF caused an increase in cell growth indicated by total cellular protein amounts with the optimal dose at 10 ng/ml (Fig. 6*A*). In contrast, the enzyme activity of cellular PAcP diminished following the dosage of EGF (Fig. 6*A*). The EGF effect in regulating tyrosine phosphorylation status of c-ErbB-2 in these cells was further examined. Results shown in Fig. 6*B* (*top*) demonstrated that the Tyr(P) level of c-ErbB-2 increased, in proportion to the EGF concentrations with the optimal induction at 10 ng/ml. The c-ErbB-2 protein level was also inspected and shown in Fig. 6*B* (*bottom*). Densitometric analyses demonstrated an over 3-fold induction of tyrosine phosphorylation of c-ErbB-2 by 10 ng/ml of EGF, in comparison with that in the control group (Fig. 6*B*). These data therefore indicate that EGF down-regulates cellular PAcP activity, thus resulting in the elevation of Tyr(P) level on c-ErbB-2 protein, which leads to an increased cell proliferation rate.

*Dephosphorylation of c-ErbB-2 by PAcP in Vitro*—To elucidate a direct interaction between these two proteins, we analyzed the dephosphorylation of c-ErbB-2 by PAcP at a neutral pH *in vitro*. c-ErbB-2 protein was immunoprecipitated from clone 81 LNCaP cells. The immunocomplexes were resuspended in Hepes-buffered saline, and purified PAcP was added. The remaining tyrosine phosphorylation level of c-ErbB-2 was

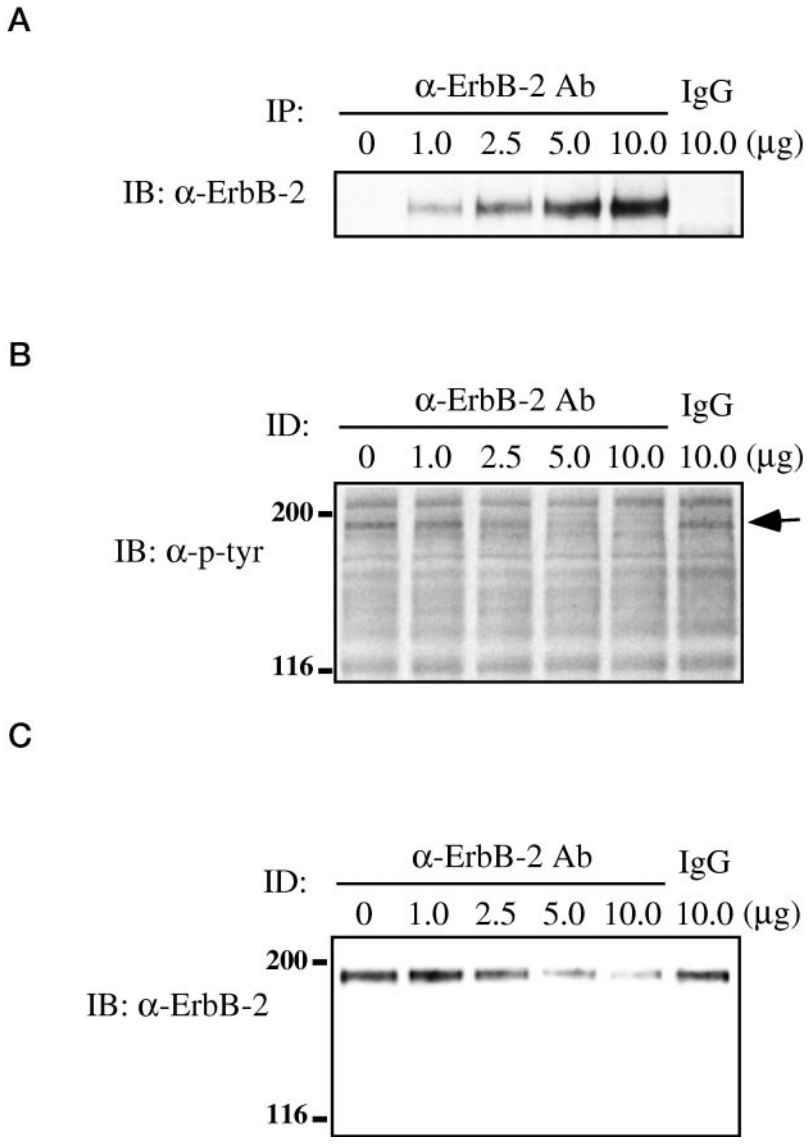


FIG. 3. **Tyrosine phosphorylation of pp185 in c-ErbB-2-depleted cell lysates.** An aliquot of 1 mg of total cell lysates from clone 81 LNCaP cells was incubated with various amounts of anti-ErbB-2 Ab or 10 $\mu$g of normal mouse IgG (IgG) as indicated in the *figure* for 1 h. Immunocomplexes were spun for experiments shown in *panel A*, and supernatants were collected for experiments shown in *panels B* and *C*, respectively. *A*, immunoblotting of immunoprecipitated proteins from 1 mg of cell lysate with anti-ErbB-2 Ab. *B*, tyrosine phosphorylation of pp185 in the immunodepleted cell lysates. 50 $\mu$g of cell lysates from the supernatant of each immunoprecipitated sample were electrophoresed and transferred to a nitrocellulose filter. Immunoblotting was performed with anti-Tyr(P) (*α-p-tyr*) Ab. *Arrow*, the position of pp185. *C*, c-ErbB-2 protein levels remained in the immunodepleted cell lysates. The same filter shown in *B* was rehybridized with anti-ErbB-2 Ab. *IP*, immunoprecipitation; *IB*, immunoblotting; *ID*, immunodepletion.

examined by immunoblotting with anti-Tyr(P) Ab. As shown in Fig. 7*A*, PAcP dephosphorylated c-ErbB-2 protein on tyrosine residues following a time course. c-ErbB-2 protein amounts in each sample were analyzed by immunoblotting with anti-ErbB-2 Ab (Fig. 7*B*). The relative tyrosine phosphorylation level of c-ErbB-2 was semiquantified. The results indicated that PAcP dephosphorylated c-ErbB-2 protein in a time-dependent fashion (Fig. 7*C*). However, in the presence of a PTPase inhibitor, sodium orthovanadate, no dephosphorylation of c-ErbB-2 by PAcP was observed (Fig. 7*C*). As a negative control, there was no dephosphorylation in the absence of PAcP protein (Fig. 7*C*).

*The Relationship among the Growth Rate, PAcP Expression, and Phosphotyrosine Level of c-ErbB-2 in Prostate Cancer Cells*—Our data indicate that tyrosine phosphorylation levels of c-ErbB-2 can be regulated by cellular PAcP in human prostate cancer cells. Since c-ErbB-2 is one of the major protein-tyrosine kinases involved in growth factor-stimulated proliferation of mammalian cells including prostate cells (32, 33), we investigated the biological significance of decreased tyrosine phosphorylation of c-ErbB-2 protein by analyzing the cell proliferation rate. We first examined the growth rate of different LNCaP subcloned cells. As shown in Table I, the doubling time of clone 33, 51, and 81 cells in a steroid-reduced medium was approximately 112, 57, and 49 h, respectively. Similar studies indicated that growth rates of PAcP cDNA-transfected LNCaP subcloned cells (LNCaP-28 and -40) and cDNA-transfected PC-3 subcloned cells (PC-412 and -416) were lower than that of their parental control cells (Table I). The cellular PAcP activity in each cell type was further measured. Data taken collectively show that in all three independent prostate cancer cell systems, the higher the activity of cellular PAcP is, the lower the tyrosine phosphorylation levels of c-ErbB-2 and the slower the cell growth rates (Table I).

## DISCUSSION

The protein-tyrosine phosphatase activity of PAcP has been demonstrated in many biochemical studies over the last decade (17–19, 22–25, 28). In normal canine prostate glands, well differentiated epithelia expressed a high level of PAcP and a low level of tyrosine-phosphorylated proteins (34). In contrast, poorly differentiated canine prostate basal cells did not express a detectable PAcP, while the Tyr(P) level was high (34). Treatment of canine differentiated prostate tissue with orthovanadate resulted in an increase in cellular Tyr(P) level and a decrease in PAcP activity (35). Additionally, in human prostate cancer cell cultures, the tyrosine phosphorylation of cellular proteins inversely correlated with cellular PAcP activity (20, 23). These studies imply that PAcP is involved in regulating protein tyrosine phosphorylation in differentiated prostate ep-

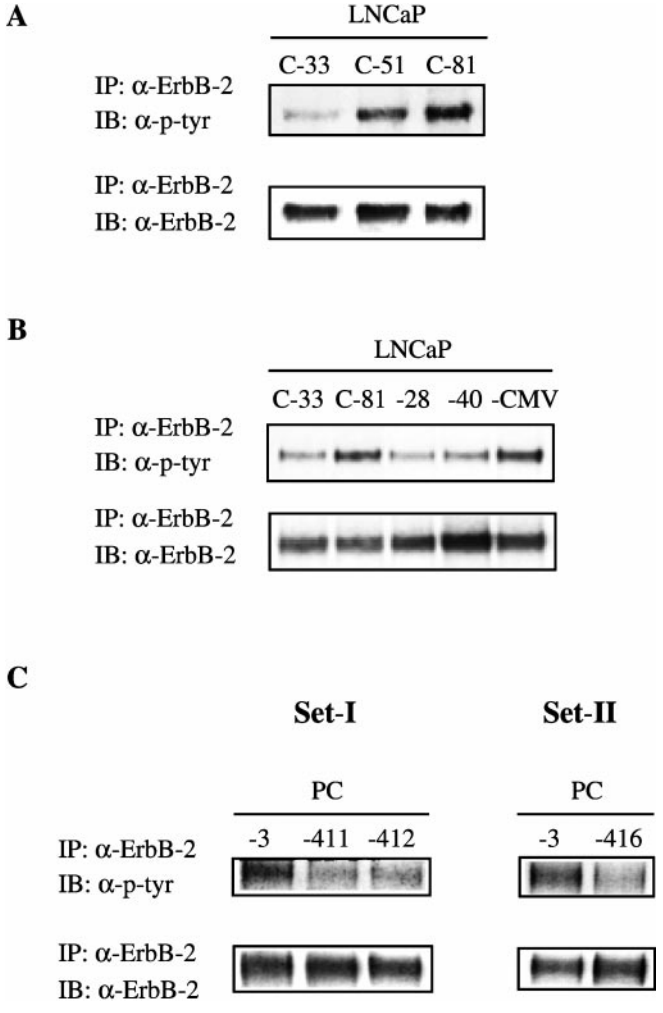


FIG. 4. **Effect of PAcP expression on tyrosine phosphorylation of c-ErbB-2 in prostate cancer cells.** An aliquot of 1 mg of total cell lysates from each cell line was incubated with 5 $\mu$g of anti-ErbB-2 Ab for 2 h at 4 °C. The immunocomplexes were washed, and immunoprecipitated proteins were electrophoresed on a 7.5% SDS-gel. Immunoblotting was performed with anti-Tyr(P) (*α-p-tyr*) Ab followed by anti-ErbB-2 Ab. Tyrosine phosphorylation and protein levels of c-ErbB-2 in different subcloned LNCaP cells (*A*), PAcP cDNA-transfected clone-81 LNCaP cells (*B*), and PAcP cDNA-transfected PC-3 cells (*C*) were shown. LNCaP-CMV cells, a subclone of LNCaP clone 81 cells transfected with the vector alone. *Set-I* and *Set-II* in *C* represent two sets of independent experiments. *IP*, immunoprecipitation; *IB*, immunoblotting.

ithelia. Nevertheless, the *in vivo* substrate(s) of PAcP has not yet been identified.

In the current study, our results clearly indicate that the tyrosine phosphorylation status of c-ErbB-2 protein is regulated by PAcP in prostate cancer cells. For example, our initial experiments using clone 81 LNCaP and PC-3 cells lacking PAcP expression demonstrated a poor immunoprecipitation of c-ErbB-2 by an Ab (C-18) against its C terminus, but not by an Ab (9G6) recognizing its N terminus (data not shown). Perhaps the inaccessibility of C-18 Ab is due to the high extent of tyrosine phosphorylation on C terminus of c-ErbB-2 protein in these two cell lines. Among different subclones of LNCaP cells, loss of PAcP expression correlated with an elevation of Tyr(P) level on c-ErbB-2 (Figs. 1 and 4). PAcP cDNA transfection in clone-81 LNCaP and PC-3 cells concurred with a diminished tyrosine phosphorylation of c-ErbB-2 (Figs. 2 and 4). When clone 33 LNCaP cells were exposed to a PAcP inhibitor, L-(+)-tartrate, a significant induction of tyrosine phosphorylation on c-ErbB-2 protein was observed (Fig. 5). Furthermore, EGF treatment on the same cells caused a decrease in cellular PAcP

TABLE I
*The cell growth rate, cellular PAcP activity, and c-ErbB-2 phosphorylation level in different prostate cancer cells*

| Cell lines and subclones | Doubling time[a] | PAcP activity[b] | Tyr(P) level of c-ErbB-2[c] |
|---|---|---|---|
| | *h* | $A_{410}/mg/min$ | *-fold* |
| LNCaP | | | |
| Clone 33 | 111.7 | 0.636 ± 0.019 | 1.00 |
| Clone 51 | 56.8 | 0.197 ± 0.007 | 2.47 |
| Clone 81 | 48.8 | 0.118 ± 0.001 | 5.03 |
| PAcP cDNA-transfected LNCaP | | | |
| Clone 81 | 51.7 | 0.141 ± 0.002 | 1.00 (2.85)[d] |
| PAcP-28 | 86.5 | 0.872 ± 0.002 | 0.39 (1.11) |
| PAcP-40 | 90.3 | 0.514 ± 0.013 | 0.35 (1.00) |
| CMV-13 | ND[e] | 0.193 ± 0.004 | 1.14 (3.26) |
| PAcP cDNA-transfected PC-3 | | | |
| PC-3 | 32.2 | 0 | 1.00 (3.03) |
| PC-411 | ND | 0.181 ± 0.008 | 0.52 (1.57) |
| PC-412 | 49.2 | 0.139 ± 0.001 | 0.67 (2.03) |
| PC-416 | 53.5 | 0.223 ± 0.007 | 0.33 (1.00) |

[a] For doubling time determination, cells were seeded at a density of $5 \times 10^4$ cells/well in a steroid-reduced medium in six-well plates. The cell counting method was described under "Experimental Procedures." The data shown indicated the average of duplicate wells, and similar results were observed in two sets of independent experiments.

[b] PAcP activity assay was performed as described under "Experimental Procedures." The results represent the L-(+)-tartrate-sensitive AcP activity. The data shown are the average of two sets of independent experiments.

[c] The relative level of Tyr(P) on c-ErbB-2 was semiquantified by normalizing to its protein level after densitometric analyses from the data shown in Fig. 4.

[d] The data indicate the relative Tyr(P) level of c-ErbB-2 by designating the lowest point as 1.0 in each cell system.

[e] ND, not determined.

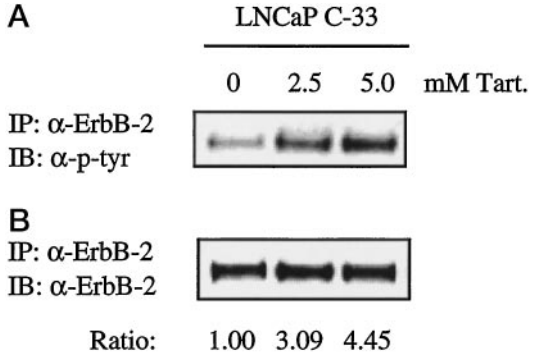


FIG. 5. **Effect of L-(+)-tartrate on tyrosine phosphorylation of c-ErbB-2.** Subconfluent clone 33 LNCaP cells were exposed to 0, 2.5, and 5.0 mM of L-(+)-tartrate, respectively, for 16 h. An aliquot of 1 mg of total cell lysates was reacted with 5 $\mu$g of anti-ErbB-2 Ab for 2 h at 4 °C. Immunocomplexes were washed, and proteins were electrophoresed on a 7.5% SDS-gel. *A*, immunoblotting of immunoprecipitated c-ErbB-2 proteins with anti-Tyr(P) (*α-p-tyr*) Ab. *B*, immunoblotting of the same filter with anti-ErbB-2 Ab after stripping. The ratio indicates the relative Tyr(P) level of c-ErbB-2 protein after densitometric analyses. *IP*, immunoprecipitation; *IB*, immunoblotting.

activity, with a parallel increase in Tyr(P) level of c-ErbB-2 (Fig. 6). Biochemically, PAcP dephosphorylated c-ErbB-2 on tyrosine residues in a time-dependent fashion (Fig. 7). To the best of our knowledge, this is the first demonstration of a putative *in vivo* substrate of PAcP, a differentiation-associated PTPase.

The inverse correlation is exhibited between cellular PAcP expression and the Tyr(P) level of c-ErbB-2 as well as cell growth rate in different LNCaP subcloned cells and PAcP cDNA-transfected PC-3 cells (Table I). However, compared with LNCaP-40 subcloned cells, LNCaP-28 cells express a higher level of PAcP (Fig. 2*A* and Table I), while the tyrosine phosphorylation level of c-ErbB-2 as well as the cell growth rate in these two cell lines are very similar (Fig. 4*B* and Table

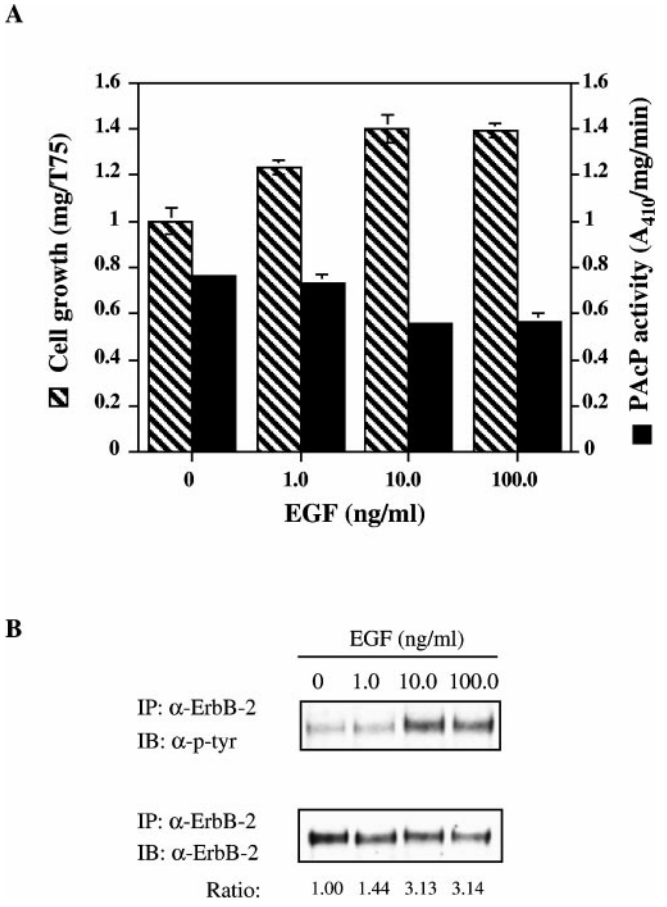


FIG. 6. **Effect of EGF on cell growth, cellular PAP activity, and tyrosine phosphorylation of c-ErbB-2.** Subconfluent LNCaP clone 33 cells were fed with medium containing 1% charcoal-treated FBS for 24 h before exposed to 0, 1, 10, and 100 ng/ml of EGF, respectively, for 16 h. Cells then were harvested, aliquoted, and lysed by the lysis buffer with orthovanadate (for immunoprecipitation) or without orthovanadate (for PAcP activity assay). PAcP activity assay was performed as described under "Experimental Procedures." An aliquot of 1 mg of total cell lysates was reacted with 5 $\mu$g of anti-ErbB-2 Ab for 2 h at 4 °C, and immunoprecipitated proteins were electrophoresed on a 7.5% SDS-gel. *A,* cellular PAcP activity and total protein amount (mg/T75 flask) of cells exposed to different doses of EGF. *Bar*, the range of results from two sets of samples. *B,* immunoblotting of c-ErbB-2 in immunocomplexes with anti-Tyr(P) (*α-p-tyr*) Ab (*top*), or with anti-ErbB-2 Ab (*bottom*). The relative level of Tyr(P) on c-ErbB-2 was semiquantified by normalizing to its protein level after densitometric analyses. *IP*, immunoprecipitation; *IB*, immunoblotting.

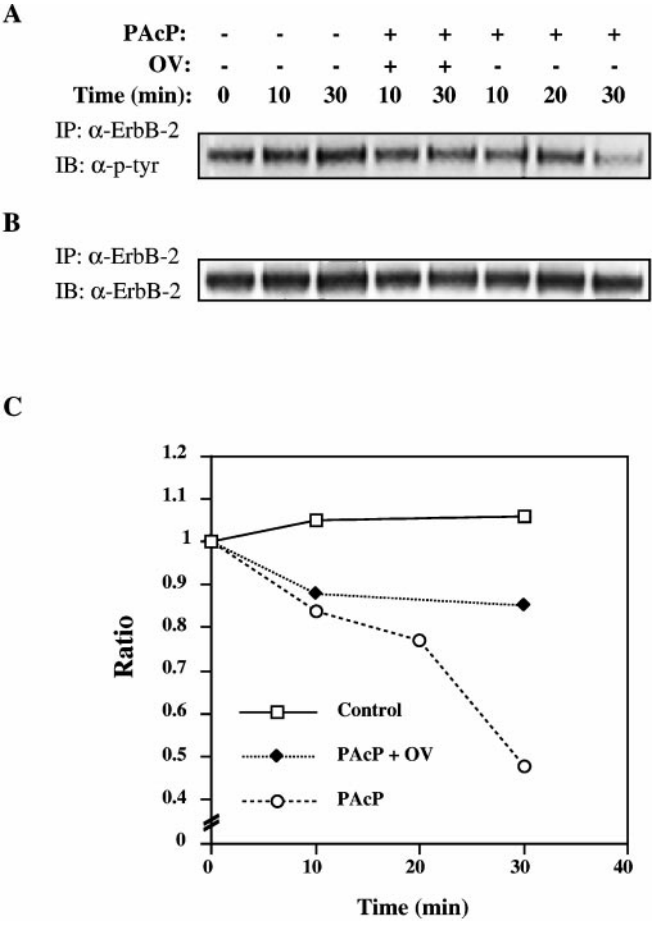


FIG. 7. **Tyrosine phosphorylation level of PAcP-treated c-ErbB-2.** c-ErbB-2 protein was immunoprecipitated from 1 mg of clone 81 LNCaP cell lysates and washed thoroughly to remove sodium orthovanadate in the lysis buffer. Immunocomplexes were then incubated with purified PAcP (3 $\mu$g/sample) in the presence or absence of 200 $\mu$M sodium orthovanadate at room temperature for 10, 20, and 30 min. In control experiments, immunocomplexes were incubated with Hepes-buffered saline alone. Tyrosine phosphorylation (*A*) and protein levels (*B*) of c-ErbB-2 after incubation with PAcP are shown. *OV*, sodium orthovanadate; *IP*, immunoprecipitation; *IB*, immunoblotting. *C*, the summary of densitometric analyses of Tyr(P) (*p-tyr*) level on c-ErbB-2. Tyrosine phosphorylation level of each sample (*A*) was normalized to its corresponding protein level (*B*). The ratio of the sample in the absence of PAcP without sodium orthovanadate at 0 min was defined as 1.0. Similar results were obtained from three sets of independent experiments.

I). To address this question, we analyzed the PAcP level in their conditioned media, since there are two forms of PAcP (14, 20). The results indicated that LNCaP-28 cells secreted a significantly higher amount of PAcP than LNCaP-40 cells ($A_{410}$ = 0.209 of LNCaP-28 *versus* 0.002 of LNCaP-40). Thus, we proposed that a fraction of PAcP in LNCaP-28 cells is directed to the constitutively secretory pathways. Therefore, the high PAcP level in total cell lysates of LNCaP-28 cells includes the intermediate form of secretory PAcP, as described in our previous studies (20). Alternatively, the inhibition of the Tyr(P) level of c-ErbB-2 may reach to the optimum by the exogenous PAcP in LNCaP-40 cells. Although LNCaP-28 cells produce increased amounts of PAcP, the excess PAcP cannot function to further dephosphorylate c-ErbB-2 *in vivo*.

c-ErbB-2 is a transmembrane glycoprotein of $M_r$ 185,000 that has an extensive sequence homology to EGFR (36, 37). Similar to EGFR, c-ErbB-2 is a putative growth factor receptor with an intrinsic tyrosine kinase activity, yet its ligand has not been identified (38). Amplification or overexpression of the c-*erbB-2* gene has been found in approximately 20–30% of human breast cancers (39). Patients with breast tumors overexpressing the c-*erbB-2* gene have a significantly lower survival rate and a shorter time to relapse than patients with tumors lacking overexpression of the c-*erbB-2* gene (39). In prostate cancer, however, studies on the role of c-ErbB-2 have yielded conflicting results (40–43). Therefore, c-ErbB-2 has not been accepted as a marker for prostate tumor progression (44, 45). In the present study, we utilized a variety of prostate cancer subcloned cells as model systems to investigate not only the interaction between PAcP and c-ErbB-2, but also a possible functional role of c-ErbB-2 in regulating the growth of prostate cancer cells. Our results clearly demonstrate that the tyrosine phosphorylation level of c-ErbB-2 correlates with the proliferation rate of prostate cancer cells (for a summary, see Table I). Since cellular PAcP activity in prostate carcinomas is lower than that in noncancerous prostate cells (15, 16),[2] we hypothesize that cells with a low level of PAcP have an unregulated

[2] J. Andresson, P. S. Rao, and M. F. Lin, unpublished data.

tyrosine phosphorylation on c-ErbB-2, which eventually leads to the uncontrolled proliferation of cancers.

Several lines of evidence indicate that PTPases interact with protein-tyrosine kinases intracellularly and regulate kinase signal transduction pathways. For example, a physical association between LAR and IR was shown by coimmunoprecipitation from Chinese hamster ovary cells, which overexpressed the human IR gene (8). When rat fibroblast cells with an overexpression of the human IR gene were transfected with protein-tyrosine phosphatase 1B cDNA, insulin- and insulin-like growth factor I-stimulated signalings were inhibited (9). Subsequently, it was found that protein-tyrosine phosphatase 1B was coprecipitated with the IR $\beta$-subunit by specific Abs from insulin-stimulated cells (46). SHP-1, an Src homology 2 domain-containing PTPase, bound selectively to the tyrosine-phosphorylated erythropoietin receptor through its Src homology 2 domain and henceforth inactivated JAK2 by dephosphorylation (9, 10). Thus, a physical association indeed exists between PTPases and their *in vivo* substrates. In previous studies, we observed that the majority of cellular PAcP resides in the cytosol and that a fraction of it could associate with the plasma membrane using immunofluorescent staining with a confocal microscopy (21).[3] It indicated that cellular PAcP and c-ErbB-2 are localized favorably for interaction. However, we have had limited success in demonstrating a physical interaction between PAcP and c-ErbB-2 by coimmunoprecipitation techniques. Several plausible reasons could explain this phenomenon. For example, since there is no significant sequence homology between PAcP and other members of the PTPase family (47, 48), the interaction mechanism of PAcP with its *in vivo* substrate(s) could be different from other PTPases. Alternatively, it is possible that the association between PAcP and c-ErbB-2 is very labile and that the binding is highly sensitive to the experimental conditions. Thus, the dissociation between these two proteins occurs during experimental procedures. Crystallographic results clearly demonstrated that PAcP has two critical free sulfhydryl groups at cysteine residues that could serve as phosphate acceptors (24). Subsequently, titration experiments revealed a "S-$^{32}$P" intermediate product of PAcP occurring during the dephosphorylation reaction (25). Thus, substrate-trapping mutants generated by site-directed mutagenesis of these cysteine residues should provide us with new tools for studying *in vivo* association between PAcP and c-ErbB-2.

The treatment of LNCaP cells with L-(+)-tartrate and EGF resulted in an increased tyrosine phosphorylation level of c-ErbB-2 protein. Since L-(+)-tartrate is a classic inhibitor of PAcP and also since its inhibition effect on cellular PAcP in LNCaP cells has been illustrated (22, 23), we propose that the induction of the Tyr(P) level of c-ErbB-2 by L-(+)-tartrate is directly due to the suppression of cellular PAcP activity. However, the molecular mechanism of EGF effect on tyrosine phosphorylation of c-ErbB-2 is not completely understood. It is possible that the suppression of cellular PAcP activity by EGF leads to a hyperphosphorylated c-ErbB-2. Other possibilities also exist. For example, we observed a coimmunoprecipitation of c-ErbB-2 protein by anti-EGFR Ab in clone 33 LNCaP cells (data not shown), indicating that a heterodimerization occurs between these two receptors. Since EGF treatment stimulates the tyrosine kinase-specific activity of EGFR, the increase of Tyr(P) level of c-ErbB-2 is possible via a cross-phosphorylation mechanism (49). Furthermore, the PAcP activity could be suppressed by direct phosphorylation through the active EGFR because of favorable subcellular localization, or by shutdown of its *in vivo* synthesis machinery. Detailed investigations are needed to delineate the molecular mechanism of interaction between cellular PAcP and c-ErbB-2 in LNCaP cells.

In conclusion, our data clearly demonstrated that cellular PAcP can dephosphorylate c-ErbB-2 oncoprotein on tyrosine residues in prostate cancer cells. This dephosphorylation results in a diminished proliferation rate of these cells. Further studies will help to elucidate their *in vivo* interactions and molecular sites of dephosphorylation of c-ErbB-2 by PAcP. These results will aid in understanding the mechanism of one step in multistage prostate carcinogenesis.

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[3] M. F. Lin, M. L. Chen, and J. K. Christman, unpublished data.

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