Analysis of pp60$^{c\text{-}src}$ Tyrosine Kinase Activity and Phosphotyrosyl Phosphatase Activity in Human Colon Carcinoma and Normal Human Colon Mucosal Cells

Virginia DeSeau, Neal Rosen, and Joseph B. Bolen

Laboratory of Tumor Virus Biology (V.D., J.B.B.) and Medicine Branch (N.R.), National Cancer Institute, Bethesda, Maryland 20892

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$^{c\text{-}src}$. 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$^{c\text{-}src}$. 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$^{c\text{-}src}$ derived from the normal colon cells in immune-complex kinase assays, while having no detectable effect on the activity of pp60$^{c\text{-}src}$ from the colon tumor cells. The in vitro protein kinase activity of pp60$^{c\text{-}src}$ derived from RIPA buffer lysates of colon carcinoma cells was found to be elevated five- to sevenfold when compared with pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ from normal colon mucosal cells. These results provide additional evidence that the activity of pp60$^{c\text{-}src}$ may be regulated differently in colon carcinoma and normal colon mucosal cells.

Key words: pp60$^{c\text{-}src}$, 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

to the specific activity of the mutated c-src-encoded pp60$^{c\text{-src}}$ protein phosphotrans-ferase [3–7]. The specific activity of pp60$^{c\text{-src}}$ 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 pp60$^{c\text{-src}}$ 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 pp60$^{c\text{-src}}$ has been demonstrated by site-specific mutation of tyrosine 527 to phenylalanine which results in transformation-competent pp60$^{c\text{-src}}$ molecules possessing elevated protein kinase activity [11–13]. Since pp60$^{c\text{-src}}$ does not appear to autophosphorylate tyrosine 527 residues at a significant rate in in vitro protein kinase assays [9,14], it is thought that phosphory-lation of this amino acid within cells represents a function of cellular tyrosine kinases distinct from pp60$^{c\text{-src}}$. Thus, regulation of pp60$^{c\text{-src}}$ 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 pp60$^{c\text{-src}}$ protein kinase activity.

Recently, we reported that the tyrosine-specific protein kinase activity of pp60$^{c\text{-src}}$ 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 pp60$^{c\text{-src}}$ kinase activity did not correlate with a proportional increase in the synthesis rate or abundance of pp60$^{c\text{-src}}$, suggesting that the specific activity of the c-src-encoded phosphotransferase might be enhanced. Additional com-parison of pp60$^{c\text{-src}}$ molecules from colon carcinoma cells lines and normal colon mucosal cells reveals that they possess indistinguishable sites and quantities of phos-phorylated serine and tyrosine residues and are not stably complexed with other cellular proteins [14]. However, the elevated protein kinase activity of pp60$^{c\text{-src}}$ 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 phosphoty-rosyl 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 phosphoty-rosyl 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], stim-ulates the kinase activity of pp60$^{c\text{-src}}$ isolated from the normal colon cells but has no effect on the activity of pp60$^{c\text{-src}}$ from the colon carcinoma cells. Additionally, our results demonstrate that the level of pp60$^{c\text{-src}}$ 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

(Rockville, MD). NIH 3T3 cells. Rous sarcoma virus-transformed NIH 3T3 cells (RSV 3T3), and NIH 3T3 (pMcsrc/cos)A cells, which are NIH 3T3 cells that over-express normal avian pp60^{c-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 morpholine-propanesulfonic 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 pp60^{c-src} was conducted as previously described [14] following addition of MAb 327, a monoclonal antibody that recognizes avian and mammalian pp60^{c-src} [19], or EC10, a monoclonal antibody that recognizes only avian pp60^{c-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 MgCl$_2$) containing 20 μCi gamma ^{32}P 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 ^{35}S methionine or ^{32}P orthophosphate has been previously described [14]. Immunoblot analysis of pp60^{c-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 ^{32}P-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 pp60^{c-src} isolated from HT29 and WiDr colon carcinoma cells is elevated approximately 50- to 60-fold over that of pp60^{c-src} isolated from normal colon mucosal cells [14,15]. To determine whether the elevation in tumor-derived pp60^{c-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}

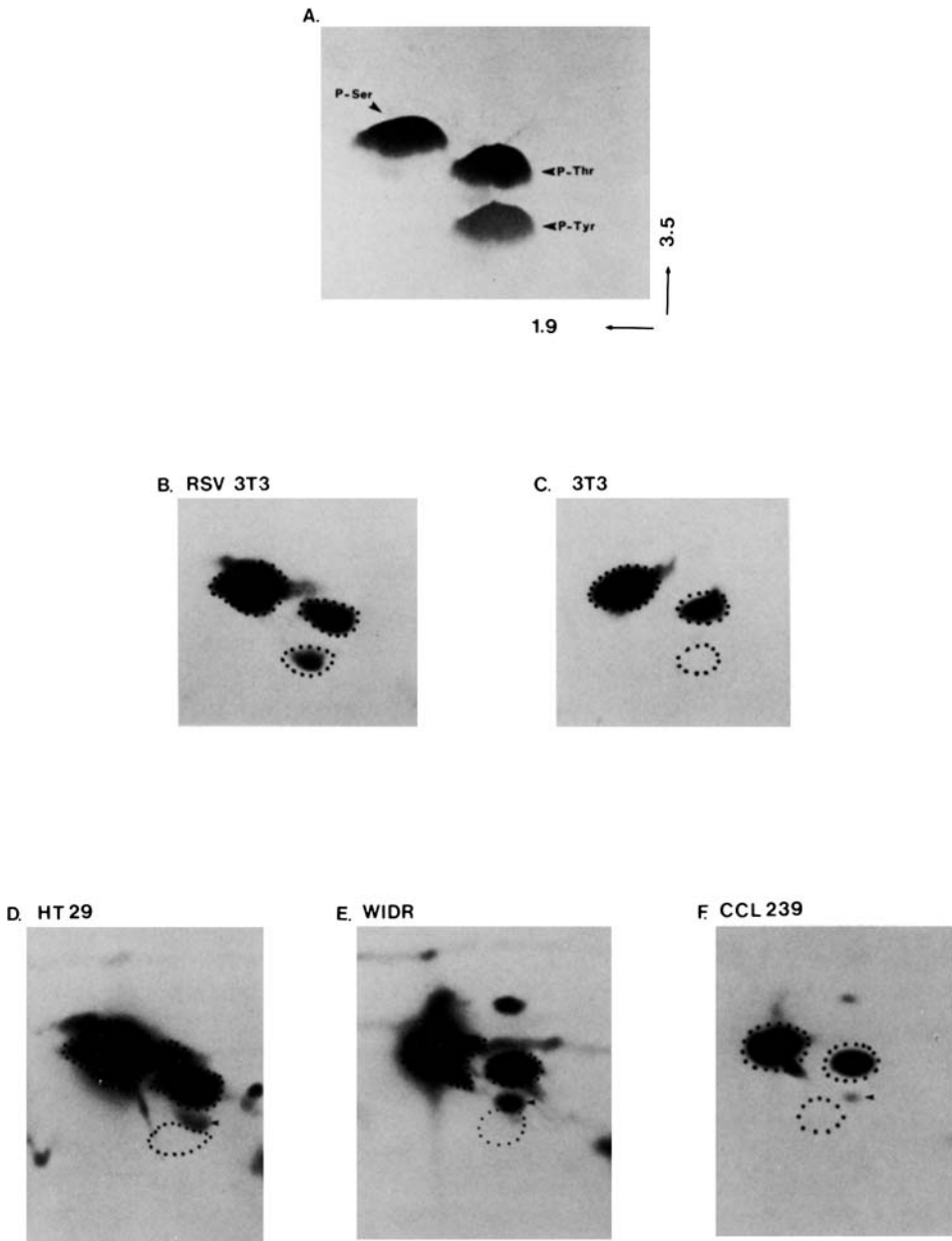Fig. 1.   Total cell phosphoamino acid analysis. Cell lines were labeled for 18 hr with ^{32}P orthophosphate and prepared for analysis of acid-stable phosphoamino acids as described in Materials and Methods. (A) Pattern of ninhydrin-stained phosphoamino acid standards following two-dimensional electrophoresis. 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.
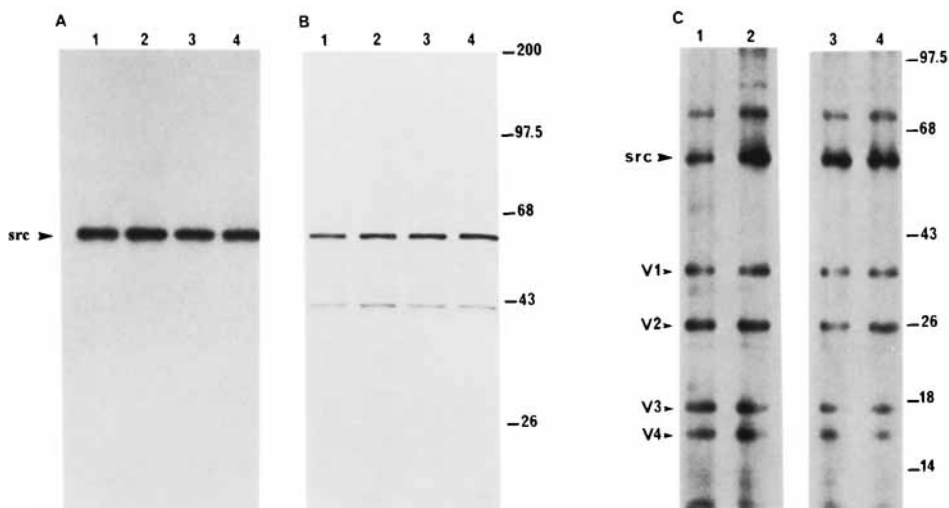
Fig. 2. Effect of immune-complex kinase assays on the level of ^{32}P orthophosphate-and ^{35}S methionine-labeled pp60$^{c\text{-}src}$ from normal colon cells and colon carcinoma cells; ^{32}P orthophosphate (A) and ^{35}S methionine (B) labeled CCL 239 cells (lanes 1, 2) or HT 29 cells (lanes 3,4) were lysed in RIPA buffer and immunoprecipitated by MAb 327. The amount of lysate from each cell was adjusted so that approximately equal amounts of ^{32}P or ^{35}S counts were present in the pp60$^{c\text{-}src}$ bands. Immune-complex protein kinase assays using 10 μM unlabeled ATP were conducted on the immunoprecipitates represented in lanes 2 and 4. (C) The ^{32}P-labeled pp60$^{c\text{-}src}$ bands shown in panel A analyzed by limited proteolysis using V8 protease. The samples represented in each lane are the same as given in panel A. The position of pp60$^{c\text{-}src}$ (src), various V8 phosphopeptides (VI-V4), and prestained molecular mass markers (BRL, Gaithersburg, MD) are indicated.

molecules, partial V8 proteolytic peptide analysis of the ^{32}P pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ is not present in the MAb 327 immune-complexes.

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

To determine whether incubation of the cells with vanadate would alter immune-complex pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ 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$^{c\text{-}src}$ 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
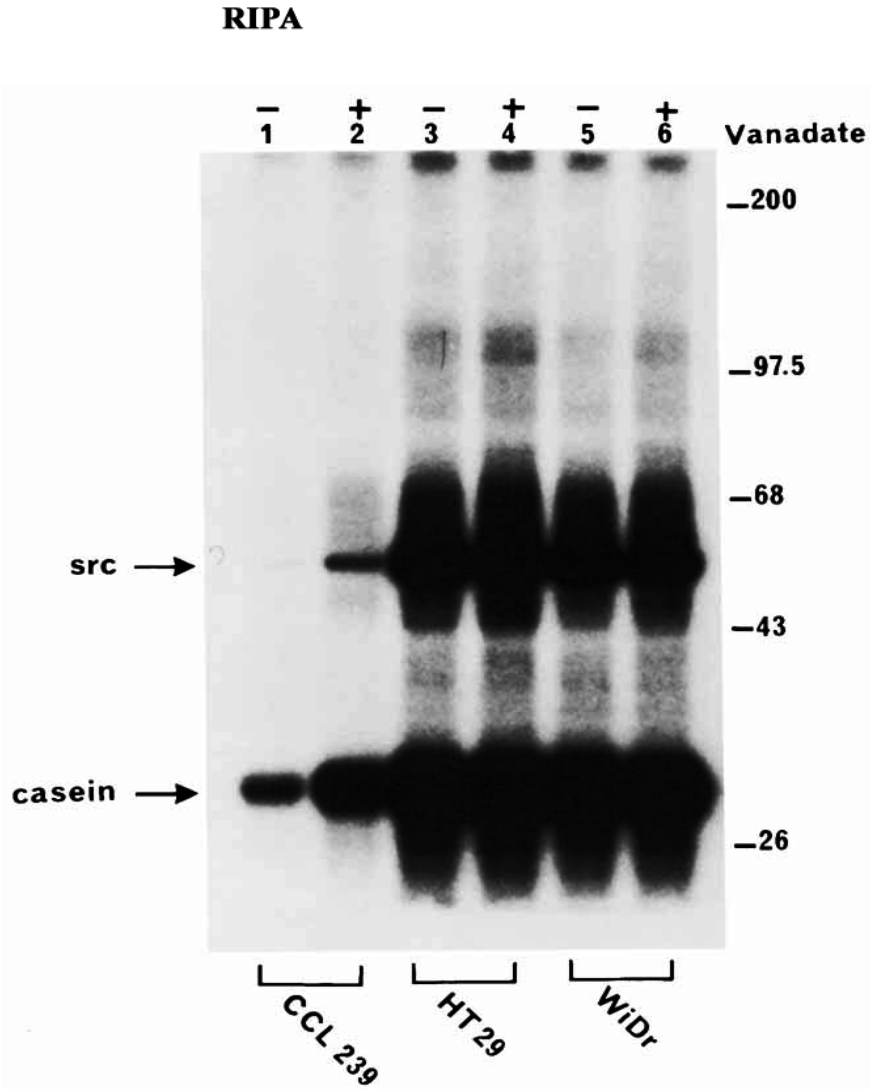
RIPA

Fig. 3. Protein kinase activity of pp60$^{c\text{-}src}$ 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 pp60$^{c\text{-}src}$ (src), casein, and prestained molecular mass markers are indicated.

5 mM MgCl$_2$ on pp60$^{c\text{-}src}$ immune-complex protein kinase activity. The results of this experiment demonstrate that addition of ATP and Mg^{2+} to the MNNE lysis buffer decreases the activity of pp60$^{c\text{-}src}$ derived from in both the HT29 and WiDr cells (Fig. 4, lanes 3–6), while having no detectable effect on the activity of pp60$^{c\text{-}src}$ derived from the normal colon mucosal cells (Fig. 4, lanes 1, 2).
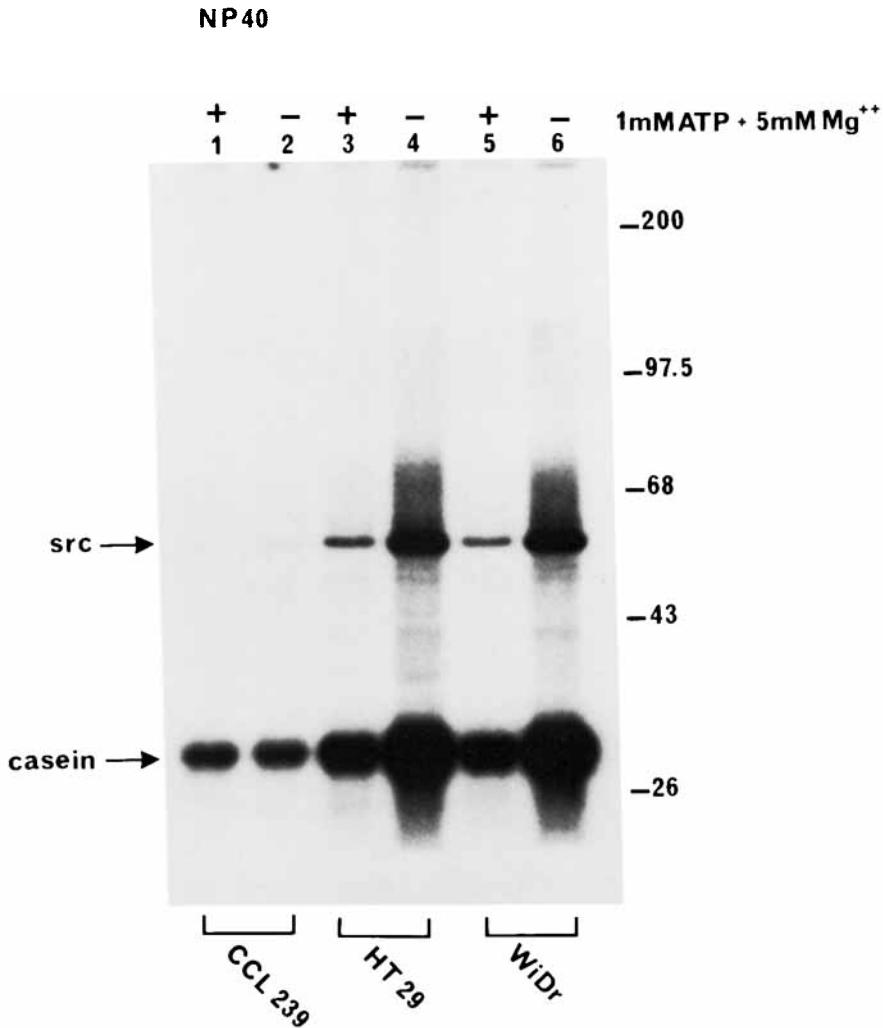
NP40

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 (data not shown). It should be noted that all of the immune-complexes represented in the experiments shown in Figures 3 and 4 were washed in RIPA buffer prior to performing the immune-complex protein kinase assays.

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 $pp60^{c-src}$ 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 ^{32}P-labeled $pp60^{c-src}$ 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 ^{32}P from the $pp60^{c-src}$ molecules in the immune-complexes is not apparently the result of protein degradation by proteases in the cellular lysates since avian $pp60^{c-src}$ molecules metabolically labeled with ^{35}S methionine and used in a parallel experiment did not reveal time- and concentration-dependent losses of ^{35}S from the immunoprecipitates (Fig. 5C,D). Additional support for this conclusion is provided by the finding that the amount of avian $pp60^{c-src}$ 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 $pp60^{c-src}$ 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 $pp60^{c-src}$ molecules has been increased during the course of the experiment since no additional $pp60^{c-src}$ 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 $pp60^{c-src}$ 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 $pp60^{c-src}$ 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, ^{32}P-labeled avian $pp60^{c-src}$ 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 ^{32}P from $pp60^{c-src}$ in these assays. The results of other experiments using the same ^{32}P-labeled $pp60^{c-src}$ substrate where the protein concentration of the CCL 239 and HT29 lysates was varied also demonstrated that the maximum detectable
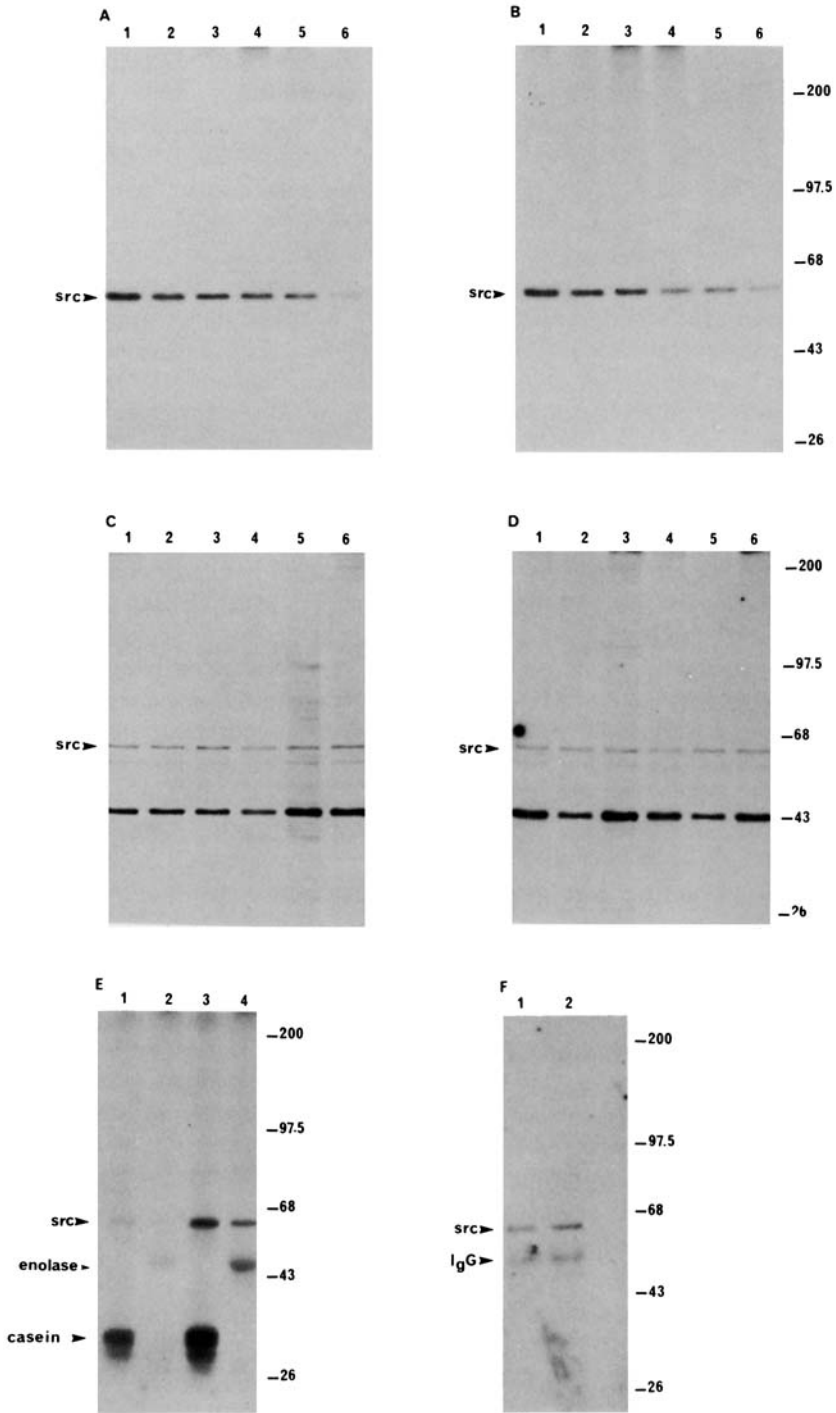
Fig. 5. Phosphotyrosyl phosphatase activity in HT29 cell lysates. (A) Equal amounts of in vitro phosphorylated avian pp60$^{c\text{-}src}$ in EC10 immune-complexes were incubated for 0 (lane 1), 5 (lane 2), 10 (lane 3), 15 (lane 4), 20 (lane 5), or 30 (lane 6) min with 150 μg of HT29 MNNE cell lysate prior to analysis on SDS gels. (B) Equal amounts of in vitro phosphorylated avian pp60$^{c\text{-}src}$ 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 pp60[c-src] molecules autophosphorylate tyrosine residue 416 in immune-complex kinase reactions [25,26]. Thus, it is conceivable that phosphotyrosyl phosphatases 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 (pMcsrc/cos)A cells were metabolically labeled with ^{32}P orthophosphate for 4 hr; the ^{32}P-labeled avian pp60[c-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 ^{32}P-labeled avian pp60[c-src] molecules
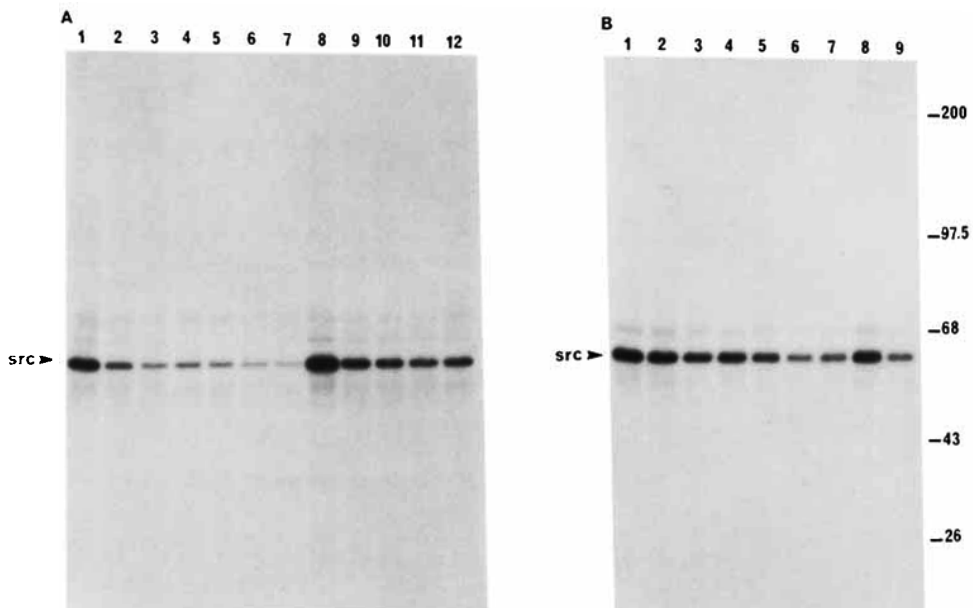
Fig. 6. Effect of vanadate on phosphotyrosyl phosphatase activity in HT29 cell lysates. (A) Equal amounts of in vitro phosphorylated avian pp60[c-src] in EC10 immune-complexes were incubated with 200 μg of MNNE HT29 lysate for 0 (lanes 1, 8), 5 (lanes 2, 9), 10 (lanes 3, 10), 15 (lane 4), 20 (lanes 5, 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 pp60[c-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 phosphothreonine (lane 6), 500 μg/ml phosphotyrosine (lane 7), 10 μM ZnCl (lane 8), or 500 μg/ml phosphoserine (lane 9). The positions of pp60[c-src] (src) and prestained molecular mass markers are indicated.

^{35}S-methionine-labeled avian pp60[c-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 pp60[c-src] in EC10 immune-complexes were incubated in the absence (lanes 1, 2) or presence (lanes 3, 4) of 300 μg HT29 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 pp60[c-src] in EC10 immune-complexes prior to (lane 1) or following (lane 2) incubation with 300 μg HT29 MNNE lysate for 15 minutes.
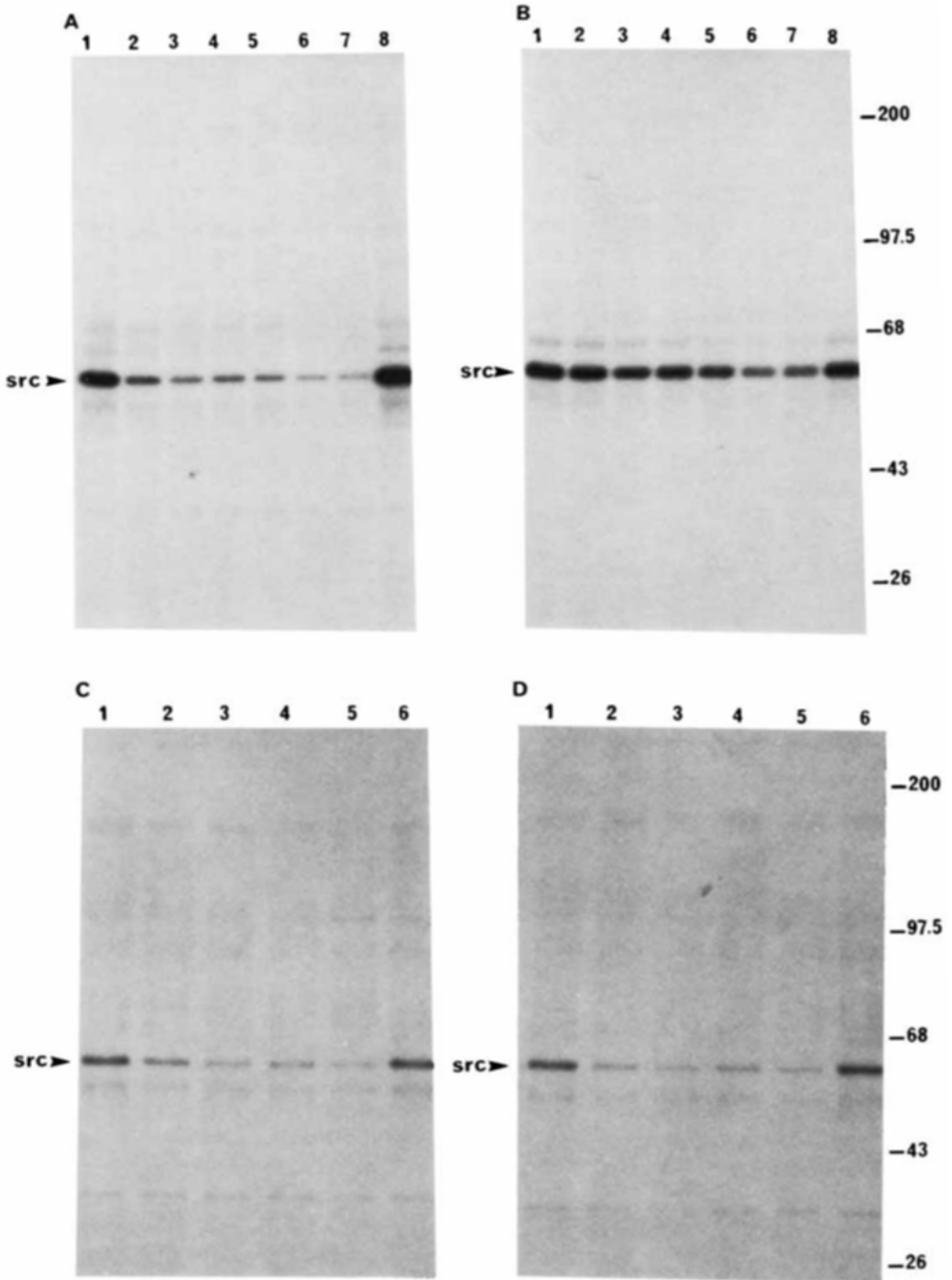
Fig. 7. Comparison of phosphatase activity in lysates of normal colon mucosal cells and colon carcinoma cells. Equal amounts of in vitro phosphorylated (**A, B**) or in vivo phosphorylated (**C,D**) avian pp60$^{c\text{-src}}$ in EC10 immune-complexes were incubated with 200 μg of HT29 (A, C) or CCL 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$^{c\text{-src}}$ (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
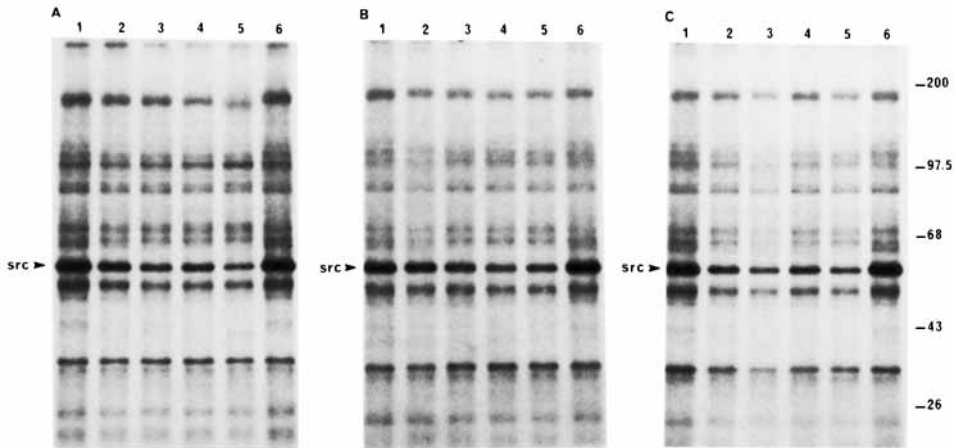

Fig. 8. Comparison of phosphatase activity in lysates of normal colon mucosal cells and colon carcinoma cells using soluble pp60^{c-src} substrate. Equal amounts (50 μg) of ^{32}P-orthophosphate-labeled (pMcsrc/cos)A NIH 3T3 MNNE cell lysates were incubated for 20 min with 0 (lane 1), 50 (lane 2), 100 (lane 3), 200 (lane 4), or 300 μg (lanes 5, 6) of MNNE lysate from CCL 239 (A), HT29 (B), or WiDr cells (C) in the presence (lane 6) or absence (all other lanes) of 100 μM vanadate prior to addition of 100 μM vanadate to all reactions and immunoprecipitation of avian pp60^{c-src} with EC10 antibody. The positions of pp60^{c-src} (src) and prestained molecular mass markers are shown.

protein kinase assays 50- to 60-fold over that of $pp60^{c-src}$ derived from normal mucosal cells [15]. Comparison of the relative abundance of $pp60^{c-src}$ in these cells by immunoblot analysis suggests that the apparent specific activity of $pp60^{c-src}$ from the colon tumor cell lines is ten- to 30-fold higher than $pp60^{c-src}$ from normal colon mucosal cells [14]. The elevated activity of $pp60^{c-src}$ 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 $pp60^{c-src}$ 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 $pp60^{c-src}$ tyrosine residues 416 (Figs. 5–7) and tyrosine 527 (Fig. 7) in immune-complexes and hydrolysis of $pp60^{c-src}$ tyrosine residue 527 in cellular lysates (Fig. 8). Similar conclusions were reached when mammalian $pp60^{c-src}$ 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 $pp60^{c-src}$/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 $pp60^{c-src}$-specific substrates in these cells might be limited in number. In addition, it is possible that potential protein substrates of $pp60^{c-src}$ 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 $pp60^{c-src}$ 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 $pp60^{c-src}$ could be demonstrated.

However, the presence of vanadate in the culture medium was found to significantly stimulate $pp60^{c-src}$ in vitro kinase activity from normal colon mucosal cells while having no detectable effect on the activity of $pp60^{c-src}$ derived from colon carcinoma cells (Fig. 3). Analysis of ^{32}P orthophosphate-labeled $pp60^{c-src}$ 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 ^{32}P pulse-chase analysis with $pp60^{c-src}$ 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 pp60^{c-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 pp60^{c-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 pp60^{c-src} kinase activity as a function of lysis buffer conditions was limited to the colon carcinoma cells. Since the same amount of pp60^{c-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 pp60^{c-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 pp60^{c-src} kinase activity following addition of ATP and Mg^{2+} to the MNNE buffer in the tumor cell lysates is also unclear. With RIPA buffer lysates, the difference in specific activty of pp60^{c-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 pp60^{c-src} between normal colon and colon carcinoma cells would be less than three- to fourfold. These observations suggest that comparing of the results of pp60^{c-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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