SRC transcriptional activation in a subset of human colon cancer cell lines

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Received 29 November 2000; accepted 30 November 2000

First published online 8 December 2000

Edited by Veli-Pekka Lehto

Abstract Human *SRC* encodes the non-receptor tyrosine kinase pp60^{c-Src}, which is activated in many human colon cancer cell lines (HCCLs) and tumors. We found that both c-Src protein and mRNA levels were elevated in a subset of HCCLs. Increased c-Src mRNA and protein levels correlated strongly with increased c-Src kinase activity. Nuclear run-on analysis and c-Src mRNA half-life determination demonstrated increased mRNA levels were due to increased transcription of the *SRC* gene. We also observed decreased c-Src mRNA stability in cell lines that displayed *SRC* transcriptional activation. Our findings provide the first evidence that *SRC* transcriptional activation is an important determinant of c-Src expression and activity in HCCLs.

Key words: c-Src; Oncogene activation; Transcription; Colon cancer

1. Introduction

Colorectal cancer (CRC) is the fourth most common malignancy affecting the Western population, and the second leading cause of deaths due to cancer [1]. The genetic events leading to malignant disease have been well documented, allowing stepwise models of colonic tumorigenesis to be elucidated [2,3]. In addition to these genetic anomalies, activation of the non-receptor tyrosine kinase pp60^{c-Src} has been a consistent early finding in CRC [4–10]. The recent report of an activating *SRC* mutation in a small subset (12%) of highly advanced colon tumors has verified an important role for c-Src in progression of CRC [11]. However, in the vast majority of CRC, c-Src is activated through non-mutagenic means. In many studies, increased c-Src kinase activity can be explained by a concomitant overexpression of pp60^{c-Src}, suggesting overexpression of pp60^{c-Src} could play an important role in colorectal tumorigenesis [4,7,8,12–14]. Indeed, overexpression of normal c-Src is able to cause transformation of mouse fibroblasts [15], with synergistic levels of transformation achieved upon co-overexpression with epidermal growth factor receptor (EGFR) [16]. In addition, antisense-mediated down-regulation of c-Src expression in the HT29 colon adenocarcinoma cell line results in severely diminished tumorigenicity and reduced vascular endothelial growth factor expression [17,18]. Activation and/or overexpression of c-Src has also been observed in other human tumors, notably those of the breast [19,20], lung [21,22], pancreas [23,24] and liver [25]. However, the precise mechanism by which c-Src activation occurs in colon and other cancers has not been determined.

Here we report a strong correlation between elevated pp60^{c-Src} kinase activity, elevated pp60^{c-Src} levels and increased c-Src mRNA levels in human colon cancer cell lines (HCCLs). Increased c-Src mRNA resulted from transcriptional activation of the *SRC* gene. Our findings therefore implicate *SRC* transcriptional activation, a previously unrecognized form of c-Src regulation, as an important determinant of c-Src expression and activity in HCCLs, as well as a potentially important event in colon tumorigenesis.

2. Materials and methods

2.1. Cell culture

The HT29, WiDr, SW480, SW620, and LS174-T, HCCLs were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium media and 10% fetal calf serum (Gibco). COLO 201, COLO 205, COLO 320, DLD-1 and HCT-15 cell lines were also from ATCC and were grown in RPMI 1640 media with 10% fetal calf serum. The HCT-116 cell line (ATCC) was grown in McCoy's 5A media with 10% fetal calf serum. The KM12C cell line was a gift of Dr. Fidler (MD Anderson Cancer Center). All cells were maintained at 37°C and 5% CO_2.

2.2. Plasmid constructs

c-Src cDNA clones have been described previously [26]. HHC189 containing a 1.1 kb insert of β-actin and pGD-P-25A containing a 1.5 kb insert of glucose-6-phosphate dehydrogenase were obtained from the ATCC. pMI containing exons 2 and 3 of the human c-Myc gene was a gift of Dr. Zheng (Department of Pathology, University of Saskatchewan). pRibo containing a 5.8 kb insert of 18S ribosomal RNA cDNA was a gift of Dr. W. Roesler (Department of Biochemistry, University of Saskatchewan).

2.3. mRNA extraction and Northern blot analysis

For mRNA half-life studies, actinomycin D was added to semi-confluent cells to a final concentration of 5 μg/ml. Following actinomycin D addition, cells were harvested at various time points and total cellular RNA was isolated using the method of Chomcyznski and Sacchi [27]. For other Northern blotting experiments, cells were grown until they were semi-confluent, then harvested; RNA was isolated using the same methodology. Equal amounts (15 μg per lane) of total cellular RNA were fractionated in 1% denaturing formaldehyde–agarose gels. RNA was transferred to Gene Screen nylon membranes (DuPont) by capillary elution, UV cross-linked, and then hybridized with cDNA probes specific for c-Src, c-Myc or β-actin labeled with [α-^{32}P]dCTP using an oligolabeling kit (Pharmacia). Autoradiography

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Abbreviations: HCCL, human colon cancer cell line; CRC, colorectal cancer; EGFR, epidermal growth factor receptor

0014-5793/01/$20.00
PII: S0014-5793(00)02354-1

was performed at −80°C using an intensifier screen (Kodak). For actinomycin D mRNA half-life studies, 1 week exposures to film were necessary to generate a signal for c-Src expression in SW480 and SW620, compared with overnight exposures for HT29, COLO 201 and COLO 205.

2.4. Immunoblot analysis

Cells from semi-confluent 10 cm plates were lyzed directly in a loading buffer containing 65 mM Tris–HCl, (pH 7.0), 2% (w/v) sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10% glycerol and 0.05% (w/v) bromophenol blue at the same time as plates used for Northern blot analysis. Following protein concentration determination using a Lowry kit (Sigma), 30 μg of total cellular protein was resolved on a 10% SDS–polyacrylamide gel. Gel transfer to nitrocellulose and membrane blocking were performed using standard procedures. The blot was incubated first with the pp60^{c-Src} specific monoclonal antibody, mAb 327 (a gift from Dr. J. Brugge) at 1.0 μg/ml, washed and probed with an anti-mouse IgG horseradish peroxidase conjugate (Santa Cruz) diluted 1:2000. Membranes were then incubated in chemiluminescence reagents (Dupont) and exposed to Kodak X-OMAT Blue XB-1 film for detection.

2.5. Cell lysate preparation, immunoprecipitations and in vitro c-Src kinase assays

Cells were lyzed in ice-cold, modified RIPA buffer. Immunoprecipitation and in vitro c-Src kinase assays were subsequently performed as described [28]. Briefly, lysate containing 500 μg of total cellular protein was incubated with excess mAb 327 for 1 h at 4°C. Immune complexes were collected by the addition of 30 μl of a 30% protein G–Sepharose slurry, incubation for 30 min at 4°C, and centrifugation at 10 000×*g* for 1 min at 4°C. Immunoprecipitate pellets were re-suspended in 40 μl of a kinase reaction buffer containing 0.25 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.8, 25 mM $MgCl_2$, 0.75 M NaCl, 1 μCi [γ-^{32}P]ATP, 10 μM ATP, 300 μM Src optimal peptide (AEEEIYGEFEAKKKK) [29], 200 μM sodium orthovanadate, and 4 mg/ml *p*-nitrophenol phosphate for 15 min at 30°C. Reactions were terminated by the addition of 25 μl of 50% (v/v) acetic acid. Aliquots from each reaction were spotted onto p81 phosphocellulose squares, which were subsequently washed three times in 0.5% (v/v) phosphoric acid, dried and counted in a scintillation counter; two independent experiments were performed.

2.6. Nuclei purification and nuclear run-on analysis

Approximately 3×10^8 cells were harvested and re-suspended in nuclear isolation buffer (60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 mM Tris–HCl, pH 7.4, 0.5 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride and 300 mM sucrose). Cells were gently lyzed by addition of Nonidet-P-40 to a final concentration of 0.5%. The crude preparation of nuclei was then layered onto the same buffer containing 1.7 M sucrose. Nuclei were pelleted through the sucrose cushion, re-suspended in nuclear storage buffer (50 mM Tris–HCl, pH 8.3, 40% glycerol, 0.1 mM EDTA and 0.1 mM DTT) and snap-frozen. Nuclear run-ons were then performed essentially as described [30] with a few minor modifications. Briefly, nuclei were thawed and an equal volume of 2× reaction buffer (10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 0.3 M KCl, 5 mM DTT, 1 mM each of ATP, GTP and UTP) and 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmol) were added. Following incubation at room temperature for 30 min, 20 U of RNase-free DNase was added, then incubation was continued for a further 5 min. Labeled RNA was isolated as described [27]. The labeled RNA (usually 5×10^6 cpm/reaction) was then hybridized to a series of DNA probes (10 μg/sample) immobilized on Zeta Probe nylon membranes (Bio-Rad) using a slot blot apparatus. Hybridization conditions were the same as described for Northern blots.

3. Results

3.1. Overexpression of c-Src mRNA results in increased c-Src protein and kinase activity in HCCLs

We have examined both the level and specific kinase activity of pp60^{c-Src} in a number of HCCLs. In agreement with reports from other groups [7,8], increased c-Src kinase activity in HCCLs could be explained by a concomitant overexpression of c-Src protein (compare Fig. 1B and C). To determine the mechanism of pp60^{c-Src} overexpression, we also examined c-Src mRNA levels in the same HCCLs (Fig. 1A). We found those cell lines that constitutively overexpressed pp60^{c-Src} (HT29, COLO 201, COLO 205, WiDr and LS174-T), and hence had increased c-Src kinase activity, also expressed higher levels of c-Src mRNA than cell lines that have lower levels of pp60^{c-Src} and lower c-Src kinase activity. The only cell line that did not follow this consistent trend was the COLO 320 cell line. These cells displayed intermediate steady state c-Src mRNA levels, low pp60^{c-Src} levels and low c-Src kinase activity. This particular cell line also displayed unusually high levels of c-Myc mRNA which resulted from a massive amplification of the *C-MYC* gene in the form of double minute chromosomes [31].

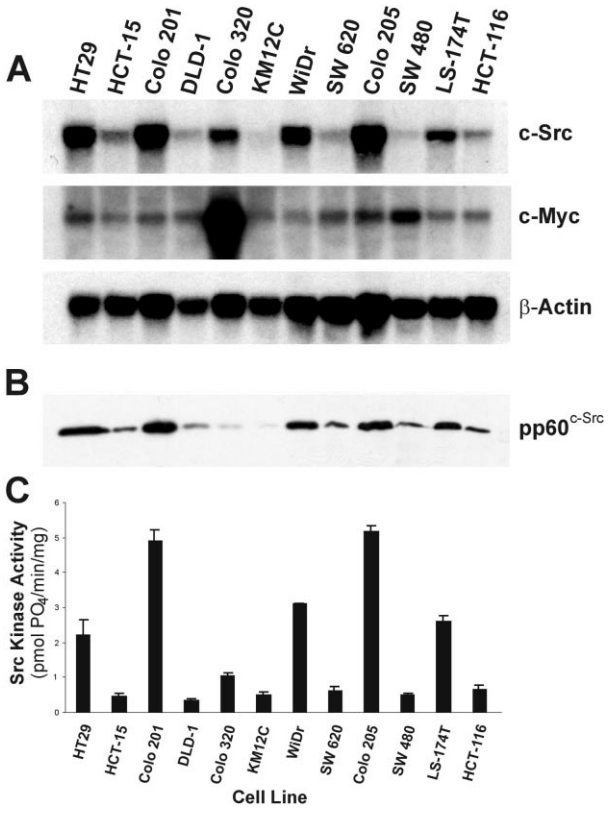

Fig. 1. c-Src mRNA, protein expression levels and kinase activity in various HCCLs. (A) Northern blot of total RNA from semi-confluent cells sequentially probed specifically for c-Src, c-Myc and β-actin as described in Section 2. (B) Western blot of total cellular proteins from the same cell lines harvested at the same time as in (A), using a specific anti-pp60^{c-Src} monoclonal antibody. (C) In vitro kinase assay of c-Src immunoprecipitated from cell lysates prepared at the same time as in (A). c-Src kinase activity is expressed as pmol of PO_4 incorporated into the synthetic Src optimal peptide AEEEIYGEFEAKKKK [29] per minute per mg of total cellular protein.

3.2. c-Src overexpression results from transcriptional activation of the SRC gene

To determine if the observed increase in c-Src mRNA and protein levels resulted from increased transcription of the *SRC* gene, we performed nuclear run-on analysis using representative cell lines as described in Section 2 (Fig. 2). We were able to reproducibly detect a higher signal for c-Src in over-

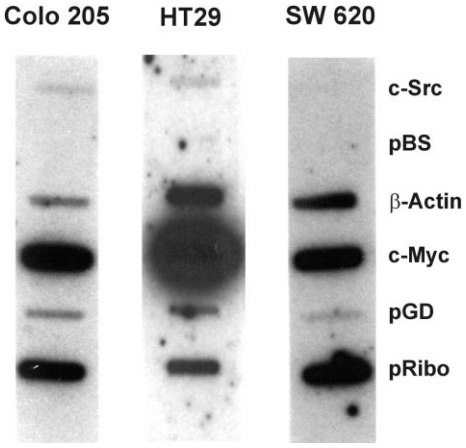

Fig. 2. Transcriptional activation in two high c-Src expressing cell lines versus a low c-Src expressing cell line. Nuclei were prepared from HT29, COLO 205 and SW620 cell lines and run-ons performed as described in Section 2. ^{32}P-labeled RNA from these nuclei was then hybridized to a nylon membrane containing 10 μg slot-blots of denatured c-Src, pBS (pBluescript), β-actin, c-Myc, pGD (glucose-6-phosphate dehydrogenase), and pRibo (18S ribosomal RNA cDNA) as indicated.

expressing cell lines, such as COLO 205 and HT29, when compared to low expressing cell lines such as SW620. We were consistently unable to detect any signal for *SRC* transcription in low expressing cell lines, such as SW620, despite transcription rates of a number of control genes being similar to other cell lines, such as HT29 and COLO 205. These results suggested that the *SRC* gene was transcribed at a relatively higher rate in the c-Src overexpressing cell lines compared to the low expressing cell lines.

However, the steady state levels of mRNA detected in the Northern blots could also be influenced by differential mRNA stability. To corroborate nuclear run-on results, we examined c-Src mRNA half-life in these HCCLs (Fig. 3). Following inhibition of cellular transcription by actinomycin D, total RNA was isolated at various time points and analyzed by Northern blotting. In all cases, we found that the ubiquitously expressed c-Myc transcripts were rapidly degraded. In contrast, we found that levels of β-actin mRNA remained constant throughout the course of the experiment. However, when we examined the stability of c-Src mRNA we found some interesting differences. In cells that expressed low levels of c-Src (SW480, SW620), the c-Src transcripts were stable over the time course of the study. Conversely, in high c-Src expressing cell lines (HT29, COLO 201, COLO 205), the c-Src transcripts were rapidly degraded with an estimated half-life of less than 2 h. We have confirmed this pattern of differential c-Src mRNA stability in other high and low expressing cell lines including WiDr and DLD-1 (S. Dehm and K. Bonham, unpublished data). Taken together, the data from mRNA stability studies and nuclear run-on experiments demonstrate that the mechanism of c-Src overexpression in this subset of HCCLs is at the level of transcription.

4. Discussion

The mechanism of c-Src activation in colon tumors and cell lines has remained elusive despite the evidence supporting an important role for c-Src in CRC. Our results showed increased c-Src kinase activity in a subset of HCCLs correlated with a concomitant increase in c-Src protein levels. Furthermore, those cell lines that exhibited elevated pp60^{c-Src} levels also had elevated c-Src mRNA levels, compared with cell lines that had lower c-Src protein and kinase activity. Thus, a strong trend existed between elevated c-Src kinase activity,

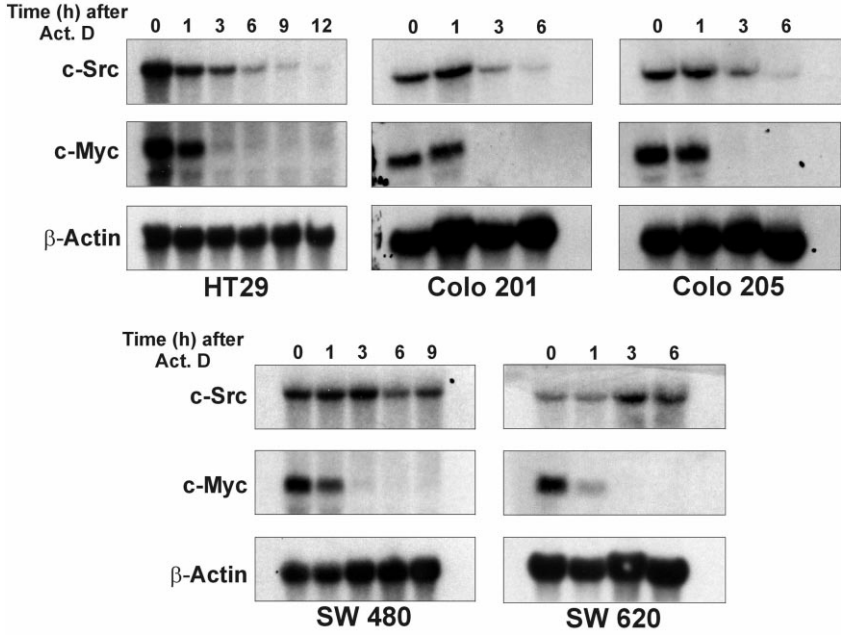

Fig. 3. Differential stability of c-Src mRNA in high c-Src expressing (HT29, COLO 201, COLO 205) versus low c-Src expressing (SW480, SW620) colon cancer cell lines. Total RNA was isolated from five colon cancer cell lines at various times following exposure to 5 μg/ml actinomycin D. Total RNA was blotted and hybridized sequentially with probes specific for c-Src, c-Myc and β-Actin. Much longer exposures to film were necessary to generate a signal for c-Src mRNA in SW480 and SW620 compared with HT29, COLO 201, and COLO 205. Lane numbering refers to hours after actinomycin D addition that cells were harvested.

increased pp60^{c-Src} levels, and increased c-Src mRNA levels. Further analysis showed increased c-Src mRNA in these cell lines was the result of transcriptional activation of the *SRC* gene. *SRC* transcriptional activation is therefore an important, yet previously unrecognized event that contributes to increased c-Src expression and kinase activity in a subset of HCCLs.

An interesting but unexpected finding resulting from this study was differential c-Src mRNA stability. Those cell lines that displayed increased c-Src mRNA, protein and kinase activity had decreased c-Src mRNA stability when compared with cell lines that had lower levels of c-Src mRNA. This finding suggests an even higher level of *SRC* transcription than previously anticipated to account for steady state c-Src mRNA levels observed during Northern blot analysis. While the mechanism of this differential stability is not yet known, we have recently identified evolutionarily conserved elements in the 3′ untranslated region of the c-Src transcript that could function as stabilizing elements (S. Dehm and K. Bonham, unpublished data). Destabilization of c-Src mRNA in cell lines where *SRC* is transcriptionally activated could represent a negative feedback form of regulation. If so, this would parallel findings where elevated pp60^{c-Src} kinase activity results in decreased c-Src protein stability [32,33], suggesting there exist multiple layers of feedback regulation to prevent inappropriate pp60^{c-Src} overexpression.

Two promoters, termed *SRC*1A and *SRC*1α, control transcription of the *SRC* gene [26,34,35]. The *SRC*1A promoter has all the hallmarks of a housekeeping promoter; it is GC rich, lacks TATA or CAAT regulatory motifs, and is regulated by the Sp-family of transcription factors as well as a novel factor termed SPy [26,34]. Conversely, the *SRC*1α promoter shows a more tissue-restricted pattern of expression and is regulated by the liver-enriched transcription factor hepatocyte nuclear factor-1α [35]. We have analyzed the relative levels of transcripts arising from these two promoters, and we have shown that they are used to different degrees in many of the HCCLs described here [35]. We are currently investigating the mechanisms determining differential promoter usage and transcriptional activation of the *SRC* gene.

In addition to transcriptional activation of the *SRC* gene, we have also noted an inverse correlation between *SRC* transcriptional activation and documented *KRAS* gene status in these same HCCLs. The SW480, SW620, HCT-116, HCT-15 and DLD-1 HCCLs have low levels of c-Src mRNA, low to intermediate levels of pp60^{c-Src}, low c-Src kinase activity and have an activated mutant *KRAS* allele [36]. Conversely, the HT29, WiDr, COLO 201 and COLO 205 cell lines display *SRC* transcriptional activation and have wild-type *KRAS* [36]. This is a particularly striking observation since both c-Src activation and *KRAS* mutation are common events in colon cancer [37,38]. Further investigation is necessary to determine whether these two events may be mutually exclusive in CRC.

In conclusion, we have demonstrated that *SRC* transcriptional activation in a subset of HCCLs resulted in increased c-Src expression and kinase activity. There are also reports that have suggested that c-Src overexpression and/or activation could play an important role in other cancers. For example, a c-Src specific antisense strategy has been employed in the SKOv-3 ovarian cancer cell line resulting in diminished anchorage-independent growth and tumor forming ability in a xenograft nude mouse model [39]. Similarly, antisense-mediated down-regulation of c-Src expression in NIH3T3 cells engineered to overexpress the EGFR or an EGFR-HER-2 chimera resulted in the reversal of the transformed phenotype of these cells [40]. Increased c-Src expression and/or kinase activity has also been reported in other cancers such as breast [19,20], lung [21,22], pancreas [23,24] and liver [25]. Since the exact mechanism of c-Src activation has not been identified in these other cancers, it is possible that *SRC* transcriptional activation is an important determinant of c-Src expression and activity in non-colon cancers as well.

Acknowledgements: We thank Dr. D. Anderson for helping to establish in our laboratory several of the techniques used in this report. This work was supported with grants from the Health Services Utilization and Research Commission of Saskatchewan and the Medical Research Council of Canada to K.B. S.D. was funded with a University of Saskatchewan College of Medicine Graduate Research Scholarship.

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