# Increase in Activity and Level of pp60$^{c\text{-}src}$ in Progressive Stages of Human Colorectal Cancer

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## Abstract

**Activation of the tyrosine kinase of the c-*src* gene product, pp60$^{c\text{-}src}$, has been shown to occur in nearly every primary colorectal carcinoma, and is found as early as in polyps of high malignant potential. However, no studies have addressed potential pp60$^{c\text{-}src}$ changes which occur during progression. To examine this question, we have studied kinase activity and protein levels in 7 colonic polyps, 19 primary lesions, and 19 liver metastases relative to normal colonic mucosa. Significant increases in tyrosine kinase activity were seen as early as in colonic polyps of high malignant potential. Further increases were observed in activity and level in primary tumors. However, the greatest increases in activity and protein levels were observed in liver metastases. Additionally, six metastatic lesions were obtained in which synchronous primary tumor was resected. In each of these liver metastases, pp60$^{c\text{-}src}$ activity and level were significantly increased relative to the corresponding primary tumor, as well as to normal colonic mucosa. Our results demonstrate that progression of colon primary tumors to liver metastases correlates with increased pp60$^{c\text{-}src}$ kinase activity and protein level. (*J. Clin. Invest.* 1993. 91:53–60.) Key words: colon polyps • hepatic metastases • oncogenesis • primary colon adenocarcinoma • tumor progression**

## Introduction

Recently, several genetic events have been implicated in the development and/or progression of human colorectal carcinoma. These genetic changes include the loss of potential tumor suppressor genes on chromosome 5 (1–5) termed MCC and APC (6–9), chromosome 18 (3, 10–12) termed DCC, and chromosome 17 (3, 11, 13), which includes the p53 gene (14). Although each of these events occurs with some frequency in colon tumors, as reviewed by Fearon and Vogelstein (15), none is characteristic of every tumor, and the effect of these cumulative mutations on the loss of growth regulation leading to frank carcinoma and its progression to metastatic disease remains unknown. In addition to changes in tumor suppressor genes, activation of proto-oncogenes has also been implicated in the development of colon cancer. Activation of the c-K-*ras* gene by point mutation is found in > 50% of colon carcinomas (16) and occurs at an early stage of tumor development (15). Activation of the tyrosine kinase of the c-*src* gene product, pp60$^{c\text{-}src}$, has also been demonstrated to occur at an early stage of tumor development, specifically, in polyps of high malignant potential but not in small benign polyps (17). Strikingly, most, if not all primary colon carcinomas and the cell lines established from them, have demonstrated activation of the pp60$^{c\text{-}src}$ tyrosine kinase (18, 19).

The mechanism of pp60$^{c\text{-}src}$ activation in colon carcinomas remains unknown, as does its potential role in tumor development and progression. However, recent studies have demonstrated that specific inhibitors of tyrosine kinases, such as herbimycin A (20), growth inhibit every colon tumor cell line with activated pp60$^{c\text{-}src}$ and that the extent of growth inhibition correlates closely with decreased pp60$^{c\text{-}src}$ activity (21). Additionally, while the role of pp60$^{c\text{-}src}$ regulation in growth and differentiation of normal colon cells remains to be understood, many studies have implicated its regulation as important to several cellular processes, including regulation of mitosis (22–24), response to growth factor-mediated cellular proliferation (25, 26), and differentiation of several mature cell types (27, 28). Furthermore, because increased pp60$^{c\text{-}src}$ tyrosine kinase activity is associated with malignant transformation, reviewed by Hunter (29), regulation of level and/or activity may be critical in growth regulation and malignant transformation of colonic epithelial cells. No studies have addressed potential pp60$^{c\text{-}src}$ changes that occur during progression of colorectal carcinoma to metastatic disease. Whether or not further alterations in pp60$^{c\text{-}src}$ activity and/or level are required for the transformed cell to gain full metastatic potential is unknown. To examine this question, we have studied tyrosine kinase activity and protein levels of pp60$^{c\text{-}src}$ in fresh tissues from a representative number of lesions comprising the spectrum of benign colon polyps to metastatic colorectal liver disease. Additionally, several synchronous primary and metastatic lesions from the same patient were examined to determine if changes in pp60$^{c\text{-}src}$ corresponded to those in isolated tumors. Our results demonstrate that colon tumor progression correlates with increased kinase activity and protein level of pp60$^{c\text{-}src}$.

## Methods

*Clinical samples.* Tissue samples were collected from 34 patients at the M. D. Anderson Cancer Center. These included five patients with synchronous colonic polyps and carcinomas and two with large, benign villous adenomas. A total of 19 primary colonic malignancies were obtained. Nineteen metastatic colon lesions were also collected, including specimens from 6 patients from whom both primary colonic tumor tissue and metastatic lesions were available and 13 patients with isolated liver metastases. Patients who had received either radiation therapy or chemotherapy within 6 mo before surgery were excluded from this study. On the basis of pathology reports, the

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stage of the tumor was determined according to the Astler-Coller modification of the Dukes' system, with designations as follows: $B_1$, lesions confined within the bowel wall; $B_2$, lesions that penetrate through all layers of the bowel; C, lesions classified as either $B_1$ or $B_2$ with the addition of lymph node involvement; and D, tumors that have metastasized beyond regional lymph nodes (30).

*Tissue lysates.* Normal colonic mucosal tissue and colon adenocarcinoma tissue were separated from underlying muscle tissue by blunt dissection. Light microscopy demonstrated that separation occurred at the level of the muscularis mucosae. Metastatic lesions were separated from the surrounding normal hepatic parenchyma by sharp dissection. Histopathologic examination was performed on all tumors, and biopsies of metastatic tissue were compared to samples from the primary site to confirm that the origin of the tumor was colon. Sections from the tumors were stained with hematoxylin and eosin, and the percent of viable tumor cells was estimated by a trained pathologist. After dissection of the samples was completed, the tissues were quick-frozen in liquid nitrogen within 15 min following surgery. Samples were homogenized in Ripa A Buffer (1% Triton-X-100, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 150 mM disodium chloride, 5 mM EDTA, 1% aprotinin, 5 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, 1 mM sodium vanadate, 5 mM sodium pyrophosphate, 20 mM sodium phosphate, pH 7.4) using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY), and the lysates were clarified by centrifuging at 15,000 rpm for 20 min at 4°C. Protein concentrations were measured in resulting lysates by the BCA protein assay (Pierce Chemical Co., Rockford, IL).

*Immune complex protein kinase assays.* Aliquots of lysate containing 200 μg of cellular protein were incubated with 3 ng of monoclonal antibody mAb 327, specific for $pp60^{c\text{-}src}$ (Oncogene Science Inc., Mineola, NY) for 1 h at 4°C (31). Because mAb 327 does not bind *Staphylococcus aureus* protein A, lysates reacted with this antibody were then incubated with 6 μg of rabbit anti-mouse IgG (Cappel Laboratories, West Chester, PA) for 1 h at 4°C. Samples were then incubated with 50 μl of 10% (vol/vol) formalin-fixed *Staphylococcus aureus* (Cowan strain) (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA) in NP-40/NEDT buffer (0.02 M Tris-HCl [pH 7.5], 150 mM NaCl, 1 M EDTA, 0.02% sodium azide [wt/vol], 0.5% deoxycholate [wt/vol], 0.5% NP-40) containing 1% bovine serum albumin. Immunoprecipitates were washed three times and then resuspended in reaction buffer containing 10 mM magnesium chloride, 20 mM Hepes, 100 μM sodium vanadate, 5 μg of acid-denatured rabbit muscle enolase (Sigma Chemical Co., St. Louis, MO), and 10 μCi [$\gamma$-$^{32}$P]ATP for 10 min at 25°C. After the incubation period, the reaction was terminated using a final buffer consisting of 2% SDS, 5% B-mercaptoethanol, 0.125 M Tris (pH 6.8), 1 mM EDTA, 10% glycerol, and 0.02% bromophenol blue. Proteins were resolved on 8% SDS-polyacrylamide gels (32) and radiolabeled proteins were detected by autoradiography with Kodak X-omatic AR film (Eastman Kodak Co., Rochester, NY) and an intensifying screen at −70°C.

*Immunoblot analysis of $pp60^{c\text{-}src}$.* Aliquots of lysate containing 200 μg of cellular protein were resolved on 8% SDS-polyacrylamide gels and transferred to a nitrocellulose filter (0.1 μm pore size) (Schleicher & Schuell, Inc., Keene, NH) at 100 V for 3 h at 4°C in transfer buffer (200 mM glycine, 25 mM Tris, 20% methanol). After the transfer was complete, the nitrocellulose filter was incubated in preabsorption buffer (3% bovine serum albumin and 5% 2%-lowfat milk in TTBS: 50 mM Tris, 0.1 M NaCl, 0.2% Tween-20, [pH 7.15]) at 37°C for 3 h with shaking. The filters were next incubated with 5 μg per lane mAb 327 at 4°C for 16 h with shaking. Filters were then washed four times for 15 min each time in TTBS and then incubated with $10^6$ cpm/lane of $^{125}$I-rabbit anti-mouse IgG for 3 h at 24°C. After four TTBS washes, the nitrocellulose was dried and exposed to Kodak X-omatic AR film with an intensifying screen at −70°C.

*Analysis of data.* Relative $pp60^{c\text{-}src}$ in vitro protein kinase activity was determined by densitometric scanning of the 60-kD band, representing autophosphorylation, and the 44-kD band, representing enolase phosphorylation, on the resulting autoradiographs. The level of $pp60^{c\text{-}src}$ protein in individual tumors was determined by densitometric scanning of the 60-kD band on autoradiographs of the immunoblots. Densitometry was done using a DU-7 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA), and results were expressed as relative changes compared to normal colonic mucosa. Changes in activity and level of polyps and primary tumors are expressed as the *n*-fold increase relative to normal colonic mucosa from the same patient for all samples. Data for liver me-

*Table I. Activity and Level of $pp60^{c\text{-}src}$ in Colonic Polyps*

| Patient | Site | Polyp size* | Histology | $pp60^{c\text{-}src}$ level‡ | $pp60^{c\text{-}src}$ autophosphorylation§ | Enolase phosphorylation‖ |
|---|---|---|---|---|---|---|
| | | *cm* | | | | |
| ST | descending | <1 | tubular adenoma | 1.7 | 1.7 | 2.0 |
| ST | descending | <1 | tubular adenoma | 1.7 | 2.4 | 4.3 |
| ST | descending | <1 | tubular adenoma | 1.3 | 3.5 | 6.6 |
| GR | rectum | <2 | villous adenoma | 0.8 | 0.9 | 0.8 |
| DE | sigmoid | >2 | tubular adenoma | 1.1 | 5.0 | |
| DU | right | >2 | villous adenoma | 0.7 | 10.2 | 4.6 |
| RA | rectum | >2 | villous adenoma | 1.9 | 4.2 | 2.7 |

* Measured by greatest central diameter. ‡ Relative to adjacent normal mucosa as determined by immunoblotting. § Relative to adjacent normal mucosa as determined by immune complex kinase assay. ‖ Relative to adjacent normal mucosa as determined by phosphorylation of 10 μg of enolase added to the immune complex kinase assay.

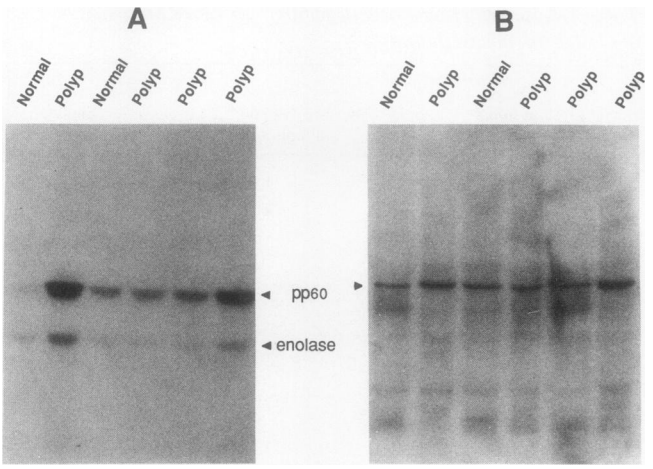


*Figure 1.* Activity and level of pp60$^{c\text{-}src}$ in colonic polyps. Polyps and adjacent normal mucosa were obtained within 15 min after resection, processed, and quick-frozen at −70°C. Cell lysates were made as described in Methods. (*A*) From lysate containing 200 μg of protein, activity of pp60$^{c\text{-}src}$ was determined by immune complex kinase assay, with the addition of enolase as an exogenous substrate. (*B*) Protein level was determined by immunoblotting. The figure displays the corresponding activity and level in normal colonic mucosa and villous adenoma (> 2 cm) from one patient (lanes *1* and *2*), normal mucosa and a 2-cm tubular adenoma from a second patient (lanes *3* and *4*), and two unpaired villous adenomas of < 2 cm (lanes *5*) and > 2 cm (lanes *6*).

tastases were compared to the corresponding normal mucosa, when available, from the same patient (6 patients). Unpaired liver metastases (13 patients) were compared to a randomized pool of normal mucosal samples. Variation among these normal mucosal samples was < 20%.

The clinical samples were characterized according to the location, histologic differentiation, Dukes' stage of the primary tumor, and patient age. The statistical significance of the differences in activity and level of pp60$^{c\text{-}src}$ between normal colonic mucosa and tumors was analyzed using the Wilcoxon signed-rank test. Thus, the value of pp60$^{c\text{-}src}$ activity and level in the presence of the other available prognostic factors was determined directly. All computations were done with the StatWorks program (Cricket Software, Philadelphia, PA).

## Results

*Activity and level of pp60$^{c\text{-}src}$ in colonic polyps.* To examine potential changes that can occur during progression to metastasis, we determined kinase activity and levels of pp60$^{c\text{-}src}$ relative to adjacent normal mucosa in a representative number of lesions comprising the spectrum of benign colon polyps to metastatic colorectal liver disease. Additionally, several primary colon tumors and synchronous metastases to the liver were examined. Histologic examination of specimens demonstrated that the normal tissue section contained primarily normal colonic mucosa, where as polyp and primary tumor sections consisted of the superficial components of the hyperproliferative polyp or invasive adenocarcinoma, respectively. The results for colonic polyps are summarized in Table I. Relative kinase activities and levels of pp60$^{c\text{-}src}$ were determined as described in Methods. When small polyps were examined, no increase was observed in autophosphorylation or enolase phosphorylation. In contrast, substantial increases in kinase activity were observed in two large (> 2 cm) benign villous adenomas. No sig-

*Table II. Activity and Level of pp60$^{c\text{-}src}$ in Primary Colon Tumors*

| Patient | Site | Dukes' stage | Histology | pp60$^{c\text{-}src}$ level* | pp60$^{c\text{-}src}$ autophosphorylation‡ | Enolase phosphorylation§ |
|---|---|---|---|---|---|---|
| HA | rectum | B1 | mod. diff. | 3.1 | 20.2 | 15.2 |
| CR | cecum | B1 | well diff. | 1.5 | 19.4 | 4.5 |
| WI | rectum | B1 | mod. diff. | 1.8 | 14.0 | 2.0 |
| BU | sigmoid | B2 | mod. diff. | 2.9 | 15.1 | 4.1 |
| ST | descending | B2 | mod. diff. | 1.5 | 10.4 | 4.1 |
| MC | cecum | B2 | mod. diff. | 1.1 | 2.3 | 2.1 |
| LA | rectum | B3 | mod. diff. | 1.3 | 22.8 | 10.5 |
| CA | descending | B3 | mod. diff. | 3.1 | 4.7 | 4.6 |
| DA | ascending | B3 | mod. diff. | 2.4 | 20.6 | 5.0 |
| HE | sigmoid | B3 | mod. diff. | 2.7 | 33.2 | 15.7 |
| FU | sigmoid | B3 | mod. diff. | 1.4 | 19.2 | 8.4 |
| MI | cecum | B3 | mod. diff. | 1.1 | 3.5 | 5.4 |
| PO | cecum | C3 | mod. diff. | 2.0 | 2.8 | 1.3 |
| DE | sigmoid | D | mod. diff. | 2.8 | 26.9 | 15.7 |
| HO | rectum | D | mod. diff. | 3.1 | 23.7 | 16.7 |
| DL | rectum | D | mod. diff. | 2.9 | 5.2 | 8.6 |
| SE | sigmoid | D | mod. diff. | 1.3 | 11.7 | 9.4 |
| AY | ascending | D | mod. diff. | 7.6 | 27.8 | 2.0 |
| JE | sigmoid | D | well diff. | 2.6 | 12.6 | 16.4 |

Abbreviations: diff., differentiated; mod., moderately. * Relative to adjacent normal mucosa as determined by immunoblotting. ‡ Relative to adjacent normal mucosa as determined by immune complex kinase assay. § Relative to adjacent normal mucosa as determined by enolase phosphorylation.

nificant increases in relative levels were observed in any colonic polyps. An example of pp60$^{c\text{-}src}$ activity and level in polyps is shown in Fig. 1. Small polyps (lanes *4* and *5*) show no increase, whereas the larger villous adenomas (lanes *2* and *6*) showed increases in autophosphorylation of 10.2- and 4.2-fold relative to adjacent normal mucosa but a less than twofold increase in relative pp60$^{c\text{-}src}$ levels. These results demonstrate that activation of pp60$^{c\text{-}src}$ in early stages of colon cancer development appears to be due primarily to an increase in specific activity of the enzyme.

*Activity and level of pp60$^{c\text{-}src}$ in primary colon tumors.* The tyrosine kinase activity of pp60$^{c\text{-}src}$ in primary colon adenocarcinomas was compared with the activity of pp60$^{c\text{-}src}$ in adjacent normal colonic mucosa in 19 separate patients as described in Methods. The results of the analyses are shown in Table II. In all 19 tumors, in vitro protein kinase assays revealed elevated pp60$^{c\text{-}src}$ activity in primary carcinomas relative to adjacent normal mucosa. Increased autophosphorylation ranged from 2.3 to 33.2 times that observed in normal mucosa, with an average increase in autophosphorylation of 15-fold and of 8-fold for enolase phosphorylation. Increases in activity appeared independent of age or sex of the patient, tumor location, or Dukes' classification of the primary tumors. The relationship to histologic differentiation could not be determined because all of the specimens in this study were classified as either well-differentiated or moderately well-differentiated tumors.

The pp60$^{c\text{-}src}$ levels in primary carcinomas ranged from 1.1 to 3.1 times that of adjacent normal mucosa and on an average were 2.4-fold higher than in normal tissues. Examples of activity and level of pp60$^{c\text{-}src}$ in primary tumors are seen in Fig. 2. This figure shows pp60$^{c\text{-}src}$ activity (panel *A*) and pp60$^{c\text{-}src}$ level (panel *B*) for three separate primary tumors (lanes *2, 4,* and *6*) versus the activity and level for the paired normal mucosa

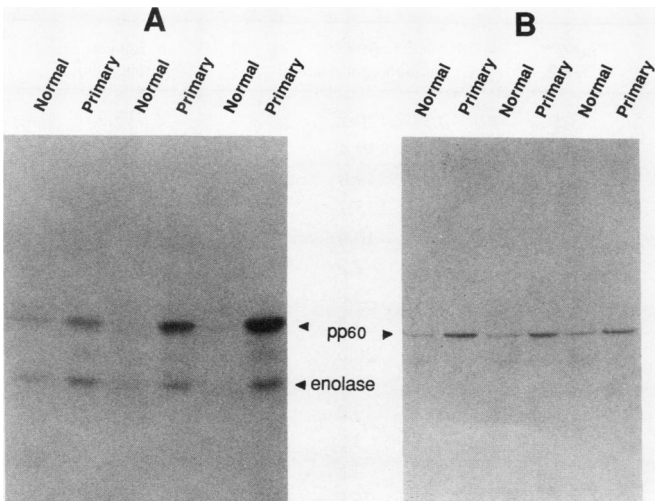


*Figure 2.* Activity and level of pp60$^{c\text{-}src}$ in primary adenocarcinomas. Specimens from tumors and adjacent normal mucosae were obtained and processed as described in Fig. 1. From lysates containing 200 μg of protein, (*A*) activity of pp60$^{c\text{-}src}$ was determined in an immune complex kinase assay with the addition of enolase as an exogenous substrate and (*B*) levels of the protein were determined by immunoblotting as described in Methods. Displayed in the figure are the corresponding activities and levels in tumor and adjacent normal mucosae of three patients.

*Table III. Activity and Level of pp60$^{c\text{-}src}$ in Liver Metastases of Colon Adenocarcinomas*

| Patient | Stage at initial presentation | pp60$^{c\text{-}src}$ level* | pp60$^{c\text{-}src}$ autophosphorylation‡ | Enolase phosphorylation§ |
|---|---|---|---|---|
| FR | B2 | 7.0 | 154.7 | 112.7 |
| PA | B2 | 8.0 | 390.4 | 145.6 |
| BA | B2 | 6.6 | 90.5 | 39.0 |
| MA | B3 | 4.8 | 50.9 | 43.7 |
| CH | B3 | 6.6 | 32.0 | 20.0 |
| PT | C2 | 5.2 | 43.7 | 26.0 |
| VA | C2 | 6.0 | 123.0 | 79.8 |
| BO | C2 | 2.2 | 52.5 | 5.0 |
| KA | C2 | 7.9 | 46.0 | 12.5 |
| LI | C2 | 7.3 | 129.0 | 28.5 |
| FO | C3 | 3.0 | 18.9 | 11.4 |
| GA | C3 | 1.0 | 10.6 | 4.5 |
| HO | D | 5.1 | 105.6 | 73.6 |
| DE | D | 2.3 | 64.9 | 23.2 |
| DO | D | 5.5 | 143.0 | 86.4 |
| DL | D | 1.0 | 4.6 | 3.4 |
| ST | D | 2.3 | 113.4 | 21.3 |
| AY | D | 11.1 | 98.2 | 2.7 |
| JE | D | 3.9 | 33.1 | 46.9 |

* Relative to adjacent normal mucosa as determined by immunoblotting. ‡ Relative to adjacent normal mucosa as determined by immune complex kinase assay. § Relative to adjacent normal mucosa as determined by enolase phosphorylation.

(lanes *1, 3,* and *5*). Each tumor demonstrates elevated autophosphorylation (tumor 1, 4.7-, tumor 2, 20.6-, and tumor 3, 33.2-fold increases) relative to the corresponding normal mucosa. Increases in enolase phosphorylation are also seen (tumor 1, 4.6-, tumor 2, 5.0-, tumor 3, 15.7-fold increases) relative to normal colonic mucosa. Changes in pp60$^{c\text{-}src}$ level are slightly higher than those observed in polyps; shown in Fig. 2 as 3.1, 2.4, and 2.7 times that of adjacent normal mucosa. These results demonstrate that pp60$^{c\text{-}src}$ is consistently activated in primary tumors and that activation may be accompanied by increases in protein level.

*Activity and level of pp60$^{c\text{-}src}$ in human colorectal liver metastases.* 19 separate liver metastases were examined, comprising a group of patients who had undergone previous primary tumor resection (13 patients) and a second group undergoing liver biopsy or resection at the time of primary tumor resection (6 patients). None had received any biologic therapy or chemotherapy within 6 mo before surgery. Total kinase activity and pp60$^{c\text{-}src}$ levels for individual metastatic lesions were compared to the corresponding normal mucosa from the same patient when available, and unpaired liver metastases were compared to the average activity or level of a randomized pool of normal mucosal samples. The pp60$^{c\text{-}src}$ activities and levels in normal hepatic parenchyma were also determined. The values were always lower than observed in normal colonic mucosa (data not shown). As shown in Table III, the greatest changes in kinase activity and protein level were consistently seen in the liver metastases. The kinase activity of metastatic lesions ranged from 4.6 to 390 times the activity in normal mucosa and on average were 90-fold higher when measured by auto-

phosphorylation and 41-fold higher when measured by enolase phosphorylation (Table III). There appeared to be no differences in pp60$^{c\text{-}src}$ activity based on age or sex of the patient or Dukes' stage at the time of initial presentation.

The levels of pp60$^{c\text{-}src}$ were also consistently increased in liver metastases. Protein levels ranged from 1.0 to 11.1 and were elevated an average of fivefold in liver metastases relative to normal mucosa. Calculation of specific activity revealed further increases of pp60$^{c\text{-}src}$ activity in metastatic disease. Thus, both specific activity and level of pp60$^{c\text{-}src}$ are significantly increased in liver metastases relative to primary tumors, resulting in highly elevated total pp60$^{c\text{-}src}$ kinase activity.

The results of pp60$^{c\text{-}src}$ activity and levels in all tissues examined are summarized in Table IV and Fig. 3. Increases in pp60$^{c\text{-}src}$ kinase activity were seen as early as in colonic polyps, and increases in both pp60$^{c\text{-}src}$ activity and level were observed in primary tumors. However, the highest pp60$^{c\text{-}src}$ activities and levels were consistently observed in liver metastases, as described above. Fig. 3 *A* demonstrates that with progression to metastatic disease there is a progressive increase in pp60$^{c\text{-}src}$ levels; however, the marked changes in total kinase activity and specific activity seen in the metastases cannot be solely accounted for by changes in the amount or levels of protein. The results of the kinase assays were also analyzed relative to the size of the polyps and the Dukes' stage of the primary tumors, as shown in Fig. 3 *B*). Polyps with greater malignant potential based on size > 2 cm were found to have higher kinase activity than smaller polyps. There were no significant differences in the activity of the primary tumors based on the Dukes' stage of the patient at the time of initial presentation. Although statistically significant increases in activity and level were observed in liver metastases, considerable differences among individual patients were apparent. As shown in Fig. 3 *B*, some metastatic lesions had lower activity than did some primary tumors.

*Activity and level of pp60$^{c\text{-}src}$ in synchronous lesions.* Although the above results demonstrate that both pp60$^{c\text{-}src}$ activity and level are increased in many liver metastases relative to primary tumors; the relationship between these increases and progression of the disease cannot be inferred by comparison of sporadic tumors. For analysis of changes during progression, synchronous lesions representing various stages of the disease were examined. Six patients with both primary and metastatic tumors obtained at the same surgery were studied. Immune complex kinase assays and immunoblots of two such patients are given in Fig. 4. As shown in the figure, increases between the primary tumors (lanes *2, 5, 8,* and *11*) and the liver metastases (lanes *3, 6, 9,* and *12*) were observed for both kinase activity and protein level. The results of this subset of patients are summarized in Table V. In every instance, both specific activity and protein level are increased in liver metastases relative to primary tumor. Thus in a given patient, progressive stages of the disease directly correlate with increased pp60$^{c\text{-}src}$ activity and level.

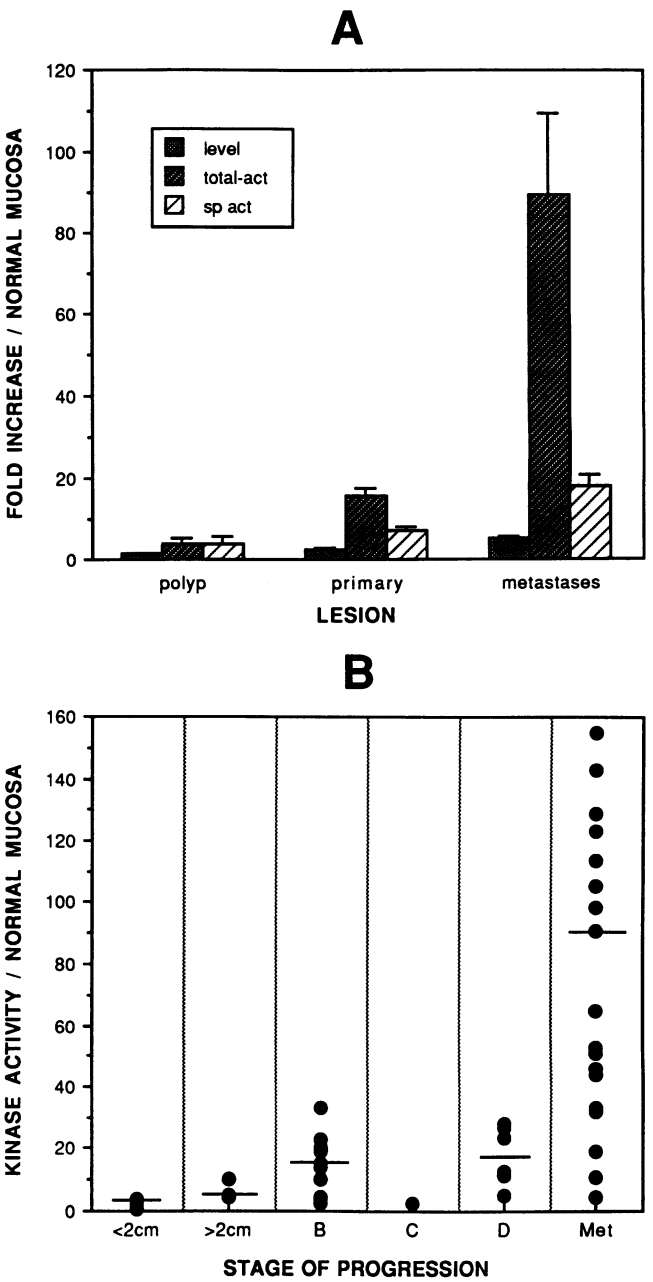


*Figure 3.* Activity and level of pp60$^{c\text{-}src}$ in progressive changes of human colon cancer. (*A*) Averages of total kinase activity as measured by autophosphorylation, specific activity, and pp60$^{c\text{-}src}$ protein levels of polyps, primary tumors, and liver metastases. (*B*) The pp60$^{c\text{-}src}$ kinase activity as measured by autophosphorylation in each of the 45 lesions examined, comprising the spectrum of disease from benign polyps through various Dukes' stages to liver metastases.

*Table IV. Activity and Level of pp60$^{c\text{-}src}$ in Progressive Stages of Colon Cancer*

| | Polyps‡ | Primary cancers‡ | Liver metastases‡ |
|---|---|---|---|
| Kinase activity (autophosphorylation) | 4.0±1.2§ | 15.6±2.1‖ | 89.7±19.8‖ |
| Protein level | 1.3±0.2 | 2.4±0.3‖ | 5.1±0.6‖ |
| Specific activity* | 3.9±1.8§ | 7.3±1.0‖ | 17.9±3.1‖ |
| Number of patients | 7 | 19 | 19 |

Mean±SEM. * Determined as ratio of autophosphorylation/protein level. ‡ Data expressed as *n*-fold increase compared to normal colonic mucosa. § $P < 0.01$; ‖ $P < 0.05$ by Wilcoxon signed-rank test vs. normal colonic mucosa.

## Discussion

Colorectal tumorigenesis is a complex, multistep process, frequently involving the deletion of tumor suppressor genes and the activation of dominant oncogenes. These changes are important events leading to unregulated growth of colon cells; however, acquisition of these malignant characteristics does not necessarily confer the ability to successfully establish distant metastases. Previous studies (33, 34) have shown that the process of metastasis consists of sequentially linked steps involving multiple host–tumor interactions (reviewed by Nicolson [35]). Tumor cell heterogeneity leads to generation of a subpopulation of cells with differences in metastatic potential (36). Therefore, tumor cells acquire the "metastatic phenotype" through additional genetic and epigenetic alterations in primary tumor cells (37, 38).

The genes involved in development of metastatic capacity are poorly understood. Certain genes, such as *nm* 23 (39), may function as potential metastasis suppressor genes (reviewed by Liotta et al. [40]). Transfection experiments with some of the known oncogenes such as *ras, src,* and *myc* have demonstrated the ability to induce metastatic competency in restricted recipient cells (41). These results suggest that specific changes do occur after acquisition of the tumorigenic phenotype. However, as yet, little is known about the changes that occur during colon tumor cell progression.

Recent studies by Bolen et al. (18) and Cartwright et al. (17, 19) have demonstrated activation of the c-*src* gene product, pp60$^{c\text{-}src}$, in human colon carcinogenesis, with activation in colonic polyps correlating with malignant potential (17). No studies have yet addressed a potential role for changes in pp60$^{c\text{-}src}$ activity or level as possible contributions to the acquisition of the metastatic phenotype in human colon cancer. To determine whether pp60$^{c\text{-}src}$ plays a role in progression of human colorectal cancer, we have examined the tyrosine kinase activity and the protein level of pp60$^{c\text{-}src}$ in colorectal polyps, primary tumors, and liver metastases. Our data demonstrate that changes in pp60$^{c\text{-}src}$ kinase in colonic polyps are primarily a result of increased specific activity. As demonstrated by Cartwright et al. (17) and confirmed by our results, polyps > 2 cm in size and of the villous type averaged 6.5- and 4.5-fold increases in autophosphorylation and enolase phosphorylation, respectively, when compared to adjacent normal colonic mucosa. When small polyps (< 2 cm) were examined, no increase was observed in autophosphorylation or enolase phosphorylation. These results establish the link between pp60$^{c\text{-}src}$ activation and malignant transformation of colon cells.

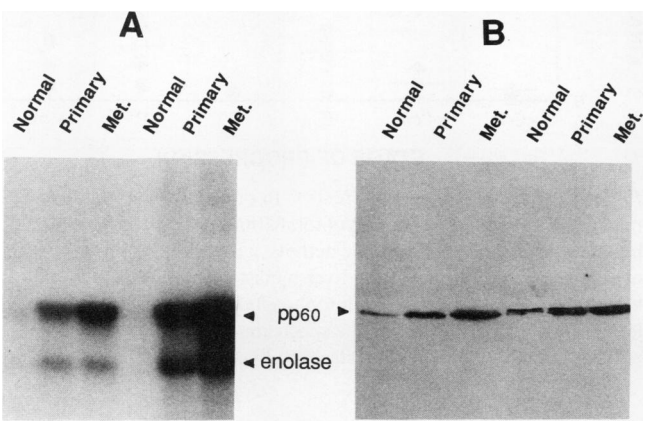


*Figure 4.* Activity and level of pp60$^{c\text{-}src}$ in synchronous lesions. Specimens of normal mucosa, primary tumor, and liver metastasis (met) were obtained from the same patient at the same surgery, and processed as described in Fig. 1. From lysates containing 200 μg of protein, (*A*) activity of pp60$^{c\text{-}src}$ was determined by immune complex kinase assay with the addition of enolase as an exogenous substrate and (*B*) level of the protein was determined by immunoblotting as described in Methods. Displayed in the figure are the corresponding activities and levels in normal mucosa, primary tumor, and liver metastases of two patients.

*Table V. Activity and Level of pp60$^{c\text{-}src}$ in Patients with Synchronous Primary Colon and Metastatic Liver Tumors*

| | Primary tumors‡ | Liver metastases‡ |
|---|---|---|
| Kinase activity (autophosphorylation) | 18.0±3.8§ | 70.0±17.9§ |
| Protein level | 3.4±0.9§ | 4.3±1.5§ |
| Specific activity* | 6.1±1.3§ | 20.0±6.9§ |
| Number of patients | 6 | 6 |

Mean±SEM. * Determined as ratio of autophosphorylation/protein level. ‡ Data expressed as *n*-fold increase compared to normal colonic mucosa. § $P < 0.05$ by Wilcoxon signed rank test vs. normal colonic mucosa.

In primary colon tumors, pp60$^{c\text{-}src}$ activity remained elevated. We have examined 19 primary colon carcinomas and have observed increased activation in all tumors. We observed an average increase in autophosphorylation of 15-fold and an average increase of 8-fold in enolase phosphorylation. Our results by immunoblot analysis in primary tumors demonstrated a 2.4-fold increase in the level of pp60$^{c\text{-}src}$ protein relative to adjacent normal colonic mucosa. These findings are consistent with results previously reported by Bolen et al. (18) and Cartwright et al. (19). Thus, increased pp60$^{c\text{-}src}$ activity in primary tumors is due mostly to increases in specific activity of the enzyme. Increases in activity appeared independent of age or sex of the patient, tumor location, or Dukes' classification of the primary tumors.

The focus of this work was to study the role of pp60$^{c\text{-}src}$ in progression of colorectal cancer. For these studies, liver metastases, the most frequent site of colorectal cancer metastasis, were studied. In every liver metastasis examined, there was a significant increase in pp60$^{c\text{-}src}$ activity and level relative to normal colonic mucosa. Increases in metastases were due to both increases in specific activity and levels of the protein. As a result (Fig. 4), the majority of liver metastases have considerably higher pp60$^{c\text{-}src}$ activity than that observed in primary tumors. The marked changes in kinase activity seen in liver metastases (Fig. 3) cannot be solely accounted for by changes in protein level. Our results suggest that other mechanisms which regulate pp60$^{c\text{-}src}$ activity, besides those controlling level, may be important for the development of metastatic lesions. Thus, these results suggest that in tumor progression, further genetic or epigenetic changes that affect pp60$^{c\text{-}src}$ may be required for the malignant, transformed cell to gain its full metastatic potential. These results are in contrast to those reported for p21$^{ras}$ in which activation occurs at an early stage of disease and expression decreases with progression (42).

That the changes are indeed reflective of tumor progression was demonstrated in synchronous lesions, i.e., primary and metastatic lesions removed from the same patient at the same surgery. In six of six patients in which primary and liver metastases were studied, both pp60$^{c\text{-}src}$ activity and level were significantly higher in the metastases than in the primary tumors. Several technical difficulties inherent in working with tumor specimens might have contributed to the changes in pp60$^{c\text{-}src}$ level and activity observed among the primary tumors and hepatic metastases, including the amount of stromal infiltration and necrotic tissue present in the samples. Indeed, differences were observed in the amount of viable tumor cells estimated from hematoxylin and eosin-stained sections of the tumor specimens analyzed for pp60$^{c\text{-}src}$. These differences are likely to account, at least in part, for the variation among individual tumors in relative increases in pp60$^{c\text{-}src}$ autophosphorylation activities for example, as shown in Fig. 3 *B*. However, no significant increases or decreases in the estimated percentage of viable tumor cells were noted between primary tumors and hepatic metastases as a group. These results suggest that pp60$^{c\text{-}src}$ changes correlate *specifically* with progression.

No studies have yet addressed a potential role for changes in pp60$^{c\text{-}src}$ activity or level as possible contributors to the acquisition of the metastatic phenotype in human colon cancer. There is, however, evidence to suggest that phosphorylation of physiologic substrates for protein tyrosine kinases may be important for production of metastases in some tumor cells (reviewed by Bishop [43]). Tyrosine kinase substrates such as the integrins, talin, and viniculin are components of cell adhesion plaques. One possible consequence of phosphorylation of these substrates could be decreased cell adhesiveness, an important step leading to the establishment of viable metastatic colonies. Immunoblotting with phosphotyrosine monoclonal antibodies has demonstrated an increase in both the degree and number of phosphorylated proteins in our metastatic lesions relative to the primary tumors and normal mucosa (G. E. Gallick, unpublished data).

Tumor progression invariably leads to increasing autonomy and heterogeneity of cell populations (44). Whether or not increased activity and level of pp60$^{c\text{-}src}$ trigger these processes or are a consequence of other events requires further study. Also, the mechanisms by which the observed changes occur remain unclear. Whatever factors are controlling pp60$^{c\text{-}src}$ specific activity and level, our data suggest that a progressive elevation in total activity may be a key component in the cascade of molecular events leading to competent metastatic colon cells. Additionally, when metastases from other primary tumors were examined, similar changes in pp60$^{c\text{-}src}$ were not observed, suggesting that these regulatory changes may be unique to progression of colon tumors (Talamonti et al., manuscript in preparation).

The consistent elevation of pp60$^{c\text{-}src}$ activity and level in colorectal liver metastases suggests possible diagnostic and therapeutic applications for this oncogene product. Identification and characterization of metastatic-specific tyrosine kinase substrates could serve as important diagnostic molecular markers of tumor progression. Finally, the development of tyrosine kinase inhibitors may provide exceptional specificity as a form of molecular antineoplastic therapy.

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