O-Phospho-l-tyrosine Inhibits Cellular Growth by Activating Protein Tyrosine Phosphatases

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ABSTRACT

O-Phospho-l-tyrosine (P-Tyr), a substrate for a wide range of protein tyrosine phosphatases, inhibited growth of human renal and breast carcinoma cells. Growth was blocked in the S phase of the cell cycle. A decrease in the amount of cyclin proteins A and B was also observed. P-Tyr incubation led to activation of cellular protein tyrosine phosphatases resulting in the inhibition of tyrosine phosphorylation of epidermal growth factor receptor as well as of p34<sup>cdc2</sup>. P-Tyr synergistically sensitized the renal carcinoma ACHN cells to killing by the chemotherapeutic agents doxorubicin and etoposide. These growth inhibitory properties of P-Tyr in <em>vitro</em> suggest its possible use as an anticancer agent.

INTRODUCTION

Protein tyrosine phosphorylation, a crucial step in cellular signal transduction, is actively regulated by PTK<sup>2</sup> and PTases. The latter enzymes have been found at both cell membrane and cytosolic sites (<sup>2</sup>). In addition to modulating the PTK function (<sup>3, 4</sup>), the PTases are also believed to participate in cellular signaling pathways (<sup>3, 5, 6</sup>). Although no known ligand for transmembrane PTases has yet been identified, evidence suggests that many ligands including cytokines and growth factors elicit their response by modulating PTase activity. For example, the proliferative response to interleukin 4 in erythroleukemic TF-1 cells was shown to be due to activation of a PTase that dephosphorylates a Mr 80,000 protein (<sup>7</sup>). PTases 1B, LAR, and LRP have been implicated in the modulation of insulin receptor tyrosine kinase activity in response to insulin action. Similarly, signal transduction via ligand activation of T-cells (<sup>9, 10</sup>) and growth factors elicit their response by modulating PTase activity and to test whether the activation of cellular PTases in turn could inhibit cell growth. P-Tyr was shown to inhibit the growth of human breast and renal carcinoma cells and to inhibit EGF receptor tyrosine phosphorylation.

MATERIALS AND METHODS

Materials. All cell lines were purchased from the American Type Culture Collection (Rockville, MD). The L-15 medium was purchased from Gibco Laboratories (Gaithersburg, MD). Fetal bovine serum was from Intercel Inc. (Purchase, NY). The Western blotting reagents and horseradish peroxidase coupled anti-mouse goat antibody were from Bio-Rad Life Science Products (Richmond, CA). Anti-phosphotyrosine antibody (PY69) was from ICN (Costa Mesa, CA). EGF was from Sigma Chemical Co. (St. Louis, MO). p43<sup>abl</sup>, Raytide<sup>TM</sup>, and antibodies to cyclins A, B, and p34<sup>cdc2</sup> were purchased from Oncogene Science (Mineola, NY). The ECL kit was from Amersham Corp. (Arlington Heights, IL). (γ-<sup>32</sup>P)<sub>ATP</sub> was supplied by Du Pont-New England Nuclear (Cambridge, MA).

Cell Culture. The human renal carcinoma cell lines ACHN, CakiII, A704, and A498 were grown in RPMI 1640, 10% fetal calf serum, 10 units/ml penicillin, and 10 µg/ml streptomycin in 5% CO<sub>2</sub> at 37°C in a humidified atmosphere. The culture medium for A498 and A704 cells was supplemented with 1 mM sodium pyruvate. The human breast carcinoma cells MDA-MB 468 were grown in L-15 medium supplemented with 10% fetal calf serum, 10 units/ml penicillin, and 10 µg/ml streptomycin at 37°C without CO<sub>2</sub>. For the cell growth inhibition assay, the cells were washed in Dulbecco's phosphate buffered saline, trypsinized, and counted in a Coulter Counter in a final volume of 10 ml.

Assay for Total Cellular PTases. Replicate semiconfluent ACHN cells were incubated with 5X concentration of P-Tyr, pH 7.4, where X represents the concentration of tyrosine in culture medium (1.67 mM) for 6 days. After P-Tyr incubation, the cells were washed in Hanks' balanced salt solution and scraped in 1 ml of buffer A [50 mM 3-(N-morpholinol)propanesulfonic acid (pH 6.0)-1 mg/ml bovine serum albumin-0.5 mM dithiothreitol-0.002% phenylmethylsulfonyl fluoride-2 µg/ml leupeptin-0.1% Triton X-100-0.01% [3-cholamidopropyl]dimethylamino]-l-propanesulfonate]. The cells were resuspended to remove clumps and the clarified supernatant was kept at -20°C prior to assaying for protein tyrosine phosphatase activity.

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3 The abbreviations used are: PTK, protein tyrosine kinase; P-Tyr, O-phospho-l-tyrosine; PTase, protein tyrosine phosphatase; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; PI, propidium iodide; SDS, sodium dodecyl sulfate; ECL, enhanced chemiluminescence; VP-16, etoposide.

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Fig. 1. Effect of P-Tyr on growth of renal and breast carcinoma cells. In a, the human renal carcinoma cells ACHN, Caki, A704, and A498 and the human breast carcinoma MDA-MB 468 cells were treated with 0, 0.1 X, 1.0X, and 10.0X P-Tyr (pH 7.4) for 6 days, where X denotes the concentration of Tyr in L-15 medium (~1.67 mM). Cell numbers are mean ± SD (bars) of three replicate readings. Similar results were obtained in three separate experiments. In b, ACHN cells were incubated in 0, 0.1 X, 1X, and 3X P-Tyr, P-Ser, P-Thr, or tyrosine and cell growth was assessed as described. Each point represents mean of triplicate cultures. Errors were <0.5%.

o-vanadate). Immunoprecipitation of the EGFR was performed using a mouse monoclonal antibody (528; Oncogene Science) and protein A-Sepharose. The immunoprecipitate was washed in 10 changes of PTPase assay buffer [50 mM 4-morpholinepropanesulfonic acid, pH 6.0–1 mg/ml bovine serum albumin-0.5 mM dithiothreitol-0.01% 1-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate] to remove all traces of o-vanadate, resuspended in 100 µl of PTPase assay buffer, and kept at 4°C. The measurement of PTPase activity in cell lysates was performed by incubating an equal amount of tyrosine phosphorylated EGFR with an appropriate dilution of cell lysates. The reaction was performed in PTPase assay buffer for 10 min and was stopped by adding Laemmli’s buffer containing 100 µM o-vanadate. Western blotting was performed on the reaction mixture to determine residual tyrosine phosphorylated EGFR as described below.

Measurement of EGFR Tyrosine Phosphorylation. To determine whether induction of cellular PTPases with P-Tyr could modulate EGF induced EGFR tyrosine phosphorylation, ACHN cells (10^4 cells/ml in 5 ml of medium)
in 25-cm² flasks were incubated with 5.0 X P-Tyr for 6 days. At the end of each day, the control and P-Tyr incubated cells were treated with EGF (50 ng/ml for 20 min). Replicates of control and P-Tyr treated samples were preincubated with o-vanadate (100 μM for 4 h) prior to EGF incubation. At the end of the EGF incubation, the culture medium was removed and the cells were washed with sterile Hanks' balanced salt solution and subsequently were lysed with 1 ml of Laemmli's buffer. The samples were briefly sonicated and stored at -20°C prior to determination of EGF induced EGFR tyrosine phosphorylation by Western blotting.

The EGFR tyrosine phosphorylation was analyzed by Western blotting using a monoclonal anti-phosphotyrosine antibody and horseradish peroxidase anti-mouse IgG second antibody using an ECL kit. Ten μl of Laemmli's lysate were resolved in a 7.5% SDS-polyacrylamide gel. The gels were washed extensively to remove residual P-Tyr. Because SDS-polyacrylamide gel electrophoresis separates proteins on the basis of molecular weight, no residual free P-Tyr could be expected to comigrate with the M, 170,000 EGFR band. The gels were blotted at 40 V for 8 h onto nitrocellulose filters. The filters were washed extensively with phosphate buffered saline to remove sodium azide that could inhibit the horseradish peroxidase reaction. The second antibody for all blots was horseradish peroxidase anti-rabbit IgG. The luminographs were developed using an ECL kit.

Tyrosine Phosphorylation of p34^{cdk2}. ACHN cells (10⁶ cells/ml in 5 ml of medium) in 25-cm² flasks were incubated with 0 and 5.0 X P-Tyr for 3 days. At the end of each day of incubation, equal numbers of control and P-Tyr incubated ACHN cells were lysed in immunoprecipitation buffer containing 100 μM o-vanadate (1× phosphate buffered saline, 1% Triton X-100, 0.5 g sodium deoxycholate, 0.1 g SDS, and 100 μM o-vanadate in 100 ml of water). The p34^{cdk2} was immunoprecipitated using anti-p34^{cdk2} antibody and protein A-Sepharose beads per the manufacturer's direction. The immunoprecipitate was resuspended in Laemmli's buffer and resolved in a 7.5% SDS-polyacrylamide gel. The Western blotting conditions were identical to those performed as detailed for EGFR tyrosine phosphorylation studies. Blots were probed with both anti-P-Tyr and anti-p34^{cdk2} antibodies.

Analysis of Cell Cycle Parameters. The distribution of cellular DNA was performed using PI fluorescence as described (28). ACHN cells were cultured in the presence of 0, 1.0 X, and 5.0 X P-Tyr for 3 days. After each day of P-Tyr incubation, the cells were trypsinized and centrifuged at 150 X g for 10 min, and the pellet was resuspended in hypotonic propidium iodide solution (0.05 mg/ml in 0.1% sodium citrate), and the cells were stained overnight at 4°C. Samples were excited at 488 nm and PI fluorescent emission (for 20,000 nuclei) was measured at the 575 band pass filter in a FACScan 80200 Becton-Dickinson flow cytometer. The data for the cell cycle analysis were analyzed using a Multicycle Cell Cycle Program (Phoenix Co., San Diego, CA).
induced EGFR tyrosine phosphorylation was inhibited in P-Tyr pretreated cells. Treatment with o-vanadate (100 μM for 4 h) prior to EGF incubation increased the tyrosine phosphorylation of EGFR and other protein substrates in control cells. EGFR mediated tyrosine phosphorylation of EGFR was enhanced to an even greater extent in P-Tyr pretreated cells when incubated with o-vanadate (100 μM, 4 h) prior to EGF incubation. The amount of EGFR proteins was unaffected by P-Tyr treatment (Fig. 2b). Thus, the EGFR tyrosine phosphorylation in response to EGF decreased in P-Tyr incubated cells and increased in P-Tyr incubated cells that were preincubated with o-vanadate. A possible explanation of the above results is that P-Tyr incubation led to activation of cellular PTPases, which in turn rapidly dephosphorylated EGFR tyrosine phosphorylation. Preincubation with o-vanadate inhibited these PTPases, restoring the ability of EGF to phosphorylate its receptor. Similar results were obtained with MDA-MB-468 cells (data not shown).

### RESULTS AND DISCUSSION

#### Inhibition of Cell Growth by P-Tyr

We determined the effect of P-Tyr incubation on the growth of both the human renal carcinoma cell lines ACHN, CakiIII, A704, and A498 and on the human breast carcinoma MDA-MB-468 cell line. P-Tyr inhibited growth of all the cell lines tested (Fig. 1a). The renal carcinoma ACHN cells were the most sensitive to P-Tyr. Cell growth was inhibited in a dose dependent manner in all cases. Neither P-Thr, P-Ser, nor tyrosine inhibited growth arrest of cells by P-Tyr was via P-Tyr mediated modulation of possible explanation of the above results is that P-Tyr incubation led to activation of cellular PTPases, which in turn rapidly dephosphorylated EGFR tyrosine phosphorylation. Preincubation with o-vanadate inhibited these PTPases, restoring the ability of EGF to phosphorylate its receptor. Similar results were obtained with MDA-MB-468 cells (data not shown).

#### P-Tyr Activates Cellular PTPase Activity

We postulated that the growth arrest of cells by P-Tyr was mediated through modulation of...
cellular PTPases. Therefore, PTPase activity in control and P-Tyr treated ACHN cells was determined by measuring dephosphorylation of immunopurified tyrosine phosphorylated EGFR at days 1–6 as described. Fig. 3 illustrates that EGFR was more rapidly dephosphorylated in the presence of lysates from P-Tyr treated cells than in that of control cells. The increased PTPase activity was observed at all time points tested. These findings suggest, but do not formally prove, that PTPases were directly activated by the presence of high concentrations of P-Tyr. Alternatively, P-Tyr may have inhibited the binding of SH2 domain containing putative inhibitors of PTPases to tyrosine phosphorylated EGFR. This failure may have led to the inability of these proteins to inhibit endogenous PTPase activity. In addition, P-Tyr induced changes in kinase activity have not been directly assessed. However, the data in Fig. 3 do suggest that lysates of P-Tyr

**Fig. 5.** Effect of VP-16 and doxorubicin in combination with P-Tyr on ACHN cell growth. a, control (C) and VP-16 (V1, 500 μM; V2, 50 μM; V3, 5 μM) incubated ACHN cells for 4 days in culture. b, control (C) and doxorubicin (D1, 500 μM; D2, 50 μM; D3, 5 μM) incubated ACHN cells for 4 days in culture. c, control (C), P-Tyr (0.5X), VP-16 (V1, 50 μM), and P-Tyr + VP-16 treated ACHN cells. d, control (C), P-Tyr (0.5X), doxorubicin (D2, 5 μM), and doxorubicin + P-Tyr treated ACHN cells. Points, mean of four replicate readings. 

Errors were <1%.
P-Tyr INHIBITION OF CELL GROWTH

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4d). The above results suggest that the incubation of ACHN cells with G2 delay after being exposed to ionizing radiation. This block was for example, Muschel et al. (33) showed that HeLa cells exhibited a late S-G2 block as noted for chemotherapeutic drugs like Adriamycin (30), vincristine (30), cisplatin (31), and VP-16 (32). The formation of mitotic promotion factor. The decreased formation of p34cdc2 and synthesis of cyclin B could have resulted in a decreased accumulation of p34cdc2 + cyclin B complex. Further evidence for a P-Tyr mediated increase in PTPase activity resulted in the inability of EOF (restorable by incubation with o-vanadate) to phosphorylate its receptor as well as the dephosphorylation of the p34cdc2 at tyrosine residues. The association of enhanced PTPase activity with the growth arrest of renal carcinoma cells. Furthermore, a constitutive dephosphorylation of p34cdc2 at tyrosine residues. Also, because p34cdc2 is specifically dephosphorylated at tyrosine residues bycdc25 (25), it is possible that incubation of P-Tyr resulted in increases in the PTPase activity ofcdc25.

Thus the inhibition of transition of ACHN cells to the G2M phase could have been due to several factors. The decreased tyrosine phosphorylation of p34cdc2 and synthesis of cyclin B could have resulted in a decreased accumulation of p34cdc2 + cyclin B complex. Furthermore, because cyclin A has recently been implicated in p34cdc2 kinase activation (29), a decreased synthesis of cyclin A would further inhibit the formation of mitotic promotion factor. The decreased formation of mitotic promotion factor could have resulted in failure to progress beyond the S phase. The P-Tyr mediated inhibition of cell growth resembles a late S-G2 block as noted for chemotherapeutic drugs like Adriamycin (30), vincristine (30), cisplatin (31), and VP-16 (32). The role of cell cycle proteins like the mitotic cyclins and p34cdc2 in mediating such S-G2 block has been investigated by several groups. For example, Muschel et al. (33) showed that HeLa cells exhibited a G2 delay after being exposed to ionizing radiation. This block was accompanied by decreased cyclin B protein levels. Similarly a G2 block accompanied by decreased cdc2 kinase activity was observed by Lock and Ross (34) in Chinese hamster ovary cells treated with etoposide. In our system, P-Tyr inhibited growth in the S phase. Furthermore, a constitutive dephosphorylation of p34cdc2 at tyrosine residue, via activation of cellular PTPases by P-Tyr, could have hindered the formation of the active kinase complex in the S phase. P-Tyr could also have mediated cell growth inhibition by decreased accumulation of cyclin A, thereby inhibiting transition to G2-M phase. The delineation of a cause and effect relationship between P-Tyr mediated cell growth inhibition and inhibition of transit into G2-M requires further investigation.

P-Tyr Sensitizes ACHN Cells to Killing by Chemotherapeutic Agents Doxorubicin and Etoposide. We determined the possible interaction of P-Tyr with doxorubicin and etoposide (VP-16) in inhibiting growth of ACHN cells. The dose-response curves for VP-16 and doxorubicin for ACHN cells are shown in Fig. 5. The 50% inhibitory concentration for P-Tyr at the same time was determined to be 0.4X. The growth curve showing the combined effect of P-Tyr (0.5X) and VP-16 (50 μM) is shown in Fig. 5c. The combination regimen resulted in complete inhibition of growth of ACHN cells. Similar results were obtained for doxorubicin (5 μM) in combination with P-Tyr (Fig. 5d). To determine the nature of P-Tyr interaction with these chemotherapeutic drugs, isobologram curves for the combination P-Tyr + doxorubicin and P-Tyr + VP-16 were plotted. P-Tyr acted synergistically with doxorubicin (Fig. 5e) and additively with VP-16 (Fig. 5f) in mediating the killing of ACHN renal carcinoma cells.

We conclude that P-Tyr decreased the growth of renal and breast carcinoma cells. P-Tyr mediated arrest of ACHN cells in S phase was associated with a concurrent decreased accumulation of the cyclin proteins A and B. The antiproliferative action of P-Tyr was accompanied by an increase in PTPase activity of these cells. The increase in cellular PTPase activity resulted in the inability of EGF (restorable by incubation with o-vanadate) to phosphorylate its receptor as well as the dephosphorylation of the p34cdc2 at tyrosine residues. The association of enhanced PTPase activity with the growth arrest of renal and breast carcinoma cells further highlights the potential tumor suppressor function of cellular PTPases. P-Tyr sensitized the ACHN cells to the cytostatic effect of doxorubicin and VP-16. The biological effects of the tyrosine analogue P-Tyr highlights its potential role as an anticancer agent.

REFERENCES


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