pp60$^{c\text{-}src}$ Activation in Human Colon Carcinoma

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Abstract

We measured the in vitro protein-tyrosine kinase activity of pp60$^{c\text{-}src}$ from human colon carcinoma cell lines and tumors. The activity of pp60$^{c\text{-}src}$ from six of nine carcinoma cell lines was higher (on average, fivefold as measured by enolase phosphorylation, or eightfold as measured by autophosphorylation) than that of pp60$^{c\text{-}src}$ from normal colonic mucosal cells, or human or rodent fibroblasts. Similarly, the activity of pp60$^{c\text{-}src}$ from 13 of 21 primary colon carcinomas was five- or sevenfold higher than that of pp60$^{c\text{-}src}$ from normal colonic mucosa adjacent to the tumor. The increased pp60$^{c\text{-}src}$ activity did not result solely from an increase in the level of pp60$^{c\text{-}src}$ protein, suggesting the specific activity of the pp60$^{c\text{-}src}$ kinase is elevated in the tumor cells. pp60$^{c\text{-}src}$ from colon carcinoma cells and normal colonic mucosal cells was phosphorylated at similar sites. We used immunoblotting with antibodies to phosphotyrosine to identify substrates of protein-tyrosine kinases in colonic cells. Three phosphotyrosine-containing proteins were detected at significantly higher levels in most colon carcinoma cell lines than in normal colonic mucosal cells or human or rat fibroblasts. All colon carcinoma cell lines with elevated pp60$^{c\text{-}src}$ in vitro kinase activity, showed increased phosphorylation of proteins on tyrosine in vivo, suggesting the presence of an activated protein-tyrosine kinase(s).

Introduction

Multiple genetic alterations appear to contribute to the malignant transformation of colonic mucosa. Allelic deletions of chromosome 5, 17, and 18 sequences have been identified in human colon carcinomas and adenomas (1–8), suggesting that tumor suppressor genes may be important regulators of cell growth. In addition, activated protooncogenes have been identified in colonic tumors, and may be involved in molecular events leading to malignant transformation of colonic epithelial cells. Transforming *ras* genes (primarily c-K-*ras*) have been detected by gene transfer experiments in the DNA of ~ 20% of colon carcinomas (9–12). Moreover, using DNA hybridization or RNAse A mismatch cleavage analyses, specific point mutations at position 12 in *ras* have been identified in ~ 40% of colon carcinomas (12, 13) and in five colon carcinoma cell lines (14–18). Several other altered protooncogenes have also been identified in colon carcinomas. For example, amplified, overexpressed and/or rearranged c-*myc* (19–25) or c-*myb* genes (26) have been observed in some colon tumors and cell lines. Recently the *lck* protein-tyrosine kinase gene (previously expressed only in tissues of lymphoid lineage) was shown to be expressed in some human colon carcinoma cell lines (27). However, a consistent or unifying pattern of activation of these altered protooncogenes has not been seen, there is no evidence that they are directly responsible for tumorigenesis, and none is present in the majority of colon carcinomas. Clearly, events in addition to the activation of these cellular genes are important to the genesis of colon carcinoma. Recent observations suggest that activation of another cellular gene, c-*src*, may be one of these events.

The protooncogene c-*src* is the cellular homologue of the Rous sarcoma virus transforming gene, v-*src*. Both c-*src* and v-*src* encode 60-kD, membrane-associated, protein-tyrosine kinases. The transforming ability of the viral protein (pp60$^{v\text{-}src}$) or of mutants of the cellular protein (pp60$^{c\text{-}src}$) is closely correlated with elevated specific activity of the enzyme (for review see references 28 and 29). All transforming mutants of pp60$^{c\text{-}src}$ tested have higher specific activity than normal pp60$^{c\text{-}src}$ (30–37).

Bolen et al. (38) reported that the in vitro protein-tyrosine kinase activity of pp60$^{c\text{-}src}$ is elevated in many human tumors including colon carcinomas, breast adenocarcinomas (39), rhabdomyosarcomas, and neuroblastomas (40). The most consistent and striking elevation in pp60$^{c\text{-}src}$ activity was observed in colon carcinoma cell lines and tumors.

We also measured the in vitro protein kinase activity of pp60$^{c\text{-}src}$ from human colon carcinoma cell lines and tumors. Our results showed that pp60$^{c\text{-}src}$ from many colon carcinoma cell lines and tumors had elevated activity. However, we observed a less dramatic increase in activity in carcinoma cell lines than has been reported, and no increase in one-third of the colon carcinoma cell lines and tumors analyzed. In contrast, Bolen et al. reported enhanced pp60$^{c\text{-}src}$ activity in all colon carcinoma cell lines and tumors tested (38). We extended these in vitro studies by analyzing proteins phosphorylated on tyrosine in vivo in colonic cells. All colon carcinoma cell lines with elevated pp60$^{c\text{-}src}$ in vitro kinase activity showed higher levels of proteins phosphorylated on tyrosine in vivo than did normal colonic mucosal cells.

Methods

Cell culture and tissues. The human colon carcinoma tumor cell lines COLO 205, COLO 201, LS 174T, LS 180, WiDr, HT-29, T84, SW 480, and HCT-8, and the normal human colonic mucosal cells, CCl 239, were obtained from the American Type Culture Collection (Rockville, MD). Tissue samples were obtained from surgical specimens collected at the University of Pittsburgh Medical Center and were frozen on dry ice within 20 min of resection.

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Protein extractions, immunoprecipitations, and in vitro kinase assays. 1.2×10^6 cells were seeded onto a 9-cm dish 12 h before lysis. Cells were washed three times in ice-cold Tris-buffered saline and lysed in a modified RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM sodium phosphate [pH 7.0], 1% aprotinin, 50 μM leupeptin, 1 mM dithiothreitol). Tissues were homogenized in Kontes glass tissue grinders in modified RIPA buffer. Lysates were clarified at 29,000 *g* for 1 h at 4°C. Protein concentrations was measured in tissue lysates by the BCA protein assay (Pierce Chemical Co., Rockford, IL) and tissue samples were standardized such that each contained 70 μg protein. Lysates were incubated with 10 μl of ascites fluid (1:500 dilution) containing a monoclonal antibody (MAb 327) specific for $pp60^{c\text{-}src}$ (41), and 8 μl of affinity-purified rabbit anti-mouse immunoglobulin G (Cooper Biomedical, Inc., Malvern, PA) as described (42–44). Under these conditions antibody was in excess of antigen. Immunoprecipitates were divided into two equal parts, one of which was used for immunoblot analysis (see below), the other of which was incubated for 20 min at 30°C in 30 μl of buffer containing 50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 7.0), 10 mM $MnCl_2$, 10 mM dithiothreitol, 1 μg of acid-denatured rabbit muscle enolase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) (45) and 40 μCi of [γ^{32}P]ATP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) (46). Proteins were resolved on 7% SDS-polyacrylamide gels (acrylamide-bisacrylamide, 39:1) and gels were stained with Coomassie brilliant blue G-250 (Bio-Rad Laboratories, Richmond, CA) to confirm that equivalent amounts of enolase were present in each lane. Radiolabeled proteins were detected with presensitized Kodak XAR film by direct autoradiography, or with an intensifying screen at −70°C. ^{32}P incorporation into proteins was quantified by Cerenkov counting of excised gel pieces, and by liquid scintillation counting after gel pieces were dissolved in 1.2 ml of 30% H_2O_2-70% perchloric acid (2:1) at 55°C for 12 h. Both methods gave similar results. $pp60^{c\text{-}src}$ in vitro protein kinase activity was linearly related to the concentration of cell protein.

Immunoblot analysis of $pp60^{c\text{-}src}$. Immunoprecipitates were divided into two equal parts, one of which was used for the in vitro protein kinase assay (see above), the other of which was directly resolved on a 7% polyacrylamide gel (acrylamide-bisacrylamide, 39:1). Proteins were transferred to nitrocellulose (0.45 μm; Schleicher & Schuell, Keene, NH) in transfer buffer (12.5 mM Tris-HCl [pH 8.3], 100 mM glycine and 20% methanol) using a Trans-Blot apparatus (Bio-Rad) for 4 h at 40 V (39, 47). Protein binding sites on the nitrocellulose were blocked by incubating the filters overnight in TNE buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 2.5 mM EDTA and 0.01% NaN_3) containing 0.1% (vol/vol) Tween 20 (Sigma Chemical Co., St. Louis, MO) and 5% nonfat, powdered milk (blocking buffer). The filters were incubated in normal sheep serum (Cooper Biomedical Corp.) (1:10 dilution in blocking buffer) for 2 h, before adding purified MAb 327 ascites (1:250 dilution) and incubating for 1 h, at room temperature (RT).[1] Under these conditions, antibody was shown to be in excess of antigen. The filters were washed in TNE buffer with changes every 10 min for 1 h before incubating with ^{125}I-labeled, affinity-purified, sheep anti-mouse immunoglobulin (New England Nuclear, Boston, MA) (1×10^6 cpm/ml blocking buffer) for 1 h, RT. The filters were washed in TNE buffer with changes every 10 min for 1 h. ^{125}I-labeled proteins were detected with presensitized Kodak XAR film by direct autoradiography, or with an intensifying screen at −70°C. The amount of $pp60^{c\text{-}src}$ detected in lysates was shown to be linear over a fivefold range of cell protein.

Radiolabeling of cells. 2×10^6 cells were seeded onto a 9 cm dish 24 h before labeling. Cells were washed three times in phosphate-free Dulbecco-Vogt modified Eagle medium (DME) and incubated for 18 h at 37°C in phosphate-free DME containing 10% dialyzed, phosphate-free, fetal bovine serum and 2.5 mCi/ml [^{32}P]orthophosphate (carrier free; ICN Pharmaceuticals, Inc.).

Two-dimensional tryptic phosphopeptide mapping. $pp60^{c\text{-}src}$ from 2×10^6 cells was resolved on a 7% SDS-polyacrylamide gel, extracted, oxidized, and digested with trypsin, as described (48). Peptides were separated in two dimensions on 100-μm cellulose thin-layer plates by electrophoresis (pH 1.9; 1 kV; 25 min) and chromatography (*n*-butanol/pyridine/acetic acid/H_2O, 75:50:15:60 [by volume]).

Immunoblotting with antibodies to phosphotyrosine (49). 10^7 cells growing on 9-cm dishes were washed twice with 10 ml of 50 mM Tris, pH 7.4; 140 mM NaCl; 3.3 mM KCl. 1 ml of protein sample buffer (5 mM sodium phosphate, pH 6.8; 2% SDS; 0.1 M DTT; 5% 2-mercaptoethanol; 10% glycerol; 0.4% bromophenol blue) at 100°C was added. The plate was swirled at an angle of 45° until the viscous cell lysate was completely free from the surface of the dish. The sample was boiled for 5 min, sheared 10 times through a 22-gauge needle, 10 times through a 27-gauge needle, and stored at −70°C before use. Immunoblotting was performed as described (50). The relative concentration of proteins in each sample was measured by PAGE in the presence of SDS followed by staining with Coomassie brilliant blue R (Sigma). Equivalent amounts of cellular protein were then fractionated on a second gel and transferred to a nitrocellulose filter by electrophoresis at 40 V for 1.5 h in transfer buffer (57.8 g glycine; 12.0 g Tris; 3.0 g SDS; 0.37 g sodium orthovanadate (Fisher Scientific Co., Pittsburgh, PA); 800 ml methanol; water to 4 liters). The filter was incubated for 16 h in blocking buffer (5% BSA [essentially fatty acid and immunoglobulin free; Sigma] and 1% ovalbumin dissolved in rinsing buffer [10 mM Tris, pH 7.2; 0.9% NaCl; 0.01% NaN_3]). The filters were incubated for 2 h at RT with affinity-purified polyclonal rabbit antibodies to phosphotyrosine (49) at a concentration of 2 μg/ml in blocking buffer and then rinsed twice for 10 min in rinsing buffer, once for 10 min in rinsing buffer supplemented with 0.05% NP-40, and twice more in rinsing buffer alone. Rabbit immunoglobulins were detected by incubating the filters with 20 μCi ^{125}I-protein A (100 μCi/μg) in 40 ml of blocking buffer for 30 min at RT, followed by two 10-min washes in rinsing buffer, one 10-min wash in rinsing buffer containing 0.05% NP-40, and concluding with two 10-min washes in rinsing buffer alone. These conditions produced the highest levels of antibody binding and the lowest levels of nonspecific binding of either antibody or protein A. Protein markers were myosin (200 kD), the upper band of a doublet representing phosphorylase b (calculated at 97 kD), BSA (69 kD), ovalbumin (46 kD), and carbonic anhydrase (30 kD).

Results

$pp60^{c\text{-}src}$ from colon carcinoma cell lines and tumors has elevated in vitro protein kinase activity. We measured the in vitro protein kinase activity of $pp60^{c\text{-}src}$ from nine human colon carcinoma cell lines (COLO 205, COLO 201, LS 174T, LS 180, WiDr, HT-29, T84, SW 480, and HCT-8) and compared it to the activity of $pp60^{c\text{-}src}$ from a normal human colonic mucosal cell line CCl 239 (HCMC), and a human (FLOW 2000) and rat (208F) fibroblast cell line. MAb 327 immunoprecipitates were divided into two equal parts, one of which was used for immunoblot analysis (see below), the other of which was incubated with [γ-^{32}P]ATP and the exogenous substrate enolase, and analyzed on an SDS-polyacrylamide gel (Fig. 1 *a*). The total $pp60^{c\text{-}src}$ protein kinase activity in normal colonic mucosal cells (HCMC), as measured either by autophosphorylation or phosphorylation of enolase, was similar to the activity in normal human (FLOW 2000) or rat (208F) fibroblasts. The $pp60^{c\text{-}src}$ activity in the different colon cell lines varied, and was generally higher than the activity in the control cell lines. Fig. 1 *b* shows the total protein kinase activity of $pp60^{c\text{-}src}$ in the tumor cell lines, relative to the activity of $pp60^{c\text{-}src}$ in the normal colonic mucosal cell line, HCMC. The $pp60^{c\text{-}src}$ activity in six tumor cell lines (COLO 205, COLO

1. *Abbreviations used in this paper:* HCMC, human colonic mucosal cell; M, mucosa; RT, room temperature; T, tumor.

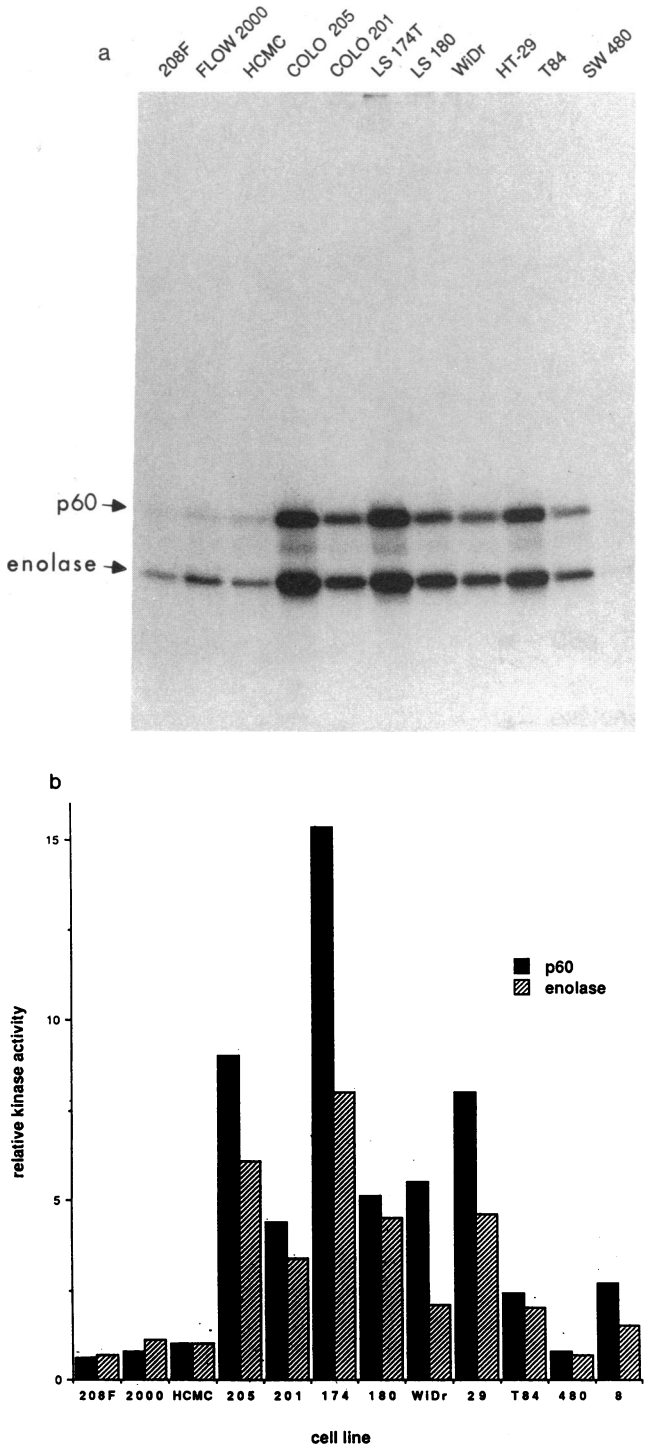

Figure 1. In vitro protein kinase activity of pp60$^{c\text{-}src}$ from colon carcinoma cell lines. Cells were seeded at a density of 1.2×10^6/9 cm dish and lysed 12 h later in RIPA buffer. Proteins were precipitated from cell lysates with excess MAb 327. Immunoprecipitates were divided into two equal parts, one of which was used for immunoblot analysis (Fig. 2), the other of which was incubated with [γ-^{32}P]ATP and enolase, and resolved on a 7% SDS-polyacrylamide gel (Fig. 1a). (*a*) A representative autoradiograph: lane 1, rat fibroblasts (208F); lane 2, human fibroblasts (FLOW 2000); lane 3, normal human colonic mucosal cells (HCMC); lanes 4–11, colon carcinoma cell lines. Exposure time, at −70°C with an intensifying screen, was 1 h. (*b*) Quantitation of the activity of pp60$^{c\text{-}src}$ from colon carcinoma cell lines relative to that of pp60$^{c\text{-}src}$ from normal colonic mucosal cells.

201, LS 174T, LS 180, WiDr, and HT-29) was significantly higher (4–15-fold higher as measured by autophosphorylation, or 2–8-fold higher as measured by enolase phosphorylation) than the activity in normal colonic mucosal cells (HCMC).

To determine whether the differences observed in pp60$^{c\text{-}src}$ kinase activity were due to differences in amount of pp60$^{c\text{-}src}$ protein and/or to changes in the specific activity of the enzyme, pp60$^{c\text{-}src}$ protein levels were measured by immunoblot analysis (Fig. 2). Two proteins were detected in each lane. The upper protein is pp60$^{c\text{-}src}$, the lower is the MAb 327 heavy chain (47). The level of immunoprecipitable pp60$^{c\text{-}src}$ in normal colonic mucosal cells (HCMC) was similar to the level in human (FLOW 2000) or rat (208F) fibroblasts. The amount of pp60$^{c\text{-}src}$ protein in the colon carcinoma cell lines varied, but was generally higher than the level present in the control cell lines. ^{125}I-labeled proteins from two to three independent experiments were quantified by counting excised bands. The amount of pp60$^{c\text{-}src}$ protein in the tumor cell lines ranged from 0.6 to 2.6 times the amount in normal HCMC. The specific activity of pp60$^{c\text{-}src}$ was estimated by dividing the total protein kinase activity by the amount of pp60$^{c\text{-}src}$ protein. The specific activity of pp60$^{c\text{-}src}$ from four tumor cell lines (COLO 205, LS 174T, LS 180, and HT-29) was higher (four- to sixfold higher as measured by autophosphorylation, or two- to fourfold higher as measured by enolase phosphorylation) than the specific activity of pp60$^{c\text{-}src}$ from the normal colonic mucosal cells. Thus, the increase in total pp60$^{c\text{-}src}$ kinase activity observed in the colon carcinoma cell lines appears to be due both to an increase in the amount of pp60$^{c\text{-}src}$ protein and to an increase in the specific activity of the kinase.

We also measured the pp60$^{c\text{-}src}$ protein kinase activity in primary colon carcinomas and adjacent normal mucosa from 21 patients (Fig. 3). Paired tissue samples of tumor (T) and normal adjacent mucosa (M) from the same patient are shown. The position of pp60$^{c\text{-}src}$ or enolase is indicated with an arrow. Three additional proteins of ~ 50, 65, and 80 kD were detected in some lanes. The 50-kD protein is probably rabbit anti-mouse IgG heavy chain. Longer film exposures show that the 65-kD protein was present in all lanes and was more heavily phosphorylated in tumor tissues than in normal mucosa. The 80-kD protein was variably present in both tumor and normal tissue. The total pp60$^{c\text{-}src}$ protein kinase activity, as measured either by autophosphorylation or enolase phosphorylation, varied somewhat between normal mucosal samples. In 13 of 21 patients studied the pp60$^{c\text{-}src}$ activity in the carcinoma was significantly higher (3–16-fold higher as measured by autophosphorylation, or 2–14-fold higher as measured by enolase phosphorylation) than the activity in the normal mucosa adjacent to the tumor.

pp60$^{c\text{-}src}$ protein levels in tissue samples (Fig. 4) were determined by immunoblotting as described above. ^{125}I-labeled proteins from duplicate experiments were quantified by

^{32}P incorporation into pp60$^{c\text{-}src}$ or enolase was quantified by liquid scintillation counting of excised gel pieces. Values represent the mean of 2–3 experiments. Actual values varied < 40% from the mean. Lane *1*, rat fibroblasts (208F); lane *2*, human fibroblast FLOW 2000 (2000); lane *3*, human colonic mucosal cells (HCMC); lane *4*, COLO 205 (205); lane *5*, COLO 201 (201); lane *6*, LS 174T (174); lane *7*, LS 180 (180); lane *8*, WiDr; lane *9*, HT-29 (29); lane *10*, T84; lane *11*, SW 480 (480); lane *12*, HCT-8 (8).

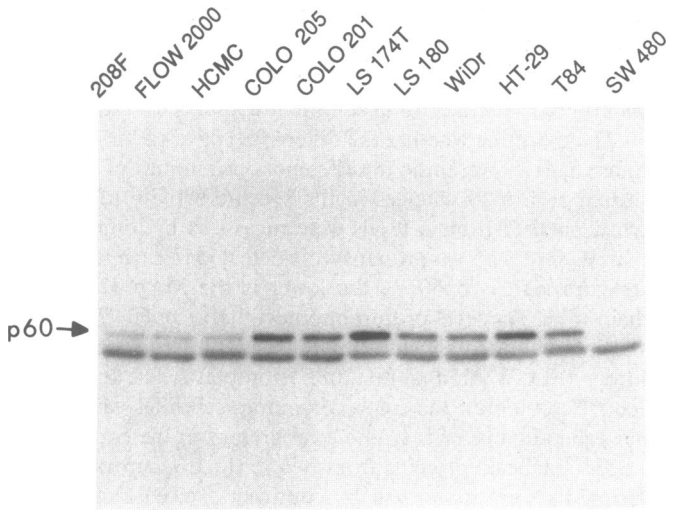

Figure 2. Immunoblot analysis of pp60$^{c\text{-}src}$ from colon carcinoma cell lines. Proteins were precipitated as described in the legend to Fig. 1. Immunoprecipitates were divided into two equal parts, one of which was used for the in vitro protein kinase assay shown in Fig. 1 *a*, the other of which was separated on an SDS gel and transferred to nitrocellulose. The filters were incubated with excess MAb 327 and ^{125}I-sheep anti-mouse immunoglobulin, and ^{125}I-labeled proteins were detected by autoradiography. The lower band is MAb 327 heavy chain. Exposure time, at −70°C with an intensifying screen, was 4 h.

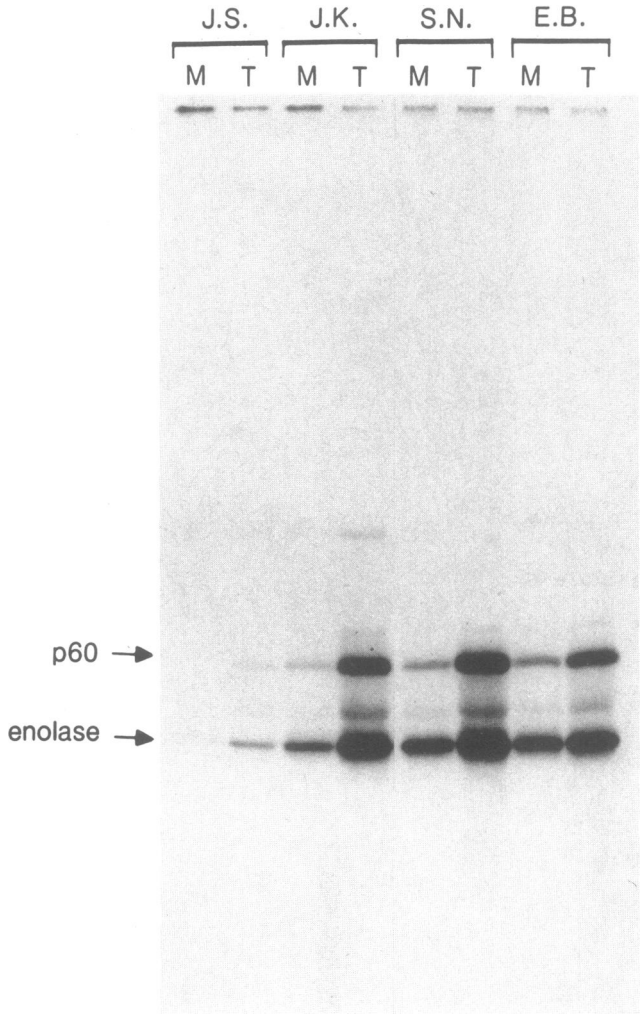

Figure 3. In vitro protein kinase activity of pp60$^{c\text{-}src}$ from colonic tumors. Tissues were homogenized in RIPA buffer and lysates were standardized such that each contained 70 μg protein. Proteins were immunoprecipitated, phosphorylated, and resolved on an SDS gel as described in the legend to Fig. 1. Paired samples of colonic tumor (T) and normal colonic mucosa (M) from the same patient are shown. Exposure time, at −70°C with an intensifying screen, was 45 min.

counting excised bands. The amount of pp60$^{c\text{-}src}$ protein varied less than 40% between different normal mucosal samples. The amount of pp60$^{c\text{-}src}$ protein in each tumor sample ranged from 0.5 to 2.8 times the amount of protein in the normal adjacent mucosa. The estimated specific activity of pp60$^{c\text{-}src}$ from 9 colon carcinomas was significantly higher (2–11-fold higher as measured either by autophosphorylation or enolase phosphorylation) than the specific activity of pp60$^{c\text{-}src}$ from normal mucosa adjacent to the tumor.

In summary, the total in vitro protein kinase activity of pp60$^{c\text{-}src}$ from six of nine colon carcinoma cell lines studied was significantly higher (on average eightfold as measured by autophosphorylation, or fivefold as measured by enolase phosphorylation) than that of pp60$^{c\text{-}src}$ from normal colonic mucosal cells, or human or rodent fibroblasts. Similarly, the total kinase activity of pp60$^{c\text{-}src}$ from 13 of 21 primary colon carcinomas was significantly higher (on average sevenfold higher as measured by autophosphorylation, or fivefold as measured by enolase phosphorylation) than that of pp60$^{c\text{-}src}$ from normal mucosa adjacent to the tumor. The increase in pp60$^{c\text{-}src}$ activity appeared to be due both to an increase in the amount of pp60$^{c\text{-}src}$ protein and to an increase in the specific activity of the kinase. Overall, we observed elevated pp60$^{c\text{-}src}$ protein kinase activity in two-thirds of colon carcinoma cell lines and tumors tested.

pp60$^{c\text{-}src}$ from colon carcinoma cells and normal colonic mucosal cells is phosphorylated at similar sites. Phosphorylation of pp60$^{c\text{-}src}$, and particularly phosphorylation of tyrosine 527, are known to regulate pp60$^{c\text{-}src}$ protein kinase activity (30, 33–37, 44, 51, 52). To determine whether altered sites of phosphorylation contribute to the activation of pp60$^{c\text{-}src}$ in colon carcinoma cell lines we compared the phosphorylation sites of pp60$^{c\text{-}src}$ from the tumor cells with those of pp60$^{c\text{-}src}$ from the normal colonic mucosal cells by two-dimensional tryptic phosphopeptide mapping. For this purpose, cultures were incubated 18 h with [^{32}P]orthophosphate and ^{32}P-labeled proteins were immunoprecipitated from lysates with MAb 327 and analyzed on an SDS-polyacrylamide gel (Fig. 5). pp60$^{c\text{-}src}$ from the colon carcinoma cell lines COLO 205, LS 174T, and HT-29 had the same mobility as pp60$^{c\text{-}src}$ from normal HCMC and as pp60$^{c\text{-}src}$ from human fibroblasts (FLOW 2000). Tryptic digestion of ^{32}P-labeled pp60$^{c\text{-}src}$ from human fibroblasts (FLOW 2000) or normal HCMC generated six major phosphopeptides (Fig. 6). Peptides 1 and 2 are phosphorylated on Tyr 527, peptide 4 on Ser 17, peptide 6 on Ser 12, and peptides 3 and 5 on amino-terminal serines (for review see reference 29). The tryptic phosphopeptides of pp60$^{c\text{-}src}$ from three colon carcinoma cell lines, COLO 205, LS 174T, and HT-29 were similar to the peptides of pp60$^{c\text{-}src}$ from HCMC and FLOW 2000 cells. There was no obvious decrease in phosphorylation of Tyr 527 with respect to Ser 17, nor an increase in Tyr 416

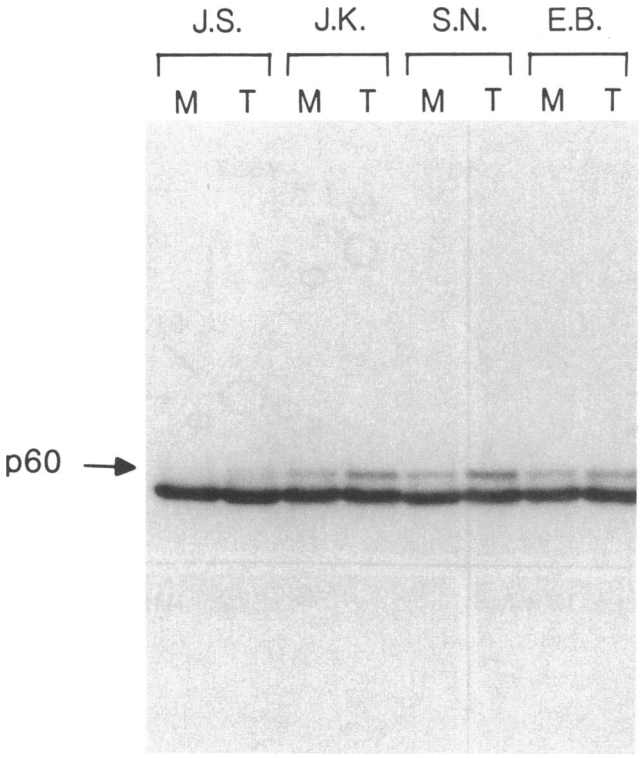

Figure 4. Immunoblot analysis of pp60$^{c\text{-}src}$ from colon carcinomas. Immunoprecipitates were divided into two equal parts, one of which was used for the in vitro protein kinase assay shown in Fig. 3, the other for immunoblotting as described in the legend to Fig. 2. Exposure time was 25 h.

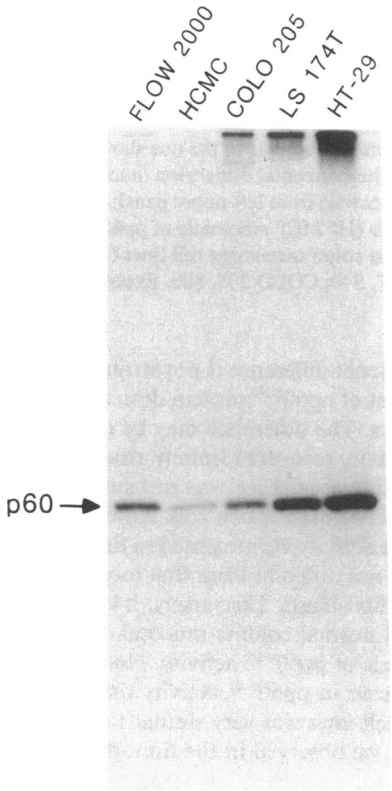

Figure 5. Phosphorylation of pp60$^{c\text{-}src}$ from colon carcinoma cell lines. Cultures were incubated 18 h with [^{32}P]-orthophosphate. ^{32}P-labeled proteins were immunoprecipitated from lysates with MAb 327 and analyzed on a 7% SDS-polyacrylamide gel. Lane *1*, human fibroblasts (FLOW 2000); lane *2*, normal human colonic mucosal cells (HCMC); lanes *3–6*, colon carcinoma cell lines. Exposure time, at −70°C with an intensifying screen, 1 h.

phosphorylation. Decreased phosphorylation of Ser 12 was noted on pp60$^{c\text{-}src}$ from LS 174T and HT-29 cells, but not significantly so on pp60$^{c\text{-}src}$ from COLO 205 cells. Because we frequently observe variation in Ser 12 phosphorylation from one experiment to another, we are uncertain about the significance of the difference observed here.

Colon carcinoma cell lines with elevated pp60$^{c\text{-}src}$ in vitro protein kinase activity show increased phosphorylation of proteins on tyrosine in vivo. To identify substrates of protein-tyrosine kinases in colonic cells we used immunoblotting with antibodies to phosphotyrosine. We compared the proteins phosphorylated on tyrosine in colon carcinoma cells with those in normal cells (Fig. 7). Three phosphotyrosine-containing proteins of ~ 145, 125, and 57 kD were prominent in eight colon carcinoma cell lines (COLO 205, COLO 201, LS 174T, LS 180, WiDr, HT-29, T84, and COLO 396). These three phosphotyrosine-containing proteins were detected at significantly lower levels in the normal HCMC, normal human mammary cells (HBL-100), normal human (FLOW 2000) or rat (208F) fibroblasts, and in three colon carcinoma cell lines (SW 480, HCT-15, and COLO 320). We measured the in vitro protein kinase activity of pp60$^{c\text{-}src}$ from SW 480 cells and observed the activity to be similar to that of pp60$^{c\text{-}src}$ from normal colonic mucosal cells (Fig. 1). All colon carcinoma cell lines with elevated pp60$^{c\text{-}src}$ protein kinase activity showed increased tyrosine phosphorylation of the 145-, 125-, and 57-kD proteins. The 57-kD protein is possibly pp60$^{c\text{-}src}$. An additional phosphotyrosine-containing protein of ~ 55 kD was detected in the colon carcinoma cell lines COLO 201, and 205, and may be p56^{lck} (27). To compare substrates of tyrosine kinases in colon carcinoma cells with substrates of tyrosine kinases in other transformed cells, we analyzed NIH 3T3 cells transformed with DNA from rat neuroblastomas (B104-1-1) (53) for proteins phosphorylated on tyrosine. B104-1-1 cells had increased tyrosine phosphorylation on the 57 but not the 145- or 125-kD proteins. Of note was a phosphotyrosine-containing protein of ~ 110 kD that was present at higher levels in normal cell lines than in most colon carcinoma cell lines.

Discussion

In summary, two-thirds of human colon carcinoma cell lines or tumors contain pp60$^{c\text{-}src}$ with elevated in vitro protein-tyrosine kinase activity. The enhanced activity appears to be due both to an increase in the level of pp60$^{c\text{-}src}$ protein and to an increase in the specific activity of the pp60$^{c\text{-}src}$ kinase. Altered phosphorylation sites on pp60$^{c\text{-}src}$ do not appear to contribute to its activation. Colon carcinoma cell lines with elevated pp60$^{c\text{-}src}$ kinase activity as measured in vitro show increased phosphorylation of proteins on tyrosine in vivo.

Our results are similar to those of Bolen et al. (38) in several ways. First, of seven carcinoma cell lines analyzed in both studies, those with relatively high activity measured in one study had relatively high activity measured in the other (COLO 205, COLO 201, LS 174T, LS 180, WiDr, and HT-29). Conversely, the cell line with relatively low activity measured in one study had relatively low activity measured in the other (SW 480). Second, although the tumors analyzed in the two studies were all different, quantitation of total activity, amount of pp60$^{c\text{-}src}$ protein, and estimation of specific activity of the pp60$^{c\text{-}src}$ kinase in the tumors compared with normal mucosa, were very similar in both studies. We found a five- to

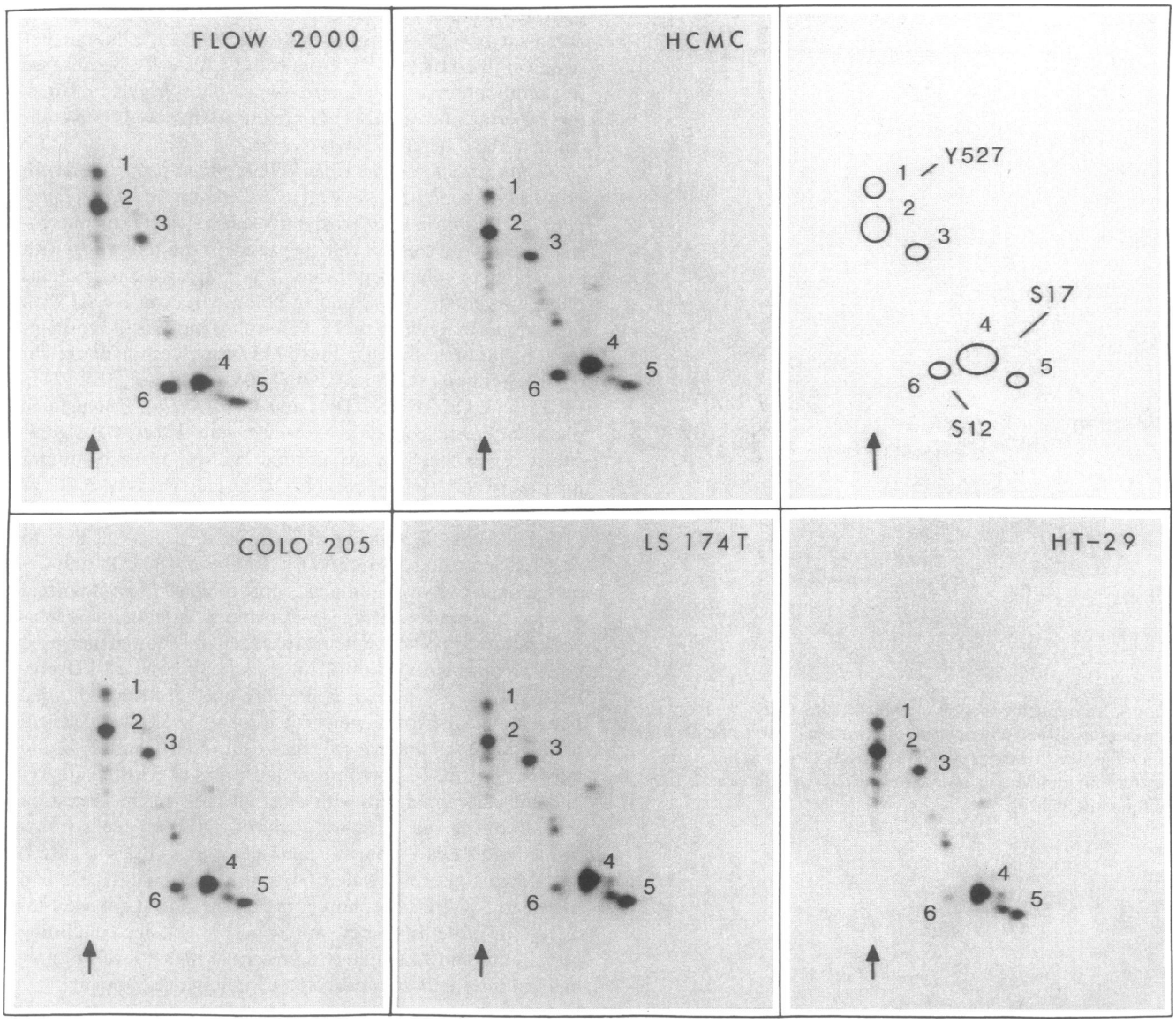

Figure 6. Tryptic digestion of pp60$^{c\text{-}src}$ from colon carcinoma cell lines. ^{32}P-labeled proteins extracted from gels similar to the one shown in Fig. 5 were oxidized, digested with trypsin, and resolved on thin layer plates by electrophoresis at pH 1.9 in the horizontal dimension (anode at the left) and by chromatography in the vertical dimension. The origin is indicated with a vertical arrow. Clockwise from left upper panel: tryptic phosphopeptides of pp60$^{c\text{-}src}$ from human fibroblasts (FLOW 2000), normal human colonic mucosal cells (HCMC), schematic of pp60$^{c\text{-}src}$ tryptic phosphopeptides and their phosphoamino acid composition, tryptic phosphopeptides of pp60$^{c\text{-}src}$ from colon carcinoma cell lines (HT-29, LS 174T and COLO 205). Cerenkov cpm loaded: FLOW 2000, 820; HCMC, 820; HT-29, 820; LS 174T, 840; COLO 205, 800. Exposure times, at −70°C with intensifying screens, 2 d.

sevenfold increase in total pp60$^{c\text{-}src}$ activity and a small increase in pp60$^{c\text{-}src}$ protein levels in tumors compared with normal mucosa, and they observed a five- to eightfold increase in total activity and no increase in amount of protein. Third, both groups showed that pp60$^{c\text{-}src}$ from colon carcinoma cells and normal colonic mucosal cells is phosphorylated at the same sites (54).

Our results differ from those of Bolen and co-workers in two ways. One, we observed elevated pp60$^{c\text{-}src}$ activity in only two-thirds of carcinoma cell lines and tumors analyzed, whereas they reported enhanced pp60$^{c\text{-}src}$ activity in all colon carcinoma cell lines and tumors tested. Two, we measured a five- to eightfold elevation of total pp60$^{c\text{-}src}$ activity in colon carcinoma cell lines, whereas they reported a 120-fold elevation in activity. This significant difference is not attributable to small differences in amount of pp60$^{c\text{-}src}$ protein detected in the cell lines in the two studies. The difference may be explained by the level of pp60$^{c\text{-}src}$ activity recovered from normal colonic mucosal cells. It is possible that there was unusually high pp60$^{c\text{-}src}$ activity in the cells we used as controls. However, this seems unlikely because the activity we measured in the normal colonic mucosal cells was not different from that measured in normal human or rodent fibroblasts. Conversely, it is possible that the other group used normal colonic mucosal cells containing unusually low levels of pp60$^{c\text{-}src}$ activity. Nonetheless, the five- to eightfold increase in pp60$^{c\text{-}src}$ activity that we observed in the carcinoma cell lines was very similar to the five- to sevenfold increase that we observed in the tumors. In con-

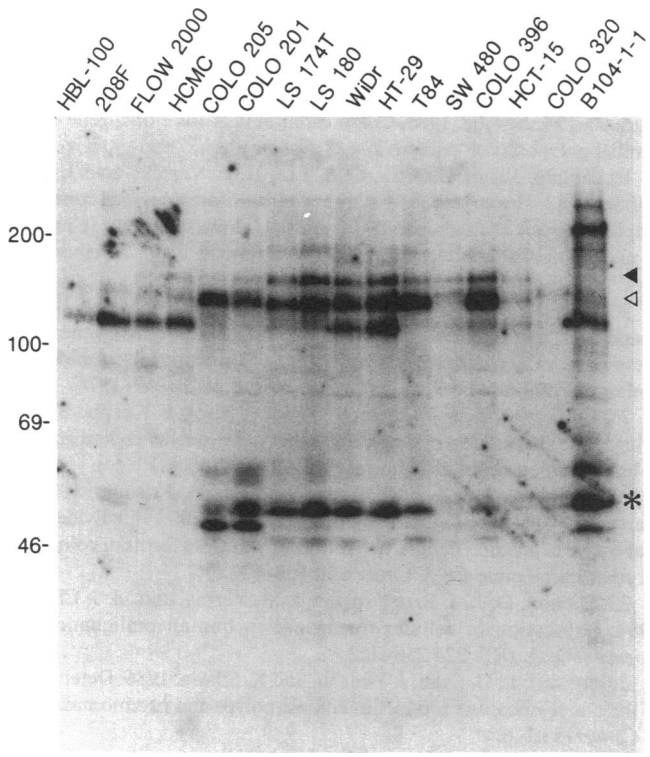

Figure 7. Comparison of proteins phosphorylated on tyrosine in normal colonic mucosal cells and colon carcinoma cells. Equivalent amounts of total cellular protein were separated by gel electrophoresis, transferred to nitrocellulose and incubated with antibodies specific for phosphotyrosine, as described in Methods. Lane *1*, normal human mammary cells (HBL-100); lane *2*, rat fibroblasts (208F); lane *3*, human fibroblasts (FLOW 2000); lane *4*, normal colonic mucosal cells (HCMC); lanes *5–15*, colon carcinoma cell lines; lane *16*, NIH 3T3 cells transformed with DNA from rat neuroblastoma (B104-1-1). (*Left margin*) Protein molecular weight markers. (*Right margin*) (◀) 145 kD protein; (◁) 125 kD protein; * 57 kD protein.

trast, Bolen and co-workers reported a 120-fold increase in pp60$^{c\text{-}src}$ activity in carcinoma cell lines and a 5–8-fold increase in tumors.

Because colon carcinoma cell lines with elevated pp60$^{c\text{-}src}$ in vitro kinase activity show increased phosphorylation of proteins on tyrosine in vivo, it is tempting to suggest that pp60$^{c\text{-}src}$ is an activated tyrosine kinase in colon carcinoma cells. However, if an activated kinase is present, it could be any one, or more than one, of 30 known mammalian protein-tyrosine kinases, including seven to eight pp60$^{c\text{-}src}$-related kinases. Furthermore, the carcinoma cells could contain a reduced level of phosphatase activity in addition to, or instead of an activated protein kinase(s). Of note is the colon carcinoma cell line T84, which has a small increase in pp60$^{c\text{-}src}$ activity, yet levels of proteins phosphorylated on tyrosine that are indistinguishable from those of cell lines with high pp60$^{c\text{-}src}$ activity. It is possible that in T84 cells the in vitro assay for pp60$^{c\text{-}src}$ activity does not accurately reflect its in vivo activity, that the kinase responsible for phosphorylating proteins on tyrosine in vivo is not pp60$^{c\text{-}src}$, that more than one tyrosine kinase is active, and/or that T84 cells contain lower levels of phosphatases than do other colon carcinoma cell lines.

Giordano and co-workers used immunoblotting with antibodies to phosphotyrosine to identify substrates of endogenous tyrosine kinases in human tumor cell lines (55). They studied one colon carcinoma cell line, HT-29, and observed two high molecular weight phosphotyrosine-containing proteins of about 130 and 110 kD. Because molecular weights can only be estimated crudely on gels, it is possible that the 130- and 110-kD proteins they observed are the same proteins as the ~ 125- and 145-kD phosphotyrosine-containing proteins that we detected at high levels in HT-29 cells and most other colon carcinoma cell lines. It will be important to identify substrates of tyrosine kinases in primary colonic tumors and in normal colonic mucosa.

A somewhat different pattern of proteins phosphorylated on tyrosine was observed in NIH 3T3 cells transformed by DNA from rat neuroblastomas (B104-1-1 cells) than in colon carcinoma cells (Fig. 7). Except for a 57-kD protein, the major substrates of tyrosine kinases in B104-1-1 cells appeared to be different than those in most colon carcinoma cell lines. It is possible that different tyrosine kinases or phosphatases are active, and/or that different substrates are present in different types of transformed cells. It is also possible that the tyrosine kinase which phosphorylates the 145- and 125-kD proteins is distinct from that which phosphorylates the 57-kD protein.

An important unanswered question is what mechanism(s) activates pp60$^{c\text{-}src}$ in colon carcinoma cells. Three mechanisms are known to regulate the protein kinase activity of pp60$^{c\text{-}src}$ in other cells: (*a*) phosphorylation, (*b*) association with another protein, or (*c*) mutation.

Lack of phosphorylation of Tyr 527 activates the protein kinase and transforming ability of pp60$^{c\text{-}src}$ (30, 33–37, 44, 51, 52). Using two-dimensional tryptic phosphopeptide mapping we did not detected decreased phosphorylation of this regulatory site on activated pp60$^{c\text{-}src}$ from colon carcinoma cells. However, it is possible that the stoichiometry of pp60$^{c\text{-}src}$ phosphorylation is altered in the colon carcinoma cells and we are not detecting the change. For example, fewer pp60$^{c\text{-}src}$ molecules may be phosphorylated on tyrosine 527, thereby activating the kinase. Examples of activated pp60$^{c\text{-}src}$ kinases that do not have detectably decreased phosphorylation on Tyr 527 are found in primary neurons (56–58), fibroblasts during mitosis (59), and fibroblasts treated with platelet-derived growth factor (60, 61). However in these cases pp60$^{c\text{-}src}$ has novel phosphorylation sites in the amino-terminal region of the molecule that may contribute to its activation.

pp60$^{c\text{-}src}$ complexed with the middle T antigen in polyoma transformed cells has elevated specific activity compared with free pp60$^{c\text{-}src}$ in the same cells (43, 51, 62, 63), suggesting that association with another protein(s) can regulate pp60$^{c\text{-}src}$ activity. However, pp60$^{c\text{-}src}$ from two closely-related colon carcinoma cell lines did not detectably complex with other cellular proteins when analyzed by density gradient centrifugation or FPLC gel filtration (54). It remains a possibility that mutations contribute to the activation of c-*src* in human colon carcinoma.

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