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Tyrosine Phosphorylation of a 185 kDa Phosphoprotein (pp185) Inversely Correlates with the Cellular Activity of Human Prostatic Acid Phosphatase

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Human prostatic acid phosphatase (PAcP), a prostate epithelium-specific differentiation antigen, was determined to exhibit the endogenous protein tyrosine phosphatase activity. We investigated the phosphoprotein(s) that might be dephosphorylated by PAcP in human prostate carcinoma cells. Several lines of evidence were presented to show that the tyrosine phosphorylation level of a 185 kDa phosphoprotein (pp185) is negatively correlated with the cellular activity of PAcP. (i) In DU145, PC-3 and high passaged LNCaP prostate carcinoma cells that have no or low PAcP expression, the phosphotyrosine (p-tyr) level of pp185 was higher than that in low passaged LNCaP cells that express an endogenous PAcP. (ii) In LNCaP cells grown in the presence of L(+)-tartrate, an inhibitor of PAcP, the tyrosine phosphorylation of pp185 was increased. (iii) Mediated by Lipofectin, a cationic liposome, the incorporation of purified PAcP protein into DU145 cells resulted in the decreased phosphorylation of pp185. Thus, the results taken collectively demonstrated that the p-tyr level of pp185 is inversely correlated with the cellular activity of PAcP and indicated that the pp185 may be a putative substrate of PAcP in prostate carcinoma cells.

Tyrosine phosphorylation of cellular proteins is instrumental in the control of cell proliferation mediated by several oncogene protein products and growth factor receptors (1,2). This however is a cyclical process of tyrosine phosphorylation and dephosphorylation, which can be regulated at either step (3,4). We investigated the regulation of phosphotyrosine (p-tyr) dephosphorylation in signal transduction in prostate epithelial cells for its potential role during multi-stage prostate carcinogenesis.

Human prostatic acid phosphatase (PAcP) has conventionally been classified as an acid phosphatase (AcP) since it hydrolyzes phosphomonoesters with an acidic pH optimum, while without known function (5). In cells, there are two forms of PAcP: one stays intracellularly and the other is secreted (6,7). Recently, the cellular form of PAcP has been implicated to be the major protein tyrosine phosphatase (PTPase) in non-cancerous prostate epithelium cells (8,9). Using the autophosphorylated epidermal growth factor (EGF) receptor as a substrate, a neutral pH optimum of p-tyr dephosphorylation by PAcP was observed *in vitro* (10). Nevertheless, the physiological role of cellular PAcP including its putative substrate in prostate epithelia remains to be established.

In this communication, we exploited the functional activity of cellular PAcP in human prostate carcinoma cells. We first examined the tyrosine phosphorylation of proteins in several prostate carcinoma cells that express different levels of PAcP. Since L(+)-tartrate is a classical inhibitor of PAcP (5), and since over 90% L(+)-tartrate-sensitive AcP activity in LNCaP cells is PAcP (11), we investigated the effect of L(+)-tartrate on protein tyrosine phosphorylation

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in those cells. Additionally, we have successfully utilized Lipofectin, a commercially available cationic liposome, to introduce functional PAcP protein into DU 145 cells (12). We identified the phosphoprotein(s) that has a decreased phosphorylation in the PAcP-incorporated DU145 cells. The results taken collectively demonstrated that the tyrosine phosphorylation level of a 185 kDa phosphoprotein (pp185) inversely correlated with the cellular activity of PAcP.

MATERIALS AND METHODS

Cell lines. Human prostate carcinoma cells, including DU145, PC-3, and different passages of LNCaP cells, were routinely maintained in RPMI-1640 medium containing 7% fetal bovine serum (FBS), 1% glutamine, and 0.5% Gentamicin (Gibco/BRL) (10-13). It has been well established that low passaged LNCaP (passage 31, p31) cells express an endogenous PAcP and their high passaged counterparts (p90) have a diminished expression of PAcP with a level approximately 10% of that in p31 cells (14). While, DU145 and PC-3 cells lack the expression of the enzyme (12,13).

It has been demonstrated that growth conditions including the cell density and harvesting methods may contribute to the differences in p-tyr in proteins (15). Since different prostate carcinoma cells have different growth rates (13), for experiments, cells were seeded at different cell densities in the same set of experiments and then harvested simultaneously when they all reached a similar 85% confluency.

Acid phosphatase assay. For experiments, cells were scraped, rinsed three times with 20 mM Hepes, pH 7.2, containing 0.9% NaCl, and lysed in a cell lysis buffer (20 mM Hepes, pH 7.4, containing 0.5% NP40, 0.5 mM DTT and protease inhibitors: 2 mM phenylmethylsulfonylfluoride (PMSF), 1 trypsin inhibitor unit of Aprotinin, 2 μM leupeptin, and 1μM pepstatin (all from Sigma, MO)) (11-13). The protein concentration was quantified by reaction with the BioRad Protein Dye and bovine serum albumin was the standard (10-12). The AcP activity was determined as previously described with p-nitrophenyl phosphate (PNPP) as the substrate in a citrate buffer, pH 5.5, and the released p-nitrophenol (PNP) in 0.1 N NaOH was measured spectrophotometrically at 410 nm (9,12). L(+)-tartrate is a classical inhibitor of PAcP activity (5,8,9,12). Since over 90% L(+)-tartrate-sensitive AcP activity is precipitated by anti-PAcP Ab in LNCaP cells, the L(+)-tartrate-sensitive AcP activity was used to represent PAcP activity. To analyze the significance of differences in enzyme activities, statistic analyses by the Student's *t* test (*p* value) were performed (13). $P<0.05$ is considered significant.

Tyrosine kinase assay. Angiotensin II (Sigma) was used as the exogenous substrate of tyrosine kinases with [γ-^{32}P]ATP in tyrosine kinase assays, as in our previous reports (10,11,16). The assay was conducted at the initial rate of reaction in the presence of orthovanadate ion, an inhibitor of several PTPases including PAcP (9,10). The ^{32}P-angiotensin II was separated from other radioactive materials by paper electrophoresis in a pH 4.4 buffer, localized by autoradiography, cut from the paper, and quantified by the Cerenkov counting (10,16).

In vivo protein phosphorylation. To examine protein phosphorylation *in vivo*, cells were labeled with ^{32}Pi (ICN) in a phosphate-free medium containing 5% dialyzed FBS for 16 hr (12,13). Cells were harvested, spun, and lysed in 20 mM Hepes, pH 7.4, containing 1% NP40, 0.5% deoxycholate, 0.1% SDS, 0.15 M NaCl, 4 mM EDTA, 2 mM sodium orthovanadate, 10 mM NaF, 100 μM $ZnCl_2$, 10 mM pyrophosphate and various protease inhibitors as described above (12,13). An aliquot of total cell lysate proteins was electrophoresed in SDS-(7.5%)-polyacrylamide gel and followed by autoradiography.

Western blot analysis. Total cell lysate proteins (200 μg) were electrophoresed in SDS-(7.5% or 5%)-polyacrylamide gels and then electroblotted to nitrocellulose membranes (MSI, MA) (17). The membrane was reacted with mouse anti-p-tyr monoclonal Ab (UBI, NY) and then with horseradish peroxidase-conjugated goat anti-mouse IgG Ab (Gibco/BRL). The p-tyr proteins were visualized by detecting the peroxidase activity utilizing an enhanced chemiluminescence (ECL) reagent kit from Amersham.

Lipofectin-mediated incorporation of PAcP. A commercially available cationic liposome, Lipofectin (Gibco/BRL), was used to introduce purified PAcP protein into prostate carcinoma cells as previously described (12). Briefly, cells were maintained in a 60-mm polystyrene tissue culture dish until they were 80 to 85% confluent. For each dish, 50 μg PAcP in 50 μl 4 mM Hepes, pH 7.4, and 50 μg Lipofectin in 50 μl H_2O were mixed gently, and stored at room temperature for 15 min. Cells were rinsed with Opti-MEM medium (Gibco/BRL) and then maintained in 3 ml of the same medium. The PAcP/Lipofectin complex was added to the cells dropwise and uniformly. After incubation at 37°C for 16-20 hr, 3 ml of RPMI 1640 medium containing 20% FBS was added and the cells were incubated for an additional 20 hr before experiments.

RESULTS

PAcP Expression and Protein Tyrosine Phosphorylation

To investigate the functional activity of cellular PAcP in human prostate carcinoma cells, we analyzed the tyrosine phosphorylation in proteins in LNCaP cells (p31) that express an

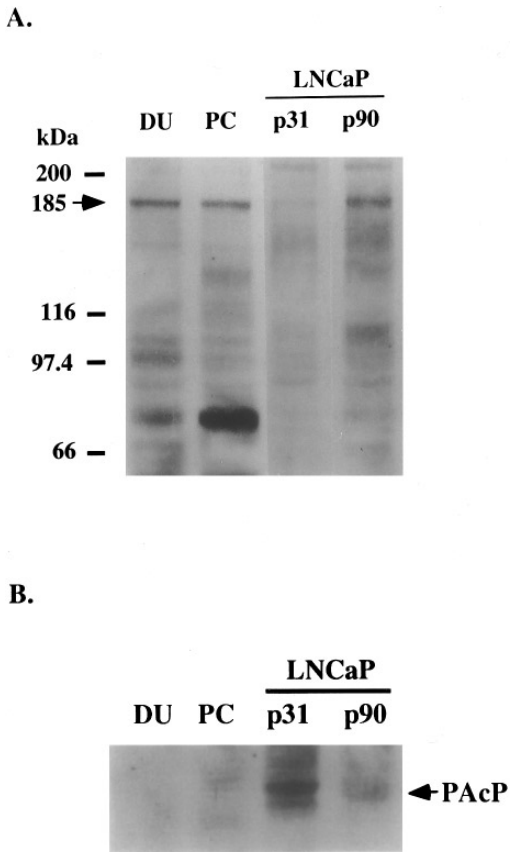

FIG. 1. Protein tyrosine phosphorylation in different prostate carcinoma cells. An aliquot of total cell lysate proteins was electrophoresed in a SDS (7.5%)-polyacrylamide gel and then electroblotted to nitrocellulose membranes. The membranes were reacted with an anti-p-tyr monoclonal Ab (A) or a rabbit anti-PAcP serum (B). The reaction was detected and visualized by an ECL method.

endogenous PAcP in comparison with that in PC-3 as well as DU145 cells those do not express the enzyme. Since the cell density and growth conditions could modulate the p-tyr levels in cells, all cells were plated in the same set of experiments and harvested simultaneously when they all reached to approximately 85% cell confluency . Western blot analyses with anti-p-tyr Ab demonstrated that the p-tyr level of a pp185 was higher in PC-3 and DU 145 cells than in p31 LNCaP cells although the p31 LNCaP cells had less p-tyr in general (Fig. 1A).

To examine further the relationship of tyrosine phosphorylation of pp185 with PAcP expression, we analyzed the p-tyr level of pp185 in the high passaged LNCaP cells that had a diminished expression of the enzyme (14). In p90 of LNCaP cells, although several proteins had elevated p-tyr, the p-tyr of pp185 increased to a similar level as in PC-3 and DU145 cells (Fig.1A). The same blotted membranes were subsequently reacted with an anti-PAcP serum. As shown in Fig. 1B, the p31 LNCaP cells expressed an endogenous PAcP, p90 cells had a decreased expression, while PC-3 and DU 145 cells lacked the expression of the enzyme.

To address further the effect on p-tyr levels by the cell growth conditions, all cells were seeded at the same density and harvested when they were confluent to reduce the cell density effect. A similar result that the p-tyr of pp185 is higher in PAcP-lack cells than in PAcP-expressing cells was observed, while the overall p-tyr level decreased in all cells (data not

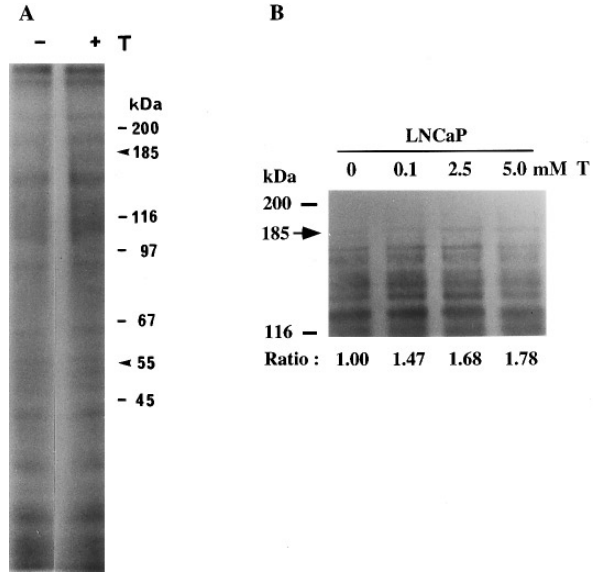

FIG. 2. L(+)-Tartrate effect on the p-tyr level of the 185 kDa protein in LNCaP cells. (A) LNCaP cells (p31) were labeled with ^{32}Pi in the presence or absence of 5 mM L(+)-tartrate in 20 mM Hepes, pH 7.4, for 16 hr. An aliquot of total cell lysate proteins (25μg each) was electrophoresed in a SDS (7.5%) gel and followed by autoradiography. T, L(+)-tartrate. (B) Cells were grown in the presence or absence of various concentrations of L(+)-tartrate in 20 mM Hepes, pH 7.4, for 16 hrs. For Western blot analyses with anti-p-tyr Ab, an aliquot of total cell lysate proteins each was electrophoresed in a SDS (5%) gel to increase the resolution of separation. The level of p-tyr in pp185 was semi-quantified by densitometric analyses on autoradiograms with Molecular Dynamics equipment and its software program. Ratio, the relative level of p-tyr in pp185. T, L(+)-tartrate.

shown). Thus, among different human prostate carcinoma cells, the tyrosine phosphorylation level of pp185 inversely correlated with the expression of PAcP.

L(+)-Tartrate Effect on Protein Tyrosine Phosphorylation

We further examined the effect of L(+)-tartrate by inhibiting PAcP on protein tyrosine phosphorylation in LNCaP cells (p31). In preliminary experiments, we did not observe a significant effect by 5 mM L(+)-tartrate on the cellular morphology after 16 hr treatment (data not shown). Cells were then *in vivo* labeled with ^{32}Pi in the presence or absence of 5 mM L(+)-tartrate followed by electrophoresis in a SDS-(7.5%) gel. In the presence of L(+)-tartrate, the phosphorylation level of pp185 was increased (Fig. 2A). This increased phosphorylation was much evident after an alkali treatment (data not shown). As controls, L(+)-tartrate did not have an effect on the phosphorylation of pp185 in PC-3 or DU 145 cells (data not shown).

To clarify that the increased phosphorylation of pp185 in cells grown in the presence of L(+)-tartrate was contributed by tyrosine phosphorylation, Western blot analyses were performed. Total cell lysate proteins were electrophoresed in a SDS-(5%) gel to increase the resolution of separation and the blotted membrane was reacted with an anti-p-tyr Ab. The p-tyr level of pp185 elevated following the dosage of inhibitor although the overall p-tyr level was also increased (Fig. 2B). In cells grown in the presence of 5 mM L(+)-tartrate, the p-tyr level of pp185 was increased by approximately 2 fold.

Since cellular PAcP may have a direct interaction with tyrosine kinase(s) (10,11), we analyzed the L(+)-tartrate effect on the tyrosine kinase specific activity at the initial rate of kinase reaction in those cells. As shown in Table 1, in the presence of 5 mM L(+)-tartrate, the cellular PAcP specific activity decreased by approximately 20% and the angiotensin phosphorylation

TABLE 1
Effect of L(+)-Tartrate on the Tyrosine Kinase and PAcP Specific Activities

Enzyme activity	L(+)-Tartrate −	L(+)-Tartrate +
^{32}P-Angiotensin II (cpm/10 μg protein)	1,144 (1x)	2,368 (2.01x)*†
PAcP (A_{410}/50 μ protein)	0.234 (1x)	0.190 (0.81x)†
L(+)-Tartrate-insensitive AcP (A_{410}/50 μg)	0.346 (1x)	0.359 (1.04x)

LNCaP cells (p31) in duplicate flasks were maintained in the presence or absence of 5 mM L(+)-tartrate in 20 mM Hepes, pH 7.4, for 16 hr. An aliquot of total cell lysates was used for analyzing the PAcP activity, while another aliquot of lysate proteins was used for quantifying the tyrosine kinase specific activity. All assays were performed at the initial rate of reaction. The data shown were the results of duplicate flasks and similar results were obtained from two independent experiments.

* Relative to control.

† $p < 0.05$ versus control by Student's t test (n = 4).

specific activity increased by two folds. L(+)-tartrate-insensitive AcP activity was analyzed as an internal control for the integrity of plasma membrane in those tartrate-treated cells. There was no significant effect on this activity. Thus, in LNCaP cells grown in L(+)-tartrate, the cellular PAcP activity was inhibited that correlated with an increase of the tyrosine phosphorylation specific activity as well as the p-tyr level in pp185.

Effect of Incorporated PAcP on Protein Phosphorylation in DU145 Cells

To exploit directly the effect of PAcP on protein phosphorylation, we incorporated PAcP protein into DU145 cells via Lipofectin (12). The incorporated PAcP retained the enzymatic activity (Table 2) as in our previous report (12). Significantly, in those PAcP-incorporated

TABLE 2
Tyrosine Kinase Specific Activity in PAcP-Incorporated DU 145 Cells

Treatment	^{32}P-Angiotensin II (cpm/10 μg)	AcP (A_{410}/100 μg)
Control	3,426 (100%)*	0.037
PAcP	3,040 (89%)	0.104
Lipofectin	2,814 (82%)	0.056
(PAcP + lipofectin)	2,015 (59%)†	0.289

Cells in duplicate flasks were exposed for various treatments as shown in the table for 16 hr and then fed with 10% FBS for an additional 24 hr. An aliquot of total cell lysate proteins was used for analyzing AcP activity, while another aliquot of cell lysate proteins was for quantifying the tyrosine kinase specific activity. All assays were performed at the initial rate of reaction. The data shown were the results from duplicate flasks.

* The number in the parentheses is the percentage of activity relative to the control.

† $p < 0.05$ versus control (n = 4).

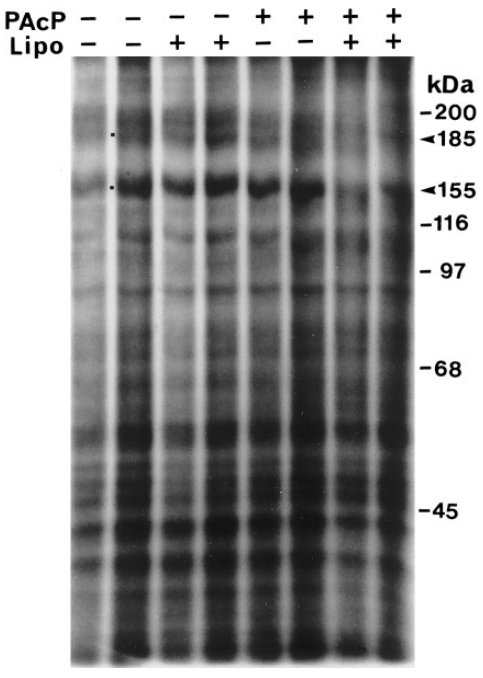

FIG. 3. Effect of PAcP on protein phosphorylation in DU145 cells. DU145 cells in duplicate flasks were exposed to different conditions as indicated in the figure for 16 hr and then fed with 10% FBS for 8 hr. Cells were labeled with ^{32}Pi in a phosphate-free medium containing 5% dialyzed serum for an additional 16 hr. An aliquot of total cell lysate proteins with 15 and 20 μg for flasks #1 and #2, respectively, from each treatment was electrophoresed in a SDS (7.5%) gel and then autoradiographed.

DU145 cells, the phosphorylations of pp185 and pp155 decreased (Fig. 3) and the tyrosine kinase specific activity diminished by 40% (Table 2). Thus, PAcP could function as a protein phosphatase in cells.

DISCUSSION

Several lines of evidence support the notion that cellular PAcP could function as a PTPase in cells although *in vitro* PAcP functions as a dual-specificity protein phosphatase (9,18). For example, cellular PAcP has been co-purified with the major PTPase activity in non-cancerous prostate cells (8). A neutral pH optimum is obtained when the EGF receptor is used as the substrate (10). PAcP does not belong to protein phosphatase 1 or 2A family since 20 nM okadaic acid has no effect on PAcP activity *in vivo* and *in vitro* (Lin, M. F. and Tsao, J., unpublished results.). Results from crystallographic studies demonstrate that PAcP has two active sulfhydryl groups per subunit (19). The ''S-^{32}P'' intermediate product has been isolated following titration experiments (20), indicating the ''cysteine'' residue in PAcP molecule could function as the phosphate acceptor, as other members of ''cysteine'' PTPases (21). Among different human prostate carcinoma cell lines, the expression of PAcP correlates with a decreased p-tyr level in cells (11,13). Driven by a cDNA expression vector, the expression of an exogenous, cellular PAcP is associated with a decreased p-tyr level in PAcP cDNA transfectants (13). Additionally, in the canine prostate gland, cells that express PAcP have low levels of p-tyr; while, inhibition of PAcP in those cells correlates with an increase of p-tyr level (22,23). Nevertheless, it is not known that the regulation of p-tyr level involving PAcP is due to a direct dephosphorylation by PAcP or via an indirect regulatory pathway nor the possible functional role of PAcP in cells.

In this communication, the results presented clearly demonstrate that the p-tyr level of pp185 is inversely correlated with the cellular activity of PAcP. In high passaged LNCaP cells, for example, a decreased expression of PAcP correlates with an increased p-tyr level of pp185, higher than that in the low passage (p31) of the same cells. Additionally, the differences in p-tyr of pp185 could not be merely due to different cell growth conditions since those cells were grown and harvested in the same set of experiments. Although there are other differences in p-tyr among these blots, the relationship between the p-tyr level of pp185 and the expression of PAcP is consistent among different cell lines and in different passages of the same cell line. This notion of inverse correlation is supported by the observations in the L(+)-tartrate-treated LNCaP cells. Alternatively, in L(+)-tartrate-treated cells, it is possible that the increased p-tyr in pp185 is due to the L(+)-tartrate inhibition on other PTPases than PAcP. Nevertheless, in prostate cells, PAcP is the major PTPase and the only known PTPase which is sensitive to L(+)-tartrate inhibition (5,8,11). The dephosphorylation of pp185 possibly by PAcP is further demonstrated by the incorporation of PAcP protein into DU 145 cells although PAcP is partially inactivated by this approach (12). Thus, different levels of p-tyr in pp185 are strongly correlated with the different cellular activities of PAcP.

The data taken collectively also support the notion that cellular PAcP might directly interact with tyrosine kinase(s) in the tyrosine phosphorylation signal transduction pathway. Significantly, the approximate 20% inhibition of cellular PAcP activity by L(+)-tartrate corresponds to the reported ratio of putative cellular form of PAcP (5-7), that may indicate a tartrate-specific inhibition on the cellular PAcP activity. Similarly, the incorporated PAcP, via Lipofectin, may also have a specific interaction with cellular proteins since a vast increase of PAcP activity in DU145 cells only correlates with a 40% decrease of tyrosine phosphorylation specific activity and the decreased phosphorylation of two proteins. Interestingly, the 40% decrease of tyrosine kinase specific activity by PAcP incorporation is the same as that was obtained after the pre-treatment of total cell lysate proteins with PAcP (11). It is also noted that, in the PAcP-incorporated DU 145 cells, the p-tyr level is diminished (12).

It would be interesting clarifying the identity of pp185. In the cell growth signal transduction pathway, there are several species of phosphoproteins that have a molecular weight (m.wt) of approximately 185 kDa and are tyrosine phosphorylated. For example, a pp185 has been determined to be a substrate of the insulin receptor tyrosine kinase (24). The neu oncoprotein (185 kDa) is expressed in human prostate carcinomas including LNCaP, DU145 as well as PC-3 cells (25). Additionally, the platelet growth factor receptor (180 kDa) and the EGF receptor (170 kDa) each exhibits a m.wt. close to the proximity of 185 kDa (26). Thus, there is a high possibility that the pp185 is the same one that has been reported.

The function of cellular PAcP as a PTPase could be biologically significant since a reduction of its activity should result in an increased level of p-tyr in proteins, and possibly the malignant growth of the cells. Significantly, it has been demonstrated in prostate carcinomas that a decreased expression of PAcP correlates with the progression/stage of the disease (27-31). Nevertheless, more experiments are required to demonstrate a direct interaction between the cellular PAcP and the 185 kDa protein and to clarify the identity of the 185 kDa phosphoprotein. It is also imperative to delineate the relationship of 185 kDa and 155 kDa proteins and the mechanism of p-tyr dephosphorylation by PAcP that may be resulted in cell growth regulation.

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