Differential pp60$^{c\text{-}src}$ Activity in Well and Poorly Differentiated Human Colon Carcinomas and Cell Lines

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Abstract

The results presented in this report demonstrate increased pp60$^{c\text{-}src}$ kinase activity associated with moderate to well differentiated colon tumors, corroborating previous observations by other groups. Extension of this analysis to include a small number of poorly differentiated colon carcinomas revealed src kinase activity comparable to that observed in normal colonic mucosa, considerably less than that observed in moderate/well differentiated lesions. Correlations of src kinase activity with differentiation was confirmed within a panel of colon cell lines where increased activity, associated with moderate/well differentiated lines, was accompanied by increased expression of pp60$^{c\text{-}src}$ protein. Use of an antiphosphotyrosine antibody in immunoprecipitation revealed the presence of novel phosphotyrosyl cellular substrates in human colon cell lines displaying elevated pp60$^{c\text{-}src}$ kinase activity. These observations suggest a role for the src protooncogene in colonic differentiation pathways. (*J. Clin. Invest.* 1992. 90:815–821.) Key words: pp60$^{c\text{-}src}$ • colon carcinoma • differentiation

Introduction

Adenocarcinoma of the large bowel remains a major public health problem affecting 1 in 20 people in the U. S. with approximately 151,000 new cases per year (1). However, the precise etiology of human colon carcinoma remains obscure. Normal colonic mucosa is maintained by continuously dividing stem cells at the base of the crypts of Lieberkuhn. As these cells migrate toward the apex they differentiate into ion-transporting enterocytes, mucous-secreting goblet cells, or enteroendocrine cells (2).

The molecular events underlying neoplastic progression in human colon represent multiple genetic alterations contributing to the malignant transformation of normal colonic mucosa. Such events implicate both the activation (2–7) and inactivation (8–15) of oncogenes and tumor suppressor genes, respectively, suggesting a role for both in the regulation of cell growth. In this respect the protein products encoded by cellular protooncogenes are believed to play a role in normal growth and differentiation pathways.

The protooncogene c-src has been implicated in both differentiation and neoplastic pathways in human tumors, where increased protein kinase activity was found associated with the differentiation status in tumors of neuroectodermal origin (16, 17) and in neoplastic progression in colon carcinomas (2, 3). The involvement of the src protooncogene in differentiation events of the nervous system, emanates from the observation of elevated expression of pp60$^{c\text{-}src}$ in the developing vertebrate nervous system (18–20). Differentiated neurons from embryonic rat brain express high levels of two forms of pp60$^{c\text{-}src}$ which display elevated specific activity (21). In addition, primary neurons induced to differentiate in culture, display increased expression of pp60$^{c\text{-}src}$ with elevated kinase activity (22). A correlation of the level of pp60$^{c\text{-}src}$ expression with differentiation events in this tissue is further strengthened by the observation of similar biochemical events in human tumors of neuronal and neuroendocrine origin (16, 17, 23). In contrast, human tumor cells of neuroectodermal origin, which do express neural characteristics, were found to have moderate to low levels of pp60$^{c\text{-}src}$ kinase activity (17). More recently, activation of pp60$^{c\text{-}src}$ protein kinase has been reported associated with preneoplastic colonic adenomatous lesions (3) and in 70% of colon carcinomas (2, 24) suggesting a role for src activation in neoplastic progression in this organ. A more extensive analysis of human tumor cell lines, derived from tissues other than those of neuroectodermal origin, has revealed low src kinase activity in most cases, with the exception of rhabdomyosarcomas, osteogenic sarcoma, Ewing's sarcoma, and colon carcinomas (17). To further evaluate the relationship between c-src activation and neoplastic progression in human colon carcinoma we measured the in vitro pp60$^{c\text{-}src}$ kinase activity, levels of pp60$^{c\text{-}src}$ protein expression, and characterized the phosphotyrosyl protein substrates in human colon tumors and a panel of human colon cell lines representing a range of differentiation states. In this report we demonstrate a strong correlation between differentiation status and relative kinase activity, raising questions regarding the role of pp60$^{c\text{-}src}$ activation in transformation and/or differentiation.

Methods

Cell lines. All cells were maintained in DME supplemented with 7.5% FCS. The panel of cell lines included the following: CX-1, HT29, CCL 238, CL 188, CaCo-2, CCL 233, CL 187, CCL 231, CCL 221, Moser, Clone D, CCL 227, CCL 228, CCL 222, Clone A, MIP 101 and CCL 220.1; all purchased from American Type Culture Collection, Rockville, MD.

Preparation of tumor material. Human colon tumors were snap frozen in liquid nitrogen and stored at −70°C before use. Tissue segments were embedded in OCT compound and a frozen tissue section was stained to assess the tumor content of material. Blocks were trimmed to remove stromal elements and only tissue with > 70% tumor content was included in this study. On average 50 frozen sections were required for each assay and in all cases sections 1, 51, 101, and 151 were stained to continuously monitor the tumor profile of the material. Preparation of tumor in this manner was considered critical for histological evaluation of tissue and subsequent interpretation of results.

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Received for publication 18 December 1991 and in revised form 30 March 1992.

J. Clin. Invest.

0021-9738/92/09/0815/07 $2.00
Volume 90, September 1992, 815–821

Immunoprecipitation of pp60$^{c\text{-}src}$. Subconfluent dishes of cell lines or frozen tissue sections from tumors were lysed in PBSTDS buffer (PBS pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, 10 U/ml aprotinin). Lysis was carried out on ice for 20 min followed by clarification in a microfuge at 4°C. Supernatants were removed and a 10-μl aliquot was taken for protein estimation using the bovine serum albumin protein assay system (Pierce Chemical Co., Rockford, IL). For immunoprecipitation, protein concentrations were standardized between samples, using 200 μg protein from each preparation. Lysates were incubated overnight at 4°C with mAb 327 (Oncogene Science, Inc., Manhasset, NY), followed by the addition of protein A Sepharose beads and a further 90-min incubation. Immune complexes were washed three times in PBSTDS, three times in 0.1 × PBS.

pp60$^{c\text{-}src}$ Western blot analysis. Precipitations for Western blot analysis were run in 7.5% polyacrylamide gels followed by overnight transfer to nitrocellulose using standard procedures. Nitrocellulose blots were blocked in triethanolamine buffered saline (TBS) buffer (10 mM Tris, 150 mM NaCl, pH 7.5) containing 3% gelatin followed by incubation with mAb327 for 90 min at room temperature. Blots were washed in three changes of TBS-Tween 20 (0.5% vol/vol) and incubated with phosphatase-labeled goat anti-mouse (Kirkegaard and Perry Labs, Gaithersburg, MD) followed by extensive washing in TBST. Blots were developed using the BCIP/NBT phosphatase substrate system (Kirkegaard and Perry Labs).

pp60$^{c\text{-}src}$ kinase assay. After immunoprecipitation of c-src, precipitates were incubated with ^{32}P-ATP in kinase buffer (50 mM Hepes, pH 7.4, 5 mM $MnCl_2$, 5 mM $MgCl_2$) for 20 min at room temperature. The antibody bead complex was washed three times in PBS, dissociated in sample buffer, and run in 12.5% polyacrylamide gels. Gels were dried and exposed to x-ray film for 2–4 h. When α-casein was included as a substrate (1 μg/sample), sample buffer was added immediately following incubation of precipitates with ^{32}P-ATP.

Immunoprecipitation of phosphotyrosyl substrates. Subconfluent monolayers of cells, incubated overnight in ^{32}P-orthophosphate, were washed twice with PBS and lysed in 2 ml NP-40 lysis buffer (20 mM Tris pH 8.0; 137 mM NaCl; 10% glycerol; 1% NP-40, 1 mM PMSF, 10 U/ml aprotinin, 1 mM sodium vanadate) on ice for 20 min followed by clarification in a microfuge at 4°C. Supernatants were removed and a 10-μl aliquot taken for protein estimation as described above. Protein concentrations were standardized between samples using 400 μg protein from each lysate, incubated at 4°C overnight with an antiphosphotyrosine monoclonal antibody (25). Addition of protein A Sepharose beads followed by a further 90-min incubation. Precipitated immune complexes which were washed three times in NP40 lysis buffer and three times in 0.1× PBS. The antibody–bead complex was dissociated in sample buffer and run in 12.5% polyacrylamide gels. Gels were dried and exposed to x-ray film for 2–4 h.

Results

pp60c-src in human colon carcinoma. Tumor lysates were prepared from frozen tissue sections, which were used to assess the tumor content and differentiation status of the tissue. Only tissue with > 70% tumor content was included in this study. Fig. 1 shows pp60$^{c\text{-}src}$ kinase activity associated with four moderate to well (Fig. 1, lanes *1–5*) and five poorly (Fig. 1, lanes *6–10*) differentiated colon carcinomas. Adjacent normal tissue, N2 and N4, from two of these individuals is also included (Fig. 1), corresponding to tumors 2 and 4, respectively. The increased kinase activity observed in the moderate and well differentiated tumors compared to normal elements is consistent with previously reported results showing activation of src kinase activity associated with human colon tumors (2, 24). Five poorly differentiated tumors were analyzed as an extension of this study with the finding that all five displayed levels

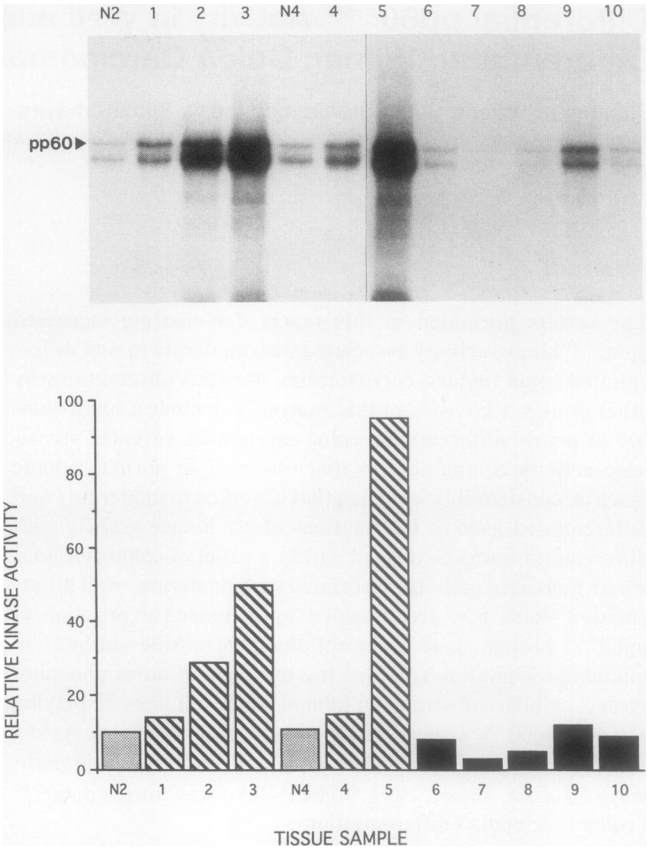

Figure 1. In vitro protein kinase activity of pp60$^{c\text{-}src}$ in moderate to well and poorly differentiated human colon carcinomas. Tissues were homogenized in lysis buffer and lysates standardized such that each contained 200 μg of protein. Proteins were precipitated from lysates with excess mAb 327 and then incubated with ^{32}P-ATP in kinase buffer before resolution on a 12.5% polyacrylamide gel. Representative autoradiograph: Four moderate to well (lanes *1–5*) and five poorly (lanes *6–10*) differentiated colon tumors with adjacent normal tissue from two of these individuals (N2 and N4 corresponding to tumors 2 and 4, respectively). Corresponding histogram: Cross-hatched columns, moderate to well differentiated tumors; dotted columns, adjacent normal tissue; solid columns, poorly differentiated tumors.

of src kinase activity comparable to that observed in normal mucosa, considerably less than that observed in moderately well differentiated lesions. The results presented in Fig. 1 represent immunoprecipitation of pp60$^{c\text{-}src}$ from tumor lysates, run on separate gels within the same experiment, enabling direct comparison of kinase levels between tumors. Densitometry scanning of the autoradiographs is presented in histogram format (Fig. 1).

To establish src kinase in both autophosphorylation and phosphotransfer events associated with pp60$^{c\text{-}src}$, we have analyzed a panel of colon tumors, representing a range of differentiation status, in kinase assays in the presence of α-casein. Fig. 2 shows phosphorylation of the exogenous substrate casein was two to six times greater in the moderate to well differentiated tumors than that measured in adjacent normal mucosa. These findings are consistent with results previously reported in colon carcinomas (2, 24). In an identical study performed simultaneously, all five poorly differentiated tumors displayed

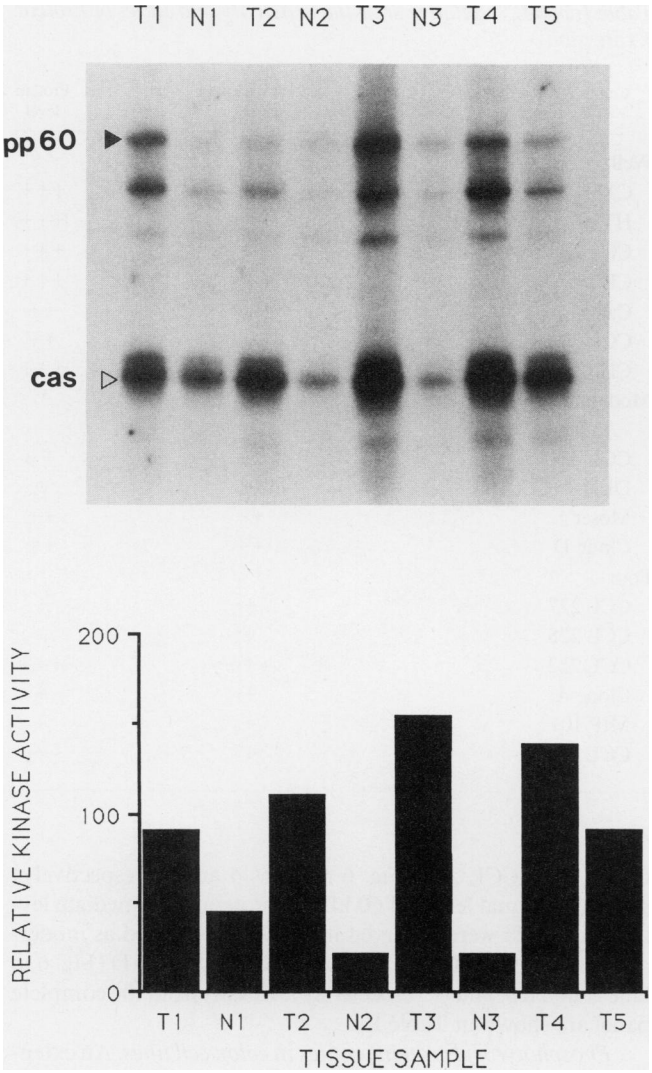

Figure 2. In vitro protein kinase activity of pp60$^{c\text{-}src}$ in the presence of α-casein in moderate to well differentiated colon tumors and adjacent normal tissue. Tissues were prepared for analysis as described in Fig. 1. The in vitro kinase assay was conducted in the presence of α-casein. Autoradiograph: Paired samples of tumor (*T*) and adjacent normal mucosa (*N*). The positions of pp60$^{c\text{-}src}$ and α-casein are indicated. Corresponding histogram shown below.

pp60$^{c\text{-}src}$ kinase activity less than that seen in the moderate to well differentiated tumors (Fig. 3).

pp60$^{c\text{-}src}$ activity in colon carcinoma cell lines. Although great care was taken to standardize and monitor the tumor content of the tissues analyzed, it is impossible to counter the heterogeneity found within a tumor and difficult to generate significant numbers of poorly differentiated colon carcinomas for analysis. The differential levels of pp60$^{c\text{-}src}$ kinase activity observed between moderate to well and poorly differentiated colon carcinomas is unlikely to be accounted for by differences in tumor content although review of the slides demonstrates variable stromal representation within the tumor group. To overcome this potential variable and to clarify a possible relationship between pp60$^{c\text{-}src}$ and differentiation, we have analyzed a panel of human colon carcinoma cell lines using the same assay systems. Although some heterogeneity exists in cultured cell lines, they comprise a more homogeneous cell population than the tumors as they are free of mesenchymal and lymphocytic elements. In these experiments 200 μg of total protein from lysates were immunoprecipitated with mAb 327 and assayed for levels of src kinase activity. The differential levels of kinase activity detected in this panel (Fig. 4) shows a strong correlation with differentiation where cell lines classified as moderate to well differentiated (CCL 231, CCL 238, HT-29, CX-1) display 5 to 10 times the kinase activity of that associated with poorly differentiated cell lines (Clone A, MIP 101, CCL 228). Results for the complete panel of colon cell lines analyzed is shown in Table I. Repetition of this experiment in the presence of α-casein demonstrated phosphotransfer activity relative to the kinase activities recorded in autophosphorylation of pp60$^{c\text{-}src}$. Relative kinase activity recorded within the cell panel shows levels in moderate to well differentiated cells

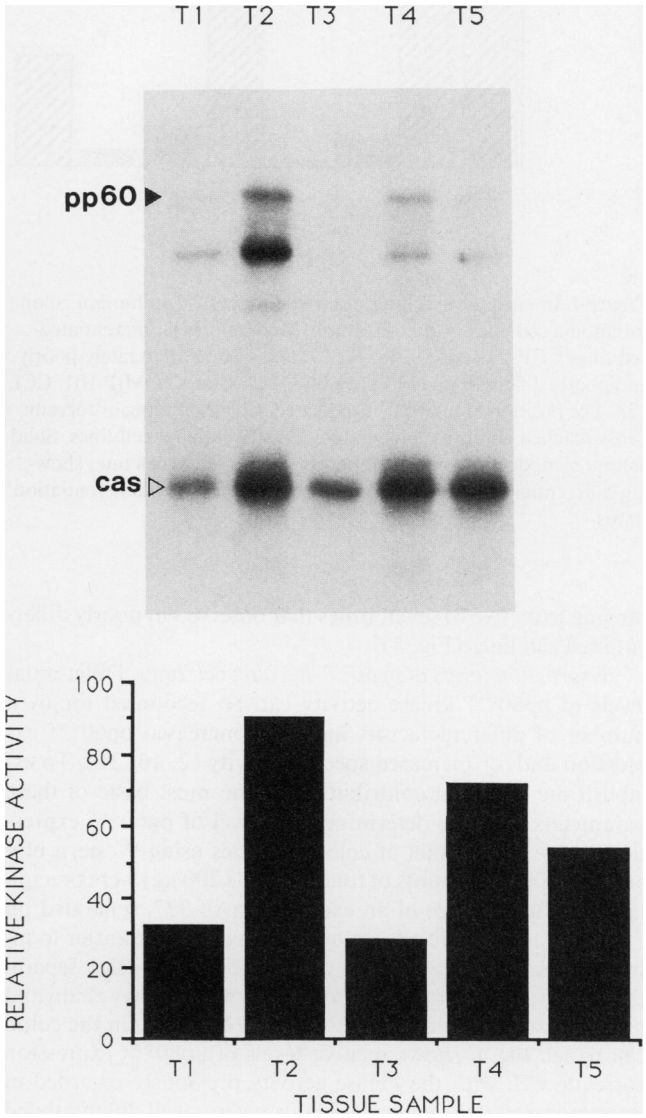

Figure 3. In vitro protein kinase activity of pp60$^{c\text{-}src}$ in the presence of α-casein in poorly differentiated colon tumors. Tissue lysates were prepared as described in Fig. 2. Autoradiograph: Lanes *T1–T5* are poorly differentiated tumors. The positions of pp60$^{c\text{-}src}$ and α-casein are indicated. Corresponding histogram shown below.

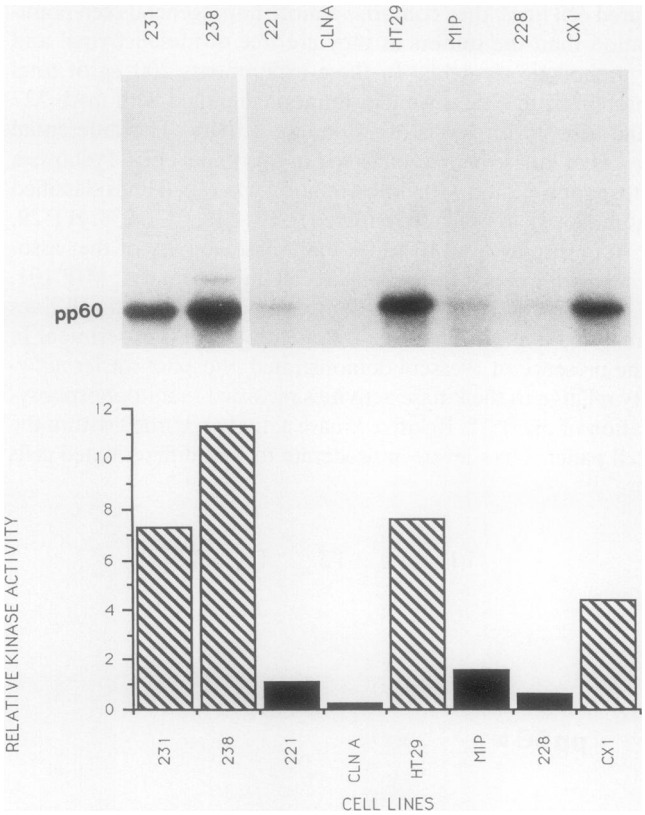

Figure 4. In vitro protein kinase activity of pp60$^{c\text{-}src}$ in human colon carcinoma cell lines. Autoradiograph: Moderate-well differentiated cell lines CCL 231, CCL 238, HT29, and CX-1. Moderately-poorly and poorly differentiated cell lines CCL 221, Clone A, MIP 101, CCL 228. The position of pp60$^{c\text{-}src}$ is indicated. Corresponding histogram: Cross-hatched columns, moderate-well differentiated cell lines. Solid columns, moderately-poorly or poorly differentiated cell lines showing differential level of kinase activity associated with differentiation status.

Table I. pp60$^{c\text{-}src}$ Relative src Kinase Activity and Level of Protein Expression

Cell line differentiation status	Relative kinase activity	Protein level
Well		
CX-1	+++	+++
HT29	+++	+++
CCL 238	+++	+++
CL 188	+++	+++
CaCo	+++	++
CCL 233	+++	++
CL 187	+++	+++
Moderate and moderate to poor		
CCL 231	++	++
DLD-1 (CCL 221)	+	+
Moser	++	++
Clone D	++	++
Poor		
CCL 227	+	+
CCL 228	+	+
CCL 222	+++	+++
Clone A	+	+
MIP 101	+	+
CCL 220.1	+	+

ranging from five to seven times that observed in poorly differentiated cell lines (Fig. 5).

Expression levels of pp60$^{c\text{-}src}$ in colon cell lines. Differential levels of pp60$^{c\text{-}src}$ kinase activity can be accounted for by a number of different factors including increased pp60$^{c\text{-}src}$ expression and/or increased specific activity (2, 16, 24). To establish the potential contribution of the most basic of these parameters we have determined the level of pp60$^{c\text{-}src}$ expression in the same panel of colon cell lines using Western blot analysis. Equal amounts of total protein (200 μg) were precipitated in the presence of an excess of mAb 327, separated on 7.5% polyacrylamide gels followed by overnight transfer to nitrocellulose. Probing of blots with mAb 327 revealed separation of the pp60$^{c\text{-}src}$ from the immunoglobulin heavy chain and resolution of variable amounts of src protein within the colon cell panel. Fig. 6 shows relative levels of pp60$^{c\text{-}src}$ expression correlate well with the kinase activity previously recorded in immunoprecipitation assays. In this regard, well differentiated cell lines CCL 238, HT-29, CX-1 (Fig. 6 *a*, lanes *1*, *4*, and *7*, respectively), and CCL 238, CL 187, CL 188 (Fig. 6 *b*, lanes *1*, *4*, and *5*, respectively) displayed the highest levels of src protein expression while the poorly differentiated cell lines Clone A, MIP 101, CCL 228 (Fig. 6 *a*, lanes *3*, *5*, and *6*, respectively) and CCL 220.1, CCL 227 (Fig. 6 *b*, lanes *6* and *8*, respectively) showed minimal levels of 60 kD src protein. Intermediate levels of pp60$^{c\text{-}src}$ were observed in cell lines classified as moderate-poorly differentiated, e.g., CCL 221 and Clone D (Fig. 6 *a*, lane *2*, and 6 *b*, lane *3*, respectively). Results from the complete panel are shown in Table I.

Phosphotyrosyl protein profiles in colon cell lines. An extension of the hypothesis involving pp60$^{c\text{-}src}$ in colon differentiation proposes increased, or novel, phosphorylation events associated with cellular substrates in vivo. To identify substrates of

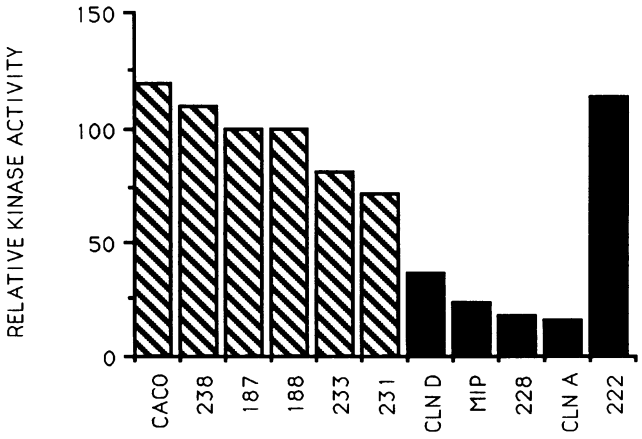

Figure 5. Histogram depicting in vitro protein kinase activity of pp60$^{c\text{-}src}$ in the presence of α-casein in human colon carcinoma cell lines. Cross-hatched columns indicate well and moderate to well differentiated cell lines. Solid columns indicated poorly differentiated cell lines.

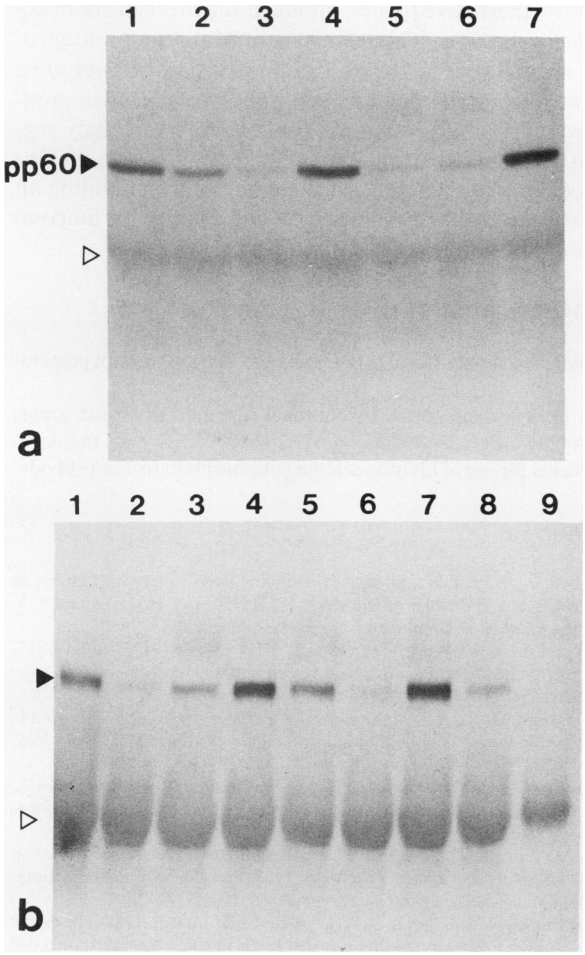

Figure 6. Western blot analysis of pp60$^{c\text{-}src}$ from human colon carcinoma cell lines. (*a*) Lane *1*, CCL 238; lane *2*, DLD-1; lane *3*, Clone A; lane *4*, HT29; lane *5*, MIP 101; lane *6*, CCL 228; lane *7*, CX-1. (*b*) Lane *1*, CCL 238; lane *2*, CCL 228; lane *3*, Clone D; lane *4*, CL 187; lane *5*, CL 188; lane *6*, 220.1; lane *7*, CCL 222; lane *8*, CCL 227; lane *9*, control. Differentiation status of all cell lines indicated in Table I.

protein tyrosine kinase in colon cell lines, we used antiphosphotyrosine antibodies in immunoprecipitation on a representative panel of colon cell lines. Metabolic labeling of cells with ^{32}P orthophosphate followed by immunoprecipitation with an antiphosphotyrosine antibody resulted in the resolution of different phosphotyrosyl protein profiles between cells representing different differentiation statuses (Fig. 7). Although phosphotyrosyl proteins were observed in both well (Fig. 7, lanes *4* and *5*) and poorly differentiated cell lines (Fig. 7, lanes *1* and *2*), additional bands were resolved in the well differentiated cell lines. Notable in this experiment was the consistent resolution of different phosphotyrosyl protein profiles observed between the two well differentiated cell lines (Fig. 7, lanes *4* and *5*).

Discussion

In this study we have confirmed previous observations involving activation of pp60$^{c\text{-}src}$ kinase activity associated with moderate to well differentiated human colon tumors compared to

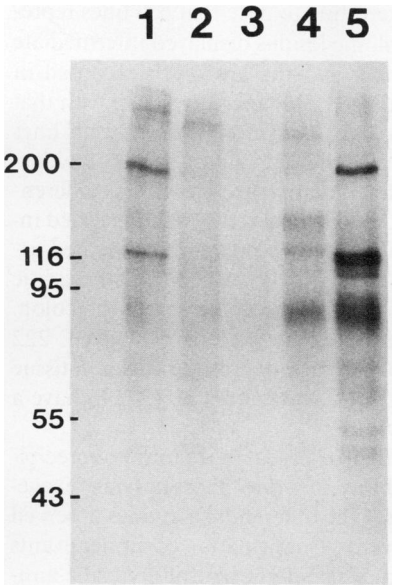

Figure 7. Immunoprecipitation of human colon carcinoma cell line phosphotyrosyl substrates. Poorly differentiated cell lines, lanes *1–3*. Well differentiated cell lines, lanes *4–5*. Lane *1*, Clone A; lane *2*, MIP 101; lane *3*, CCL 228; lane *4*, HT29; lane *5*, CL 187.

levels recorded in adjacent "normal" mucosa from the same patient (2, 24). However, extension of this study to include poorly differentiated colon tumor tissue revealed levels of pp60$^{c\text{-}src}$ kinase activity in all five tumors studied comparable to that of normal mucosa and markedly less than that observed in moderate to well differentiated colon tumors. These results suggest a role for the src protooncogene in colon differentiation pathways of colon mucosa. The involvement of pp60$^{c\text{-}src}$ in differentiation pathways of the nervous system has been established (18, 19, 20) and is maintained in neoplastic lesions, where elevated src kinase activity was observed in human tumors of neuronal and neuroendocrine origin (16, 17, 23) but not in tumor cells of neuroectodermal origin (17), which do not express neural characteristics. In contrast, activation of the pp60$^{c\text{-}src}$ protein kinase is found as an early event in colonic polyps (3) displaying a gradation in activity associated with progression and maintained in 70% of colon carcinomas studied (2, 24). In this regard src is implicated in colonic neoplastic progression. Elevated pp60$^{c\text{-}src}$ kinase activity has also been reported associated with rhabdomyosarcomas, osteosarcomas, Ewing's sarcoma, and a subset of breast carcinomas (17), although the relationship to differentiation or neoplastic progression is not clear in these cases. Consistent with findings in other organ systems the enhanced activity of pp60$^{c\text{-}src}$ kinase in moderate to well differentiated colon tumors is partly, but not wholly, due to an increase in levels of c-src protein expression suggestive of the requirement for an accompanying increase in the specific activity of the pp60$^{c\text{-}src}$ kinase (2, 3, 24).

Analysis of pp60$^{c\text{-}src}$ in an extended panel of human colon carcinoma cell lines revealed a striking correlation between the differentiation status of cells and the associated src kinase activity. In contrast to the results found in tumor material, elevated src kinase activity associated with moderate to well differentiated colon cell lines was largely accounted for by increased expression of the protooncoprotein. This could possibly be an in vitro phenomenon or accounted for by the more homogeneous phenotype displayed by cell lines, an event that is diluted out by the cellular heterogeneity found in tumor material. However, consistent with in vivo findings was the low src kinase activity and pp60$^{c\text{-}src}$ expression associated with poorly differ-

entiated cell lines. It is interesting to note that cell lines representing moderate/poor colonic lesions displayed intermediate levels of pp60$^{c\text{-}src}$ expression and the low levels recorded in SW480, a poorly differentiated cell line, is consistent with that observed by Cartwright et al. (24). Amongst the 17 cells lines tested, only one demonstrated pp60$^{c\text{-}src}$ kinase activity and levels of protein expression that did not correlate with its differentiation status. Cell line CCL 222 consistently demonstrated increased kinase activity and protein expression in repeated experiments. This cell line was originally isolated from the ascitic fluid of a 70-yr-old white male with carcinoma of the colon. Interestingly, although its xenograft grade is poor, CCL 222 secretes moderate levels of carcinoembryonic antigen in tissue culture. It is also reported by Cartwright et al. (24) to have a high pp60$^{c\text{-}src}$ kinase activity.

Phosphotransfer activity to α-casein in src immunoprecipitates demonstrated the ability of src to phosphorylate exogenous substrates maintaining the differential activities observed in autophosphorylation events. Confirmation of similar events in vivo was demonstrated using an antiphosphotyrosine antibody revealing increased phosphorylation of tyrosine on substrates 200 and 116 kD in the CCL 187 well differentiated cell line and an 80-kD substrate in both the CCL 187 and HT24 cell lines. The levels of phosphorylation on tyrosine seen in the well differentiated lines contrasts with that in the poorly differentiated lines where Clone A consistently displayed an intermediate phosphotyrosyl profile. It is tempting to conclude a direct relationship between these two events. However, as pointed out by others (24), many alternative cellular protein tyrosine kinases have been identified which could be responsible for this action.

Three main mechanisms in the regulation of pp60$^{c\text{-}src}$ kinase activity have been reported in alternative systems and include: phosphorylation (26–28); association with another protein (29–32); and mutation (27, 33–39). In a similar study involving human colon carcinomas, Cartwright (24) reported equivalent phosphorylation profiles following tryptic peptide mapping of pp60$^{c\text{-}src}$ from colon cell lines displaying differential src kinase activity and reported an absence of detectable src protein complexes in two cell lines analyzed (21). Activation of the src protooncogene via mutation remains an attractive alternative in light of the experimental data supporting activation in this manner and with the knowledge accumulated from other oncogenic elements. However, additional mechanisms of activation cannot be excluded, including amplification or regulation of c-src at the molecular level, or the influence of cellular phosphatases in regulating src kinase activity. More recently, mitotic activation of src has been shown to be modulated by the altered phosphorylation status of Tyr 527 (28) which in turn is regulated by a novel protein-tyrosine kinase activity acting at this COOH terminus residue (37). Alterations, including mutations, in such regulatory molecules are further considerations in the modulation of src kinase activity.

In this study we present data suggestive of a role for the pp60$^{c\text{-}src}$ protooncogene in differentiation events in colonic mucosa. The initial trend, observed in human colon tumor tissue, was confirmed within a panel of well and poorly differentiated human colon carcinoma cell lines. Though our results do not contradict those of others reporting in this field they do call into question the purported role of pp60$^{c\text{-}src}$ in neoplastic transformation and progression in poorly differentiated tumors. The involvement of pp60$^{c\text{-}src}$ in differentiation has been reported in alternative tissues, although the mechanism of action remains unclear. The involvement of the src protooncogene in normal colonic differentiation pathways has yet to be established along with definitive phosphotyrosyl protein products involved in differentiation. The results of this study suggest that human colon carcinoma cell lines represent a reasonable model for further study of these questions, providing an opportunity to elucidate pathways involved in colonic mucosal differentiation and neoplastic progression.

Acknowledgments

We would like to thank Carol Ann Hannan for the manuscript preparation.

This work was supported by National Institutes of Health grants RO1 CA42944, PO1 CA44704, and T32 CA09580. Special thanks to Brian Druker for use of his monoclonal antiphosphotyrosine antibody.

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