Analysis of pp60<sup>C-src</sup> Tyrosine Kinase Activity and Phosphotyrosyl Phosphatase Activity in Human Colon Carcinoma and Normal Human Colon Mucosal Cells

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We have compared the level of phosphotyrosyl phosphatase activity in lysates from normal human colon mucosal cells and human colon carcinoma cells and analyzed the effect of incubating these cells with sodium orthovanadate, an inhibitor of phosphotyrosyl phosphatase activity, on the relative abundance of acid-stable phosphotyrosine and on in vitro protein kinase activity of pp60<sup>C-src</sup>. Additionally, we compared the effect of lysing these cells in buffer containing only nonionic detergents with RIPA buffer, which contains both sodium dodecyl sulfate and deoxycholate, on the in vitro kinase activity of pp60<sup>C-src</sup>. Our results show that the level of detectable phosphotyrosyl phosphatase activity in lysates derived from normal colon cells and colon carcinoma cells is very similar. Additionally, the abundance of acid-stable phosphotyrosine in these cells cultured in the absence or presence of vanadate is not significantly different. However, incubation of these cells with vanadate significantly stimulates the activity of pp60<sup>C-src</sup> derived from the normal colon cells in immune-complex kinase assays, while having no detectable effect on the activity of pp60<sup>C-src</sup> from the colon tumor cells. The in vitro protein kinase activity of pp60<sup>C-src</sup> derived from RIPA buffer lysates of colon carcinoma cells was found to be elevated five- to sevenfold when compared with pp60<sup>C-src</sup> from these same cells lysed in buffer containing only Nonidet-P 40 as a detergent. The type of lysis buffer did not effect the activity of pp60<sup>C-src</sup> from normal colon mucosal cells. These results provide additional evidence that the activity of pp60<sup>C-src</sup> may be regulated differently in colon carcinoma and normal colon mucosal cells.

Key words: pp60<sup>C-src</sup>, tyrosine kinase, phosphotyrosyl phosphatase, human colon carcinoma, normal human colon mucosal cells

The c-src proto-oncogene encodes a 60,000-dalton membrane-associated phosphoprotein which possesses endogenous tyrosine-specific protein kinase activity [reviewed in 1 and 2]. The transforming potential of c-src mutants appears to be related

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to the specific activity of the mutated c-src-encoded pp60c-src protein phosphotransferase [3–7]. The specific activity of pp60c-src is currently thought to be negatively regulated in cells by the phosphorylation of tyrosine residues in the carboxyterminal portion of the molecule [4,8,9]. Recently, this site of tyrosine phosphorylation on avian pp60c-src molecules has been localized to tyrosine residue 527 [10].

The critical role phosphorylation of tyrosine residue 527 plays in governing the specific activity and transforming potential of pp60c-src has been demonstrated by site-specific mutation of tyrosine 527 to phenylalanine which results in transformation-competent pp60c-src molecules possessing elevated protein kinase activity [11–13]. Since pp60c-src does not appear to autophosphorylate tyrosine 527 residues at a significant rate in in vitro protein kinase assays [9,14], it is thought that phosphorylation of this amino acid within cells represents a function of cellular tyrosine kinases distinct from pp60c-src. Thus, regulation of pp60c-src tyrosine kinase activity in cells can apparently be controlled by cellular phosphotyrosyl phosphatases which function to stimulate kinase activity and cellular tyrosine kinases which act to inhibit pp60c-src protein kinase activity.

Recently, we reported that the tyrosine-specific protein kinase activity of pp60c-src molecules isolated from human colon carcinoma tissues and tumor-derived cell lines is consistently elevated over that from normal colon tissues and cultures of normal colon mucosal cells [15]. Analysis of the tumor tissues and cell lines showed that the elevation of pp60c-src kinase activity did not correlate with a proportional increase in the synthesis rate or abundance of pp60c-src, suggesting that the specific activity of the c-src-encoded phosphotransferase might be enhanced. Additional comparison of pp60c-src molecules from colon carcinoma cells lines and normal colon mucosal cells reveals that they possess indistinguishable sites and quantities of phosphorylated serine and tyrosine residues and are not stably complexed with other cellular proteins [14]. However, the elevated protein kinase activity of pp60c-src from the colon carcinoma cell lines was found to be associated with an increase in the apparent turnover rate of phosphate groups from carboxy-terminal tyrosine residues in vivo. These observations were interpreted to suggest that the level of phosphotyrosyl phosphatase activity in the colon carcinoma cell lines may be elevated [14].

In the present study, we have compared the phosphotyrosyl phosphatase activity in lysates from representative colon carcinoma cells with that from cultures of normal colon mucosal cells. Our results demonstrate that the level of detectable phosphotyrosyl phosphatase activity in these lysates is not significantly different. However, incubation of colon carcinoma cells and normal colon mucosal cells with sodium vanadate, an inhibitor of cellular phosphotyrosyl phosphatase function [16–18], stimulates the kinase activity of pp60c-src isolated from the normal colon cells but has no effect on the activity of pp60c-src from the colon carcinoma cells. Additionally, our results demonstrate that the level of pp60c-src kinase activity detected in immune-complex protein kinase assays can be substantially influenced by the type of buffer used for cell lysis.

**MATERIALS AND METHODS**

**Cell Culture**

Normal human colon mucosal cells (CCL 239) and human adenocarcinoma cell lines HT29 and WiDr were obtained from the American Type Culture Collection.
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(Rockville, MD). NIH 3T3 cells. Rous sarcoma virus-transformed NIH 3T3 cells (RSV 3T3), and NIH 3T3 (pMsrc/cos)A cells, which are NIH 3T3 cells that over-express normal avian pp60c-src [4], were obtained from David Shalloway (Pennsylvania State University).

Cell Lysis and Protein Kinase Assays

Culture cells were lysed in either a modified RIPA buffer (20 mM morpholinepropanesulfonic acid pH 7.0, 150 mM NaCl, 1% (w/v) deoxycholate, 1% (v/v) Nonidet P-40, 0.1% (w/v) sodium dodecyl sulfate, and 2 mM ethylenediaminetetraacetic acid) or MNNE buffer (100 mM morpholinepropanesulfonic acid, pH 7.0, 100 mM NaCl, 1% (v/v) Nonidet P-40, and 1 mM ethylenediaminetetraacetic acid) containing 10 µg/ml each of the following protease inhibitors—aprotinin, leupeptin, N-tosyl-L-phenylalanine chloromethyl ketone, N-p-tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride, as previously described [14]. The protein concentration of the cellular lysates was adjusted to the indicated level and immunoprecipitation of pp60c-src was conducted as previously described [14] following addition of MAb 327, a monoclonal antibody that recognizes avian and mammalian pp60c-src [19], or EC10, a monoclonal antibody that recognizes only avian pp60c-src [20]. Immune-complex protein kinase assays were performed on the washed immunoprecipitates by the addition of kinase buffer (20 mM morpholinepropanesulfonic acid pH 7.0, 5mM MgCl2) containing 20 μCi gamma 32P ATP (3,000 Ci/mmol, New England Nuclear, Boston, MA) and 10 μM unlabeled ATP. The reactions were allowed to proceed for 10 min at 23°C with constant shaking. Casein and enolase phosphorylation in immune-complex protein kinase assays was conducted by addition of either 1 mg/ml alpha-casein (Sigma, St. Louis, MO) or 100 µg/ml rabbit muscle enolase (Sigma) to the kinase buffer. The samples were analyzed on 8% sodium dodecyl sulfate (SDS) polyacrylamide gels, the radioactive bands were detected by autoradiography, and the radioactivity was quantitated as previously described [14].

Metabolic Labeling and Immunoblot Analysis

Metabolic labeling of cells with either 35S methionine or 32P orthophosphate has been previously described [14]. Immunoblot analysis of pp60c-src using MAb 327/273 has also been described previously [14, 21].

Peptide Mapping and Phosphoamino Acid Analysis

Determination of the phosphorylated amino acid products of immune-complex protein kinase reactions was conducted as previously described [22]. Analysis of the 32P-labeled proteins by limited proteolysis using Staphylococcus aureus V8 protease (Pierce, Rockford, IL) has been described [23]. Total cellular phosphoamino acid analysis was conducted as described by Cooper et al [22].

RESULTS

Comparison of Total Cellular Phosphoamino Acids

Previously we reported that the activity of pp60c-src isolated from HT29 and WiDr colon carcinoma cells is elevated approximately 50- to 60-fold over that of pp60c-src isolated from normal colon mucosal cells [14, 15]. To determine whether the elevation in tumor-derived pp60c-src kinase activity in in vitro immune-complex
protein kinase assays is potentially reflected in vivo by elevation of phosphorylated proteins within the tumor cells, we conducted total cellular phosphoamino acid analysis. For these experiments HT29, WiDr, and CCL 239 cells were labeled for 18 hr with $^{32}$P orthophosphate and the relative abundance of acid-stable phosphoserine, phosphothreonine, and phosphotyrosine was determined as described by Cooper et al [22]. We also conducted this analysis in parallel on NIH 3T3 and RSV NIH 3T3 cells. The results of these experiments are shown in Figure 1. As previously described by others [24], our results demonstrate that transformation of NIH 3T3 (Fig. 1C) cells with RSV (Fig. 1B) elevates the abundance of cellular phosphotyrosine. The increase was determined to be approximately tenfold, raising the relative abundance of phosphotyrosine in the NIH 3T3 cells from 0.1% to 0.96% in the RSV NIH 3T3 cells. The ratio of acid-stable phosphoamino acids in HT29 (Fig. 1D), WiDr (Fig. 1E), and CCL 239 (Fig. 1F) cells was found to be indistinguishable with phosphotyrosine representing approximately 0.07 to 0.12% of the detectable $^{32}$P-labeled phosphoamino acids. Similar results were obtained when shorter labeling times were used (data not shown). Addition of 50 μM sodium vanadate to the cell culture medium during the 18-hr incubation with $^{32}$P orthophosphate resulted in elevating the relative level of phosphotyrosine to approximately 0.25 to 0.35% in these cells. Significant differences in the response of the colon tumor cells and normal colon mucosal cells to the addition of vanadate were not detected.

Analysis of Phosphatase Activity in Immune-Complexes

The results obtained from phosphoamino acid analysis of normal and tumor-derived cells suggest that if pp60 c-src kinase activity is elevated in the tumor cells, the activity of cellular phosphotyrosyl phosphatases in these cells in the absence of vanadate may be sufficiently high so that no detectable elevation in cellular phosphotyrosine is evident. These observations could also indicate that the number of potential pp60 c-src substrates in the colon cells is limited. Alternatively, these results can be interpreted to suggest that pp60 c-src kinase activity in the colon tumor cells may not be elevated in vivo and becomes activated subsequent to cellular lysis. One obvious possibility is that cellular phosphatases might be nonspecifically coprecipitated in the immune-complexes derived from the tumor cell lysates thereby stimulating the activity of pp60 c-src in the immune-complex kinase assays by dephosphorylation of tyrosine residues.

To evaluate whether our immunoprecipitates contained phosphatase activity, CCL 239 and HT29 cells were labeled with $^{32}$P orthophosphate or $^{35}$S methionine for 8 hr prior to cell lysis and immunoprecipitation with MAb 327. The immune-complexes were washed according to our standard protocol; the samples were then divided into two equal aliquots and an immune-complex protein kinase assay using unlabeled ATP was conducted on one of the aliquots. The results of this experiment (Fig. 2) demonstrate that no detectable $^{32}$P was lost from the normal or tumor-derived pp60 c-src samples which participated in the kinase assays (Fig. 2A), nor was there any detectable loss of $^{35}$S-labeled pp60 c-src protein (Fig. 2B). In parallel experiments we observed that addition of 50 μM or 100 μM vanadate to the kinase buffer did not affect either the level of $^{32}$P-labeled pp60 c-src or pp60 c-src protein kinase activity in the immune-complexes (data not shown).

To examine the possibility that $^{32}$P corresponding to phosphoserine and phosphotyrosine could have been differentially lost from the $^{32}$P in vivo labeled pp60 c-src...
Fig. 1. Total cell phosphoamino acid analysis. Cell lines were labeled for 18 hr with $^{32}$P orthophosphate and prepared for analysis of acid-stable phosphoamino acids as described in Materials and Methods. (A) Pattern of ninhydrin-stained phosphoamino acid standards following two-dimensional electrophoresis. The direction of electrophoresis at either pH 1.9 or pH 3.5 is shown. Autoradiograms of the resolved $^{32}$P-labeled phosphoamino acids from RSV transformed NIH 3T3 cells (B), NIH 3T3 cells (C), HT29 cells (D), WiDr cells (E), and CCL 239 cells (F). The arrowheads in panels D–F show the position of a phosphorylated species described by Cooper et al. [19] that contains exclusively phosphoserine upon further acid hydrolysis. The positions of ninhydrin-stained phosphoamino acid standards are indicated by the dotted lines on the autoradiograms.
molecules, partial V8 proteolytic peptide analysis of the $^{32}$P pp60$^{c-src}$ bands was conducted. The results of this analysis (Fig. 2C) show that the amount of amino-terminal phosphoserine contained in the V1, V3, and V4 V8 phosphopeptides and carboxy-terminal phosphotyrosine contained in the V2 V8 phosphopeptides was similar regardless of the immune-complex reaction conditions. These results demonstrate that phosphatase activity capable of significant dephosphorylation of either phosphoserine or phosphotyrosine residues from pp60$^{c-src}$ is not present in the MAb 327 immune-complexes.

**Effect of Lysis Buffer Conditions and Vanadate on pp60$^{c-src}$ Kinase Activity**

To determine whether incubation of the cells with vanadate would alter immune-complex pp60$^{c-src}$ kinase activity, CCL 239, HT29, and WiDr cells were cultured in the presence of 50 µM vanadate for 4 hr prior to lysis in RIPA buffer containing 50 µM vanadate. MAb 327 immune-complex protein kinase assays conducted with equal amounts of cellular protein from the lysates (500 µg/ml/reaction) show that incubation with vanadate does not significantly affect the kinase activity of pp60$^{c-src}$ derived from either the HT29 or WiDr colon carcinoma cells (Fig. 3, lanes 3–6). However, incubation of the normal colon mucosal cells with vanadate stimulates immune-complex pp60$^{c-src}$ protein kinase activity approximately fivefold (Fig. 3, lanes 1, 2). In parallel experiments, we also analyzed the effect of lysing the cells in MNNE buffer containing 100 µM vanadate in the presence and absence of 1 mM ATP and
Fig. 3. Protein kinase activity of pp60c-src from vanadate-treated and untreated cells lysed in RIPA buffer. CCL 239 (lanes 1, 2), HT29 (lanes 3, 4), and WiDr cells (lanes 5, 6) were cultured with (lanes 2, 4, 6) or without (lanes 1, 3, 5) 100 μM vanadate for 4 hr prior to lysis in RIPA buffer and MAb 327 immune-complex protein kinase assays were conducted as described in Materials and Methods. The positions of pp60c-src (src), casein, and prestained molecular mass markers are indicated.

5 mM MgCl₂ on pp60c-src immune-complex protein kinase activity. The results of this experiment demonstrate that addition of ATP and Mg²⁺ to the MNNE lysis buffer decreases the activity of pp60c-src derived from in both the HT29 and WiDr cells (Fig. 4, lanes 3–6), while having no detectable effect on the activity of pp60c-src derived from the normal colon mucosal cells (Fig. 4, lanes 1, 2).
Fig. 4. Effect of ATP and Mg\(^{2+}\) on the protein kinase activity of pp60\(^{c-src}\) from cells lysed in MNNE buffer. CCL 239 (lanes 1, 2), HT29 (lanes 3, 4), and WiDr cells (lanes 5, 6) were lysed in MNNE buffer in the presence and absence of 1 mM ATP and 5 mM Mg\(^{2+}\) and MAb 327 immune-complex protein kinase assays were conducted as described in Materials and Methods. The positions of pp60\(^{c-src}\) (src), casein, and prestained molecular mass markers are indicated.

The results of the immune-complex kinase assays illustrated in both Figures 3 and 4 were derived from experiments conducted simultaneously with equal cellular protein concentrations and the same exposure time of the autoradiograms (4 hr). Quantitation of the resulting \(^{32}\)P-labeled pp60\(^{c-src}\) and casein radioactivity revealed that lysis of the colon carcinoma cells in RIPA buffer yielded pp60\(^{c-src}\) molecules with five- to sevenfold-higher levels of protein kinase activity than lysis of the same cells in MNNE buffer. Interestingly, the activity of pp60\(^{c-src}\) molecules derived from the normal colon carcinoma cells was not affected by the type of lysis buffer. Immunoblot analysis of the MAb 327 immunoprecipitates revealed that the same amount of pp60\(^{c-src}\) was present in the immune-complexes obtained by using either cell lysis buffer.
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**Analysis of Phosphatase Activity in Cellular Lysates**

To assess the activity of phosphotyrosyl phosphatases in cellular lysates, we utilized as substrate in vitro phosphorylated avian pp60⁵⁻src molecules obtained from NIH 3T3 (pMcsrc/cos)A cells following lysis in RIPA buffer, immunoprecipitation with EC10, and immune-complex kinase assays. As shown in Figure 5, addition of MNNE buffer lysates from HT29 cells to the ³²P-labeled pp60⁵⁻src molecules in the EC10 immune-complexes results in time-dependent (Fig. 5A) and concentration-dependent (Fig. 5B) loss of phosphate from tyrosine residues. The reduction in ³²P from the pp60⁵⁻src molecules in the immune-complexes is not apparently the result of protein degradation by proteases in the cellular lysates since avian pp60⁵⁻src molecules metabolically labeled with ³⁵S methionine and used in a parallel experiment did not reveal time- and concentration-dependent losses of ³⁵S from the immunoprecipitates (Fig. 5C,D). Additional support for this conclusion is provided by the finding that the amount of avian pp60⁵⁻src detected by MAb 327/273 immunoblot analysis prior to and following incubation with the HT29 cell lysates is indistinguishable (Fig. 5F).

Incubation of HT29 cell lysates with the avian pp60⁵⁻src molecules in the EC10 immune-complexes was found to increase the protein kinase activity of these molecules approximately four- to fivefold (Fig. 5E). These results suggest that the specific activity of the avian pp60⁵⁻src molecules has been increased during the course of the experiment since no additional pp60⁵⁻src was found associated with the EC10 immune-complexes (Fig. 5F). These observations are similar to those of Cooper and King [9], who recently demonstrated that the specific activity of pp60⁵⁻src molecules could be elevated in vitro following a brief treatment with potato acid phosphatase which removes phosphate from both serine and tyrosine residues.

The ability of vanadate to inhibit dephosphorylation of tyrosine on pp60⁵⁻src molecules in EC10 immune-complexes is shown in Figure 6A. In this experiment, 100 µM vanadate was capable of protecting 80–90% of the phosphorylated tyrosine residues during a 30-min incubation with 200 µg of HT29 MNNE buffer lysate. The ability of vanadate to inhibit phosphotyrosyl phosphatase activity in the HT29 lysates was found to be concentration dependent and the phosphatase activity was also found to be sensitive to inhibition by 10 µM zinc (Fig. 6B). No effect on HT29 phosphotyrosyl phosphatase activity was observed by the addition of either 500 µg/ml phosphotyrosine or phosphothreonine or phosphoserine (Fig. 6B).

To compare the relative phosphotyrosyl phosphatase activity in colon carcinoma and normal colon mucosal cell lysates, ³²P-labeled avian pp60⁵⁻src autophosphorylated in EC10 immune-complexes was incubated for different times with MNNE buffer lysates from HT29 (Fig. 7A) and CCL 239 (Fig. 7B) cells adjusted to 200 µg cellular protein per reaction in the presence or absence of 100 µM vanadate. The results of this analysis show that the rate of phosphate hydrolysis catalyzed by addition of the HT29 lysates was elevated approximately twofold compared to the rate observed in CCL 239 lysates. The addition of vanadate was found to inhibit greater than 95% of the loss of ³²P from pp60⁵⁻src in these assays. The results of other experiments using the same ³²P-labeled pp60⁵⁻src substrate where the protein concentration of the CCL 239 and HT29 lysates was varied also demonstrated that the maximum detectable
Fig. 5. Phosphotyrosyl phosphatase activity in HT29 cell lysates. (A) Equal amounts of in vitro phosphorylated avian pp60^src in EC10 immune-complexes were incubated for 0 (lane 1), 5 (lane 2), 10 (lane 3), 15 (lane 4), 20 (lane 5), or 30 (lane 6) min with 150 μg of HT29 MNNE cell lysate prior to analysis on SDS gels. (B) Equal amounts of in vitro phosphorylated avian pp60^src in EC10 immune-complexes were incubated for 15 min with 0 (lane 1), 25 (lane 2), 50 (lane 3), 100 (lane 4), 200 (lane 5), or 300 (lane 6) μg of HT29 MNNE cell lysate prior to analysis on SDS gels. (C, D) In vivo
difference in phosphatase activity from lysates of these cells was approximately
twofold (data not shown).

Avian pp60c-src molecules autophosphorylate tyrosine residue 416 in immune-
complex kinase reactions [25,26]. Thus, it is conceivable that phosphotyrosyl phosph-
ases that remove phosphate moieties from this tyrosine residue may be different
than those which hydrolyze phosphate from tyrosine residue 527. To examine this
possibility, NIH 3T3 (pMsesc/cos)A cells were metabolically labeled with 32P ortho-
phosphate for 4 hr; the 32P-labeled avian pp60c-src was immunoprecipitated with
EC10, and the washed immune complexes were incubated for different times with
equal amounts (200 µg protein/reaction) of MNNE buffer lysates from either CCL
239 (Fig. 7C) or HT29 (Fig. 7D) cells. The results of this experiment show that the
initial rate of phosphate hydrolysis from the 32P-labeled avian pp60c-src molecules

Fig. 6. Effect of vanadate on phosphotyrosyl phosphatase activity in HT29 cell lysates. (A) Equal
amounts of in vitro phosphorylated avian pp60c-src in EC10 immune-complexes were incubated with 200
µg of MNNE HT29 lysate for 0 (lanes 1, 8), 5 (lanes 2, 9), 10 (lanes 3, 10), 15 (lane 4), 20 (lanes 5,
11), 25 (lane 6), or 30 min (lanes 7, 12) in the absence (lanes 1–7) or presence (lanes 8–12) of 100 µM
vanadate. (B) Equal amounts of in vitro phosphorylated avian pp60c-src in EC10 immune-complexes
were incubated with 200 µg HT29 lysate for 0 (lanes 2, 4) or 15 min (lanes 1,3,5–9) in the presence of
100 µM vanadate (lane 1), 10 µM vanadate (lane 3), 1 µM vanadate (lane 5), 500 µg/ml phosphothreon-
ine (lane 6), 500 µg/ml phosphotyrosine (lane 7), 10 µM ZnCl (lane 8), or 500 µg/ml phosphoserine
(lane 9). The positions of pp60c-src (src) and prestained molecular mass markers are indicated.

35S-methionine-labeled avian pp60c-src in EC10 immune-complexes used in parallel experiments to those
described in panels A (C) and B (D). (E) Equal amounts of unlabeled avian pp60c-src in EC10 immune-
complexes were incubated in the absence (lanes 1, 2) or presence (lanes 3, 4) of 300 µg HT29 MNNE
lysate for 15 min prior to conducting casein (lanes 1, 3) or enolase (lanes 2, 4) kinase assays. (F) MAb
327/273 immunoblot analysis of avian pp60c-src in EC10 immune-complexes prior to (lane 1) or
following (lane 2) incubation with 300 µg HT29 MNNE lysate for 15 minutes.
Fig. 7. Comparison of phosphatase activity in lysates of normal colon mucosal cells and colon carcinoma cells. Equal amounts of in vitro phosphorylated (A, B) or in vivo phosphorylated (C, D) avian pp60<sup>c-src</sup> in EC10 immune-complexes were incubated with 200 μg of HT29 (A, C) or CCL 239 (B, D) cell lysates for 0 (lane 1), 5 (lane 2), 10 (lane 3), 15 (panels A, B, lanes 4), 20 (panels A, B, lanes 5; panels C, D, lanes 4), 25 (panels A, B, lanes 6), or 30 min (panels A, B, lanes 7, 8; panels C, D, lanes 5, 6) in the presence (panels A, B, lanes 8; panels C, D, lanes 6) or absence (all other lanes) of 100 μM vanadate. The positions of pp60<sup>c-src</sup> (src) and prestained molecular mass markers are indicated.
incubated with HT29 lysates was faster than that observed with CCL 239 lysates. However, by 10 min the rate of phosphate hydrolysis by the two cell lysates was indistinguishable. Partial proteolytic V8 peptide mapping of the $^{32}$P-labeled pp60$^{c}$-src molecules from this experiment revealed that both amino-terminal serine and carboxy-terminal tyrosine residues were being dephosphorylated at comparable rates (data not shown). Addition of 100 μM vanadate to the lysates was found to inhibit approximately 80–90% of the phosphotyrosyl phosphatase and phosphoserine phosphatase activity in the cell lysates.

The interactions of cellular phosphatases from colon cells with pp60$^{c}$-src in the immune-complexes could be altered by the association of pp60$^{c}$-src with the monoclonal antibody or other components in the immune-complexes. To examine this possibility, equal amounts of MNNE buffer lysate (50 μg protein/reaction) from $^{32}$P orthophosphate metabolically labeled NIH 3T3 (pMcsrc/cos)A cells were incubated with increasing amounts of MNNE buffer lysates from CCL 239 (Fig. 8A), HT29 (Fig. 8B), or WiDr (Fig. 8C) cells for 20 min prior to addition of 100 μM vanadate and EC10 antibody. The results of this study show that the rate of phosphate hydrolysis from the avian pp60$^{c}$-src molecules in the lysates prior to immunoprecipitation did not differ significantly between the normal colon mucosal and colon carcinoma cell lysates. As previously observed, the addition of 100 μM vanadate to the cellular lysates protected approximately 80%–90% of the pp60$^{c}$-src-associated $^{32}$P label.

**DISCUSSION**

The protein kinase activity of pp60$^{c}$-src derived from human colon carcinoma cell lines, such as HT29 and WiDr, has been observed to be elevated in in vitro
protein kinase assays 50- to 60-fold over that of pp60⁶-src derived from normal mucosal cells [15]. Comparison of the relative abundance of pp60⁶-src in these cells by immunoblot analysis suggests that the apparent specific activity of pp60⁶-src from the colon tumor cell lines is ten- to 30-fold higher than pp60⁶-src from normal colon mucosal cells [14]. The elevated activity of pp60⁶-src molecules from the colon tumor cells is associated with an apparent six-fold increase in the turnover rate of phosphate from tyrosine residues within the carboxy-terminal portion of the protein, suggesting that differences in the activity of phosphotyrosyl phosphatase may exist between the normal and tumor-derived cells [14].

The results presented in the report indicate that while phosphatase activity which acts to dephosphorylate pp60⁶-src tyrosine residues may differ between normal colon mucosal cells and colon carcinoma cells in vivo by as much as sixfold or more, less than one- to twofold differences are detectable in the in vitro activity of phosphotyrosyl phosphatases in lysates prepared from these cells. These results include analysis of phosphate hydrolysis from avian pp60⁶-src tyrosine residues 416 (Figs. 5-7) and tyrosine 527 (Fig. 7) in immune-complexes and hydrolysis of pp60⁶-src tyrosine residue 527 in cellular lysates (Fig. 8). Similar conclusions were reached when mammalian pp60⁶-src in MAb 327 immune-complexes was utilized in several of these assays (our unpublished result). We have also varied the lysis conditions of the normal and colon tumor cells by analyzing different combinations of detergent, pH, and salt concentrations and we varied the reaction conditions without successful demonstration of significant differences in phosphatase activity between lysates prepared from CCL 239 and HT29 cells (our unpublished results). Thus, most of our data suggest that differences in phosphatase activity do not exist between normal colon cells and colon carcinoma cells. We cannot rule out, however, the possibility that lysis of the cells using the condition tried to date has destroyed the normal cellular microenvironment which may be essential to the putative pp60⁶-src/ phosphatase interactions previously proposed [14].

Analysis of the relative abundance of stable phosphotyrosine-containing proteins in CCL 239, HT29, and WiDr cells revealed that significant differences are not evident (Fig. 1). As previously noted, these results could indicate that pp60⁶-src-specific substrates in these cells might be limited in number. In addition, it is possible that potential protein substrates of pp60⁶-src in these cells might have very short half-lives or are rapidly dephosphorylated. Addition of vanadate to the culture medium of these cells was found to marginally elevate the overall level of phosphotyrosine, but failed to illicit significant alterations between the normal and tumor cells. Differences in tyrosine phosphorylation of suspected pp60⁶-src substrates such as calpactin I [27–29] were also not observed in these cells in the presence or absence of vanadate (our unpublished results). Thus, no measurable effect of vanadate on the potential in vivo activity of pp60⁶-src could be demonstrated.

However, the presence of vanadate in the culture medium was found to significantly stimulate pp60⁶-src in vitro kinase activity from normal colon mucosal cells while having no detectable effect on the activity of pp60⁶-src derived from colon carcinoma cells (Fig. 3). Analysis of 32P orthophosphate-labeled pp60⁶-src molecules isolated from vanadate-treated CCL 239 and HT29 cells by V8 digestion did not reveal detectable differences in the ratio of phosphoserine to phosphotyrosine (our unpublished result). We have not conducted 32P pulse-chase analysis with pp60⁶-src molecules in the presence or absence of vanadate in these cells. While the mechanism
for these observations is currently unclear, they do demonstrate differences in the response of normal colon and colon tumor cells to vanadate. Whether the observed differences are directly related to the effect of vanadate on cellular phosphatases or other cellular functions that might modulate pp60c-src kinase activity needs to be clarified. An additional possibility that could account for the differences in the observed characteristics of pp60c-src molecules derived from the normal and tumor cells is that the c-src gene in either the tumor cells or in the cultured “normal” mucosal cells has undergone mutational alterations. We are currently analyzing other tyrosine kinases, including other members of the src “subfamily” of tyrosine kinases, in these cells to see if alterations in activity of these protein kinases or differences in phosphate turnover from these proteins can be detected.

An additional point raised by our experiments is that the in vitro kinase activity of pp60c-src derived from normal colon mucosal cells and colon carcinoma cells can vary significantly depending on the cellular lysis buffer (Figs. 3,4). In this case, the alterations in pp60c-src kinase activity as a function of lysis buffer conditions was limited to the colon carcinoma cells. Since the same amount of pp60c-src was present in the immune-complexes derived from the colon carcinoma cells lysed by either the RIPA or MNNE buffers, these results suggest that the specific activity of these molecules was altered by postlysis events. We believe that the observed activation of pp60c-src in cells lysed in RIPA buffer is independent of phosphatase interactions since 100 μM vanadate was present in both lysis buffers. The mechanism responsible for the further decrease in pp60c-src kinase activity following addition of ATP and Mg2+ to the MNNE buffer in the tumor cell lysates is also unclear. With RIPA buffer lysates, the difference in specific activity of pp60c-src between normal colon mucosal cells and colon carcinoma cells is about ten-to thirtyfold. However, with a lysis buffer which contains only Nonidet-P 40 as a detergent source, the difference in specific activity of pp60c-src between normal colon and colon carcinoma cells would be less than three- to fourfold. These observations suggest that comparing of the results of pp60c-src in vitro protein kinase assays in other cellular systems where only RIPA buffer lysis has been used should be interpreted with caution.

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