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Src Activity Increases and Yes Activity Decreases during Mitosis of Human Colon Carcinoma Cells

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Src and Yes protein-tyrosine kinase activities are elevated in malignant and premalignant tumors of the colon. To determine whether Src activity is elevated throughout the human colon carcinoma cell cycle as it is in polyomavirus middle T antigen- or F527 Src-transformed cells, and whether Yes activity, which is lower than that of Src in the carcinoma cells, is regulated differently, we measured their activities in cycling cells. We observed that the activities of both kinases were higher throughout all phases of the HT-29 colon carcinoma cell cycle than in corresponding phases of the fibroblast cycle. In addition, during mitosis of HT-29 cells, Src specific activity increased two- to threefold more, while Yes activity and abundance decreased threefold. The decreased steady-state protein levels of Yes during mitosis appeared to be due to both decreased synthesis and increased degradation of the protein. Inhibition of tyrosine but not serine/threonine phosphatases abolished the mitotic activation of Src. Mitotic Src was phosphorylated at novel serine and threonine sites and dephosphorylated at Tyr-527. Two cellular proteins (p160 and p180) were phosphorylated on tyrosine only during mitosis. Tyrosine phosphorylation of several other proteins decreased during mitosis. Thus, Src in HT-29 colon carcinoma cells, similar to Src complexed to polyomavirus middle T antigen or activated by mutation at Tyr-527, is highly active in all phases of the cell cycle. Moreover, Src activity further increases during mitosis, whereas Yes activity and abundance decrease. Thus, Src and Yes appear to be regulated differently during mitosis of HT-29 colon carcinoma cells.

Indirect evidence indicates a role for Src and the closely related tyrosine kinase, Yes, in intestinal cell growth control. First, the specific activity of cytoskeletal Src or Yes decreases as crypt cells differentiate (12, 39). Second, the specific activities of both kinases increase in malignant and dysplastic colonic cells (4, 7, 10, 13, 43, 58). Finally, infection of fetal rat colon with a retrovirus containing v-*src* induces dysplasia throughout the colon, and infection with both v-*src* and v-*myc* induces high-grade dysplasia and carcinoma (23). Together, the results suggest that downregulation of the Src and Yes kinases is important for differentiation and that upregulation is important for growth and transformation of intestinal cells.

Src activity is repressed during interphase and increases during mitosis in NIH 3T3-derived c-Src overexpressor cells (pMc-Src cells) (17, 55). Src activity also increases when fibroblasts are treated with platelet-derived growth factor or colony-stimulating factor 1 and reenter the cycle at the G_1/S transition (22, 36). Myristylation, dephosphorylation of Tyr-527, and $p34^{cdc2}$-mediated phosphorylation of Ser-72, Thr-34, and Thr-46 on chicken Src are required for mitotic activation (1, 2, 33, 40, 54). During mitosis, Src binds via its SH2 and SH3 domains to tyrosine-phosphorylated p68. p68 is related to p62, an RNA-binding protein (25, 59, 62).

Contrary to the transient increases in Src activity observed during mitosis of normal cells, cells transformed by F527 Src or polyomavirus middle T antigen (mT) maintain high levels of Src activity throughout the cell cycle (1, 32). Activation of Src, but not Yes, is required for induction of mammary tumor in mT transgenic mice (30).

The purpose of this study was to determine whether Src activity is elevated throughout the human colon carcinoma cell cycle as it is in polyomavirus mT- or F527 Src-transformed cells and whether Yes activity, which is lower than that of Src in the carcinoma cells, is regulated differently. We observed that the activities of both kinases were higher throughout all phases of the HT-29 colon carcinoma cell cycle than in corresponding phases of the fibroblast cycle. Moreover, the specific activity of Src increased further during mitosis of the HT-29 carcinoma cells, whereas Yes activity and abundance decreased.

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MATERIALS AND METHODS

Cell culture. The human colon carcinoma cell lines HT-29, WiDr, and SW 480 were obtained from the American Type Culture Collection (Rockville, Md.). WiDr, SW 480, 208F, and FLOW 2000 cells were maintained in Dulbecco-Vogt modified Eagle's medium (DMEM). HT-29 cells were maintained in McCoy's 5A medium. Unless otherwise stated, NIH 3T3 and 208F cells were grown in 10% bovine serum and other cell lines were grown in 10% fetal bovine serum (FBS).

Antibodies. Proteins were immunopurified with a monoclonal antibody (MAb) specific for Src (MAb 327) (37) or an antipeptide antibody specific for Yes (Yes 3) (36). Immunoblotting was performed with affinity-purified MAb 327 or an antipeptide antibody that recognizes the carboxy terminus of Src-related proteins (Src 2 [5]) (Santa Cruz Biotechnology, Santa Cruz, Calif.) or a monoclonal antiphosphotyrosine antibody (PY20 [26]) (Transduction Laboratories, Lexington, Ky.).

Isolation of cells. Cells in G_1 phase were prepared by placing unsynchronized cells in serum-free medium for 48 h, replating the cells in medium containing 10% serum, and harvesting cells 3 or 6 h later. Cells in G_1/S phase were obtained by treating G_1 cells for 24 h with 5 μg of aphidicolin (Sigma, St. Louis, Mo.) per ml (27, 31). Cells in S phase were prepared by washing aphidicolin-treated cells and maintaining them in drug-free medium for 3 h. Mitotic cells were prepared by incubating unsynchronized cells, or aphidicolin-treated cells that had been washed three times and placed in fresh medium, for 20 h with 0.4 μg of nocodazole {methyl[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate; Sigma} per ml (16, 42, 68). Nocodazole was added from a 4-mg/ml stock solution in dimethyl sulfoxide (stored at −20°C). The plates were rocked and gently washed with medium. Detached cells were collected, pelleted by low-speed centrifugation (4 min, 4°C), and washed three times in phosphate-buffered saline (PBS; 0.73 M NaCl, 0.018 M KH_2PO_4, 0.057 M Na_2HPO_4 [pH 7.2]). Cells exiting mitosis were prepared by washing and replating nocodazole-treated cells in drug-free medium and harvesting cells 30, 60, 90, 120, or 150 min later. For some experiments, nocodazole-induced mitotic cells were also treated with 500 μM sodium orthovanadate (Fisher Scientific, Fair Lawn, N.J.) (49, 56) or 100 nM

okadaic acid (OA; Sigma) (3, 57) for 30 min prior to cell lysis or for 60, 120, or 150 min after replating in nocodazole-free medium. Mitotic cells were also isolated without drugs, by multiple rounds (separated by 1 h) of mechanical shake-off from subconfluent plates of cells (17, 60). Shake-off cells from early rounds were stored in medium at 4°C during the collection process. Trypan blue staining (0.25% in PBS; Sigma) confirmed that 95% of mitotic cells obtained by either method were viable for up to 20 h and 90% for up to 40 h.

Analysis of cellular DNA content. Mitotic cells were collected as described above. Other cells were collected by trypsinization (0.02% trypsin, 3 min at room temperature). Cells (1×10^5 to 2×10^5) were washed three times in PBS, fixed in 70% ethanol for 30 min, collected by low-speed centrifugation, and resuspended in 1 ml of PBS (53). Following addition of 40 μg of RNase A (Sigma) per ml and 20 μg of propidium iodide (Sigma) per ml (53), cell suspensions were incubated in the dark for 30 min at room temperature and analyzed for fluorescence intensity (proportional to DNA content) in a fluorescence-activated flow cytometer (FACScan; Becton Dickinson, San Jose, Calif.) (24). Histograms of cell number versus DNA content were prepared by using CellFIT software.

Protein extractions, immunoprecipitations, and in vitro tyrosine kinase assays. Cells were lysed in ice-cold, modified radioimmunoprecipitation assay buffer (0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1% sodium deoxycholate, 0.15 M sodium chloride, 10 mM sodium phosphate [pH 7.0], 100 μM sodium vanadate, 50 mM sodium fluoride, 50 μM leupeptin, 1% aprotinin, 2 mM EDTA, 1 mM dithiothreitol). Lysates were centrifuged at 29,000 × *g* for 1 h at 4°C. Protein concentrations were measured by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.). Lysate containing 100 μg of protein was incubated with excess MAb 327 or Yes 3 antibody. Excess affinity-purified rabbit anti-mouse immunoglobulin G (Cooper Biomedical, Malvern, Pa.) was added to MAb 327 precipitates prior to collection with Pansorbin (Calbiochem, San Diego, Calif.) (9, 11, 14, 15). Immunoprecipitates were incubated for 10 min at 30°C in 30 μl of buffer containing 50 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (pH 7.0), 10 mM manganese chloride, 10 mM dithiothreitol, 1 μg of acid-denatured rabbit muscle enolase (Boehringer Mannheim, Indianapolis, Ind.) (20), and 50 μCi of [γ-^{32}P]ATP (4,000 Ci/mmol; ICN, Costa Mesa, Calif.) (9, 11, 14, 15). Repeat assays adding 20 μM unlabeled ATP to the kinase buffer gave similar results. Proteins were resolved on SDS–7% polyacrylamide gels (acrylamide-bisacrylamide, 20:1), and gels were stained with Coomassie brilliant blue G-250 (Bio-Rad, Richmond, Calif.) to confirm that equivalent amounts of enolase were present in each lane. Radiolabeled proteins were detected with Kodak XAR film and an intensifying screen at −70°C. ^{32}P incorporation into proteins was quantified by Cerenkov counting of excised gel pieces and in addition, for some autoradiograms, by scanning densitometry. The two methods gave similar results. Quantitation by liquid scintillation spectroscopy 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 gives similar results (10, 13). Src or Yes in vitro protein kinase activity is linearly related to the concentration of total cellular protein (10, 13).

Immunoblot analysis. Immunoprecipitates prepared in parallel with those used for in vitro protein kinase assays, or lysates containing 15 or 40 μg of total cellular protein, were resolved on SDS–7% polyacrylamide gels (acrylamide-bisacrylamide, 20:1). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, Mass.) in transfer buffer (25 mM Tris-HCl [pH 7.4], 192 mM glycine, 15% methanol), using a Trans-Blot apparatus (Bio-Rad) for 4 h at 40 V (10, 13–15, 48). Protein binding sites on the membranes were blocked by incubating them overnight in TNT buffer (10 mM Tris-HCl [pH 7.5], 100 mM sodium chloride, 0.1% [vol/vol] Tween 20 [Sigma]) containing 5% nonfat powdered milk (blocking buffer). Membranes containing MAb 327 immunoprecipitates were preincubated in normal sheep serum (Cooper Biomedical) (1:10 dilution in blocking buffer) for 2 h, incubated with affinity-purified MAb 327 ascites fluid (1:250 dilution) for 1 h, washed in TNT buffer with changes every 5 min for 30 min, and incubated with excess ^{125}I-labeled affinity-purified, sheep anti-mouse immunoglobulin G (New England Nuclear, Boston, Mass.) (10^6 cpm/ml of blocking buffer) for 1 h at room temperature. Membranes containing Yes 3 immunoprecipitates were incubated with excess Src 2 (1:500 dilution) for 1 h at room temperature, washed, and incubated with excess ^{125}I-labeled protein A (Amersham, Arlington Heights, Ill.). ^{125}I-labeled proteins were detected with Kodak XAR film and an intensifying screen at −70°C. ^{125}I-labeled proteins were quantified by counting excised bands. The amount of Src or Yes protein detected in lysates is linear over a fivefold range of total cellular protein (10, 13). Membranes containing total cellular protein were incubated with 1 μg of antiphosphotyrosine antibody PY20 or Src 2 per ml, washed, and incubated with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad). Proteins were detected by enhanced chemiluminescence (Amersham).

Metabolic radiolabeling of cells. In pulse-labeling experiments, unsynchronized or 20-h nocodazole-treated cells were washed twice and incubated for 30 to 60 min in methionine- and cysteine-free DMEM (Mediatech, Washington, D.C.) supplemented with 10% dialyzed FBS. The cells were then incubated in fresh medium containing 100 μCi of PRO-MIX[^{35}S] (70% L-[^{35}S]methionine and 30% L-[^{35}S]cysteine; >1,000 Ci/mmol; Amersham) per ml at 37°C for the time periods indicated. For pulse-chase experiments, unsynchronized cells were incubated with 50 μCi of PRO-MIX[^{35}S] per ml for 1 h at 37°C, washed twice, and incubated with DMEM supplemented with 10% FBS and 2 mM L-methionine (Life Technologies, Grand Island, N.Y.) in the presence or absence of 0.4 μg of nocodazole per ml for the time periods indicated. Radioimmunoprecipitation assay lysates containing 200 μg of total cellular protein were incubated with Yes 3 antibody, and immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were Coomassie blue stained and treated with autoradiography enhancer (En^{3}Hance; Du Pont, Boston, Mass.) for 1 h at room temperature. Radiolabeled proteins were visualized by autoradiography and quantified by scanning densitometry.

For ^{32}P metabolic labeling experiments, one subconfluent 10-cm dish of unsynchronized HT-29 cells was washed three times in phosphate-free DMEM and incubated for 10 h at 37°C in phosphate-free DMEM containing 10% dialyzed, phosphate-free FBS and 2.5 mCi of $^{32}P_i$ (carrier free; ICN) per ml (10, 11, 15, 43). Because of low yields of mitotic cells (about 20%), detached cells from five dishes which had been nocodazole treated for 10 h were collected into one dish and incubated with $^{32}P_i$ for 10 h. To maintain mitotic arrest, nocodazole treatment was continued during the 10-h $^{32}P_i$ incubation.

Two-dimensional tryptic phosphopeptide analysis. ^{32}P-labeled proteins from unsynchronized or nocodazole-treated HT-29 cells were immunoprecipitated with MAb 327 and resolved by SDS-PAGE. ^{32}P-labeled Src was extracted, oxidized, and digested with trypsin (6). The phosphopeptides were separated in two dimensions on 100-μm thin-layer cellulose (TLC) 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]) (6).

Phosphoamino acid analysis. Tryptic phosphopeptides were extracted from TLC plates, hydrolyzed in constant boiling HCl (5.7 M) at 110°C for 1 h, mixed with nonradioactive phosphoserine, threonine, and tyrosine standards (Sigma), and separated on TLC plates by electrophoresis in two dimensions (6).

RESULTS

Treatment of HT-29 colon carcinoma cells with an inhibitor of DNA polymerase α arrests cells at the G_1/S boundary, and treatment with an inhibitor of mitotic spindle formation arrests cells at mitosis. To synchronize cycling HT-29 cells, cells were treated with aphidicolin, an inhibitor of DNA polymerase α (31), or nocodazole, an inhibitor of mitotic spindle formation (68), and DNA content was analyzed (Fig. 1). More than 80% of cells which had been serum starved for 48 h and harvested 3 or 6 h after the addition of 10% serum were in G_1 phase. After treatment of G_1 cells for 24 h with aphidicolin, 80% of cells were arrested at the G_1/S boundary. With drug removal and maintenance of cells in drug-free medium for 3 h, 84% of cells were in S phase. When unsynchronized cells or aphidicolin-treated cells which had been washed three times and placed in fresh medium were treated for 20 h with nocodazole, 98% of cells were arrested in mitosis. Ninety minutes after replating in drug-free medium, two-thirds of cells remained in mitosis, whereas 150 min after replating, two-thirds had exited mitosis. Thus, HT-29 cells were successfully synchronized and isolated in various phases of the cell cycle.

The specific activity of Src increases during mitosis of HT-29 colon carcinoma cells. To study Src activity in HT-29 cell cycle phases, cells were harvested as described above, proteins were precipitated with MAb 327 (a MAb specific for Src) (37), and the phosphorylation of Src and the exogenous substrate, enolase, were measured in an in vitro protein kinase assay (Fig. 2A). Similar to previous findings (10, 43), the activity of Src in logarithmically growing HT-29 cells was 10- to 20-fold higher, as measured by enolase phosphorylation or autophosphorylation, than that in FLOW 2000 human fibroblasts (compare lanes 1 and 2), rodent fibroblasts, or normal colonic cells (10). Src activity during interphase (G_1 and S phases) of HT-29 cells was similar to that in unsynchronized cells (compare lanes 3 to 6 with lane 2). During mitosis, Src activity increased two- to threefold and Src protein migrated more slowly on the gel (compare lanes 7 to 11 with lanes 3 to 6). The slower-migrating form of Src appeared, on some gels, to be a doublet. Previously, a similar increase in activity and shift in mobility of Src were observed in mitotic NIH 3T3 cells which overexpress c-Src (17, 55). As HT-29 cells exited mitosis, Src activity decreased and mobility increased. One hundred fifty minutes after drug removal, two-thirds of the cells had

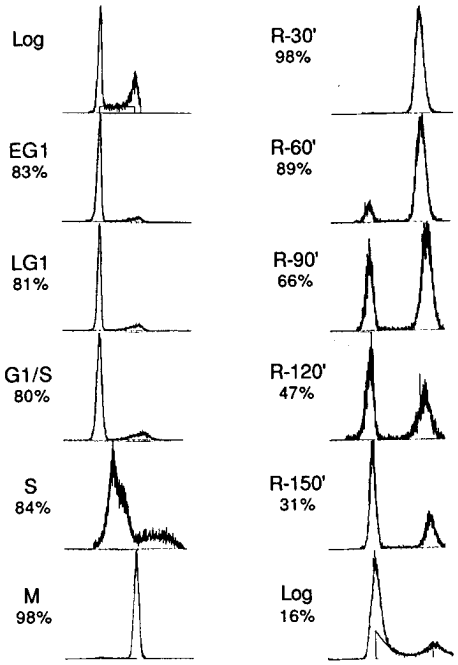

FIG. 1. Cell cycle phases of HT-29 colon carcinoma cells following treatment with aphidicolin or nocodazole. Cells in G_1 phase were prepared by placing unsynchronized, logarithmically growing cells (Log) in serum-free medium for 48 h, replating in medium containing 10% serum, and harvesting 3 (EG1) or 6 (LG1) h later. Cells in G_1/S were obtained by treating G_1 cells for 24 h with 5 μg of aphidicolin per ml. Cells in S phase were prepared by washing aphidicolin-treated cells and maintaining them in drug-free medium for 3 h. Mitotic cells (M) were prepared by washing unsynchronized or aphidicolin-treated cells and incubating them for 20 h in fresh medium containing 0.4 μg of nocodazole per ml. Cellular DNA content was analyzed by laser flow cytometry. Abscissa, DNA content; ordinate, cell number; left, percentage of cells in G_1, G_1/S, S, or M; right, percentage of cells in mitosis after replating in drug-free medium for 30, 60, 90, 120, or 150 min (R-30′ to R-150′) or in logarithmically growing cells.

exited mitosis (Fig. 1) and Src activity and migration had returned toward levels in interphase cells (Fig. 2A; compare lane 12 with lanes 3 to 6). Src activity in all phases of the HT-29 cell cycle was approximately 10-fold higher than that in corresponding phases of the fibroblast cycle (data not shown).

To measure Src protein amounts, immunoprecipitation and immunoblotting with MAb 327 were performed (Fig. 2B). Src protein levels remained unchanged throughout the HT-29 cell cycle. Thus, the specific activity of Src (a ratio of total activity to the amount of protein) was elevated two- to threefold during mitosis. The slower-migrating mitotic Src was detected on immunoblots (lane 5), as it had been in kinase assays. Thus, Src was highly active in all phases of the HT-29 cell cycle, and its specific activity further increased during mitosis.

Yes activity and abundance decrease during mitosis of HT-29 colon carcinoma cells. To study Yes activity in HT-29 cell cycle phases, cells were harvested as described above, proteins were precipitated with Yes 3 (an antipeptide antibody specific for Yes) (36), and the phosphorylation of Yes and enolase were measured in an in vitro protein kinase assay (Fig. 2C). Similar to previous observations (43), Yes activity in logarithmically growing HT-29 cells was approximately 10-fold higher than that in FLOW 2000 cells (compare lanes 11 and 12). Yes activity during interphase of HT-29 cells was similar to that in unsynchronized cells (compare lanes 1 to 4 with lane 11). In contrast to Src, Yes activity decreased threefold during mitosis and protein migration on the gel was not altered (compare lanes 5 to 9 with lanes 1 to 4). As HT-29 cells exited mitosis, Yes activity returned toward levels in interphase cells (compare lane 10 with lanes 1 to 4).

To measure Yes abundance, Yes protein was immunoprecipitated with Yes 3 and immunoblotted with Src 2 (4), an antipeptide antibody that recognizes the carboxy terminus of Src-related proteins (Fig. 2D). Yes protein levels, unlike those of Src, decreased threefold during mitosis (lane 5). Thus, the decrease in Yes protein fully accounted for the decrease in Yes activity. One hundred fifty minutes after drug removal, two-thirds of the cells had exited mitosis (Fig. 1), and Yes activity (Fig. 2C) and, to a lesser extent, Yes proteins levels (Fig. 2E) had returned toward levels in interphase cells. Thus, there is a small increase in the specific activity of Yes as cells exit mitosis.

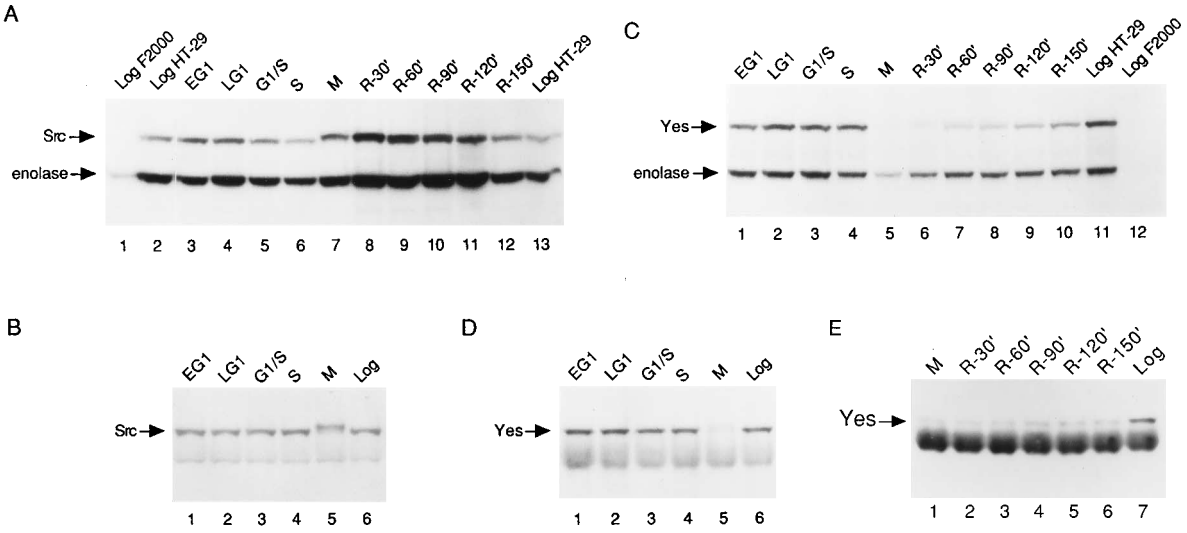

FIG. 2. Src and Yes tyrosine kinase activities and abundance during the HT-29 cell cycle. Src (A) or Yes (C) immune complex protein kinase activity was measured in cells synchronized in various phases of the cell cycle. Protein levels of Src (B) or Yes (D and E) were measured by immunoblot analysis. Exposure times at −70°C with intensifying screens: (A) 20 min; (B) 2 h; (C) 20 min; (D) 4 h; (E) 12 h. F2000, FLOW 2000 human fibroblasts. Other abbreviations are as in Fig. 1.

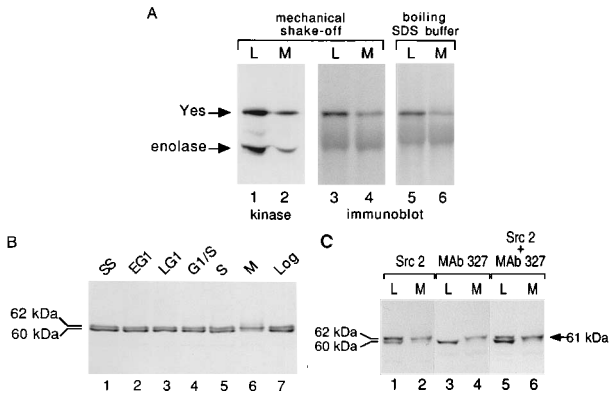

FIG. 3. (A) Comparison of Yes activity and/or abundance in unsynchronized and mitotic HT-29 cells following mechanical shake-off or after lysis of cells in boiling SDS buffer. Mitotic cells were isolated without drugs by multiple rounds of mechanical shake-off from subconfluent plates of cells (lanes 2 and 4) or following 20 h of nocodazole treatment (lane 6). Cells were lysed as described in Materials and Methods (lanes 1 to 4) or in boiling SDS buffer (lanes 5 and 6). Yes in vitro protein kinase activity was measured in unsynchronized (L; lane 1) and mitotic (M; lane 2) cells. Yes protein levels were measured by immunoblot analysis (lanes 3 to 6). Exposure times; lanes 1 and 2, 15 min; lanes 3 and 4, 2.5 h; lanes 5 and 6, 24 h. Exposures for lanes 1 to 4 were at −70°C with intensifying screens, and exposures for lanes 5 and 6 were at room temperature. (B) Immunoblot analysis of Src and Yes using Src 2, a carboxy-terminal antibody which recognizes both proteins. Lysates containing 15 μg of total cellular protein were prepared from HT-29 cells synchronized in various phases of the cell cycle, resolved by SDS-PAGE, transferred to PVDF membranes, and incubated with Src 2 (5). The membranes were washed and incubated with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase, and proteins were detected by enhanced chemiluminescence. SS, serum starved for 48 h. Other abbreviations are as in Fig. 1. (C) Comparison of mitotic proteins that are recognized by Src 2 and/or MAb 327, a MAb specific for Src. Lysates containing 15 μg of total cellular protein were prepared from unsynchronized (L) or mitotic (M) HT-29 cells. Immunoblotting was performed as described for panel B, using Src 2 (lanes 1 and 2) or MAb 327 (lanes 3 and 4). The autoradiogram shown in lanes 1 and 2 was superimposed on that shown in lanes 3 and 4 and photographed (lanes 5 and 6).

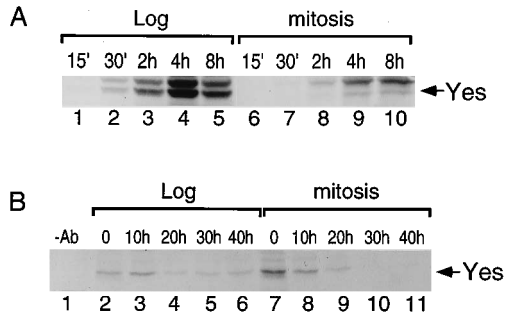

FIG. 4. (A) Comparison of the synthetic rate of Yes in unsynchronized and mitotic HT-29 cells. Unsynchronized or 20 h nocodazole-treated cells were pulse-labeled with 100 μCi of PRO-MIX[^{35}S] per ml for 15 min to 8 h. Proteins were immunoprecipitated with Yes 3 antibody from lysates containing 200 μg of total cellular protein and resolved by SDS-PAGE. Exposure time was 10 days. (B) Comparison of the degradation rates of Yes in unsynchronized and G_2/M HT-29 cells. Cells were pulse-labeled with 50 μCi of PRO-MIX[^{35}S] per ml for 1 h, washed, and maintained in the presence or absence of 0.4 μg of nocodazole per ml for up to 40 h. Yes immunoprecipitations were performed as described above except for lane 1, in which Yes 3 antibody was not added (−Ab). Exposure time was 3 days.

To determine whether lower protein levels were due to a nonspecific drug effect, we harvested mitotic cells by mechanical shake-off (Fig. 3A). Similar to nocodazole-treated cells, mitotic cells obtained by this method had a two- to threefold decrease in Yes activity (compare lanes 1 and 2) and abundance (compare lanes 3 and 4). To determine whether lower protein levels were due to poorer extraction of proteins from mitotic cells or activation of proteases during lysis, we lysed cells in boiling SDS buffer (Fig. 3A). Again, Yes protein levels decreased two- to threefold during mitosis (compare lanes 5 and 6). Thus, while Yes was highly active in all phases of the HT-29 cell cycle, its activity and abundance decreased during mitosis, and the decreases were not artifacts of drug treatment, proteolysis during lysis, or protein solubility.

Because of the possibility that the amino-terminal, antipeptide Yes antibody does not recognize mitotic Yes, we resolved cellular proteins by SDS-PAGE, transferred them to PVDF membranes, and immunoblotted them with Src 2 (5), an antipeptide antibody that recognizes the carboxy terminus of Src-related proteins (Fig. 3B). (Previously we determined that of seven Src family member tested, only Src and Yes are expressed at detectable levels in colon carcinoma cells [43].) Two protein bands of the appropriate molecular masses for Src (60 kDa) and Yes (62 kDa) were observed in serum-starved (lane 1), unsynchronized (lane 7), and interphase (lanes 2 to 5) cells. Three different bands ranging in size from 60 to 63 kDa were present in mitotic cells (lane 6). Of these, the major 61-kDa band appeared to migrate slower than Src, slightly faster than Yes, and in a similar location to mitotic Src (Fig. 2B). The intensity of the band was less than or equal to that of Yes during interphase. The band could represent mitotic Src alone or together with comigrating Yes. If the latter and Yes protein levels during mitosis were equivalent to those in interphase, then we would have expected to see an increase in the intensity of this band compared with interphase Yes levels. That we did not suggested that Yes protein levels, as measured by using the carboxy-terminal Src 2 antibody, were decreased during mitosis.

To determine whether the 61-kDa protein comigrated with mitotic Src, we compared the proteins recognized by Src 2 (Fig. 3C, lanes 1 and 2) with those recognized by MAb 327 (Fig. 3C, lanes 3 and 4) in unsynchronized and mitotic HT-29 cells. Again, the mitotic 61-kDa protein detected with Src 2 (lane 2) had a mobility similar to that of mitotic Src (lane 4). When the autoradiogram for lanes 1 and 2 was superimposed on that for lanes 3 and 4, and the lower band in lane 1 was aligned with Src in lane 3, the 61-kDa protein comigrated with mitotic Src (lane 6). Thus, a significant portion of the 61-kDa band (lane 2) is mitotic Src. Because the amount of the 61-kDa protein is less than that of Yes in unsynchronized cells (compare lanes 1 and 2), we conclude that Yes protein levels, as measured by using the carboxy-terminal Src 2 antibody, are decreased during mitosis.

Synthesis of Yes decreases and degradation increases during G_2/M of HT-29 cells. To determine whether altered rates of synthesis and/or degradation contributed to the decreased steady-state protein levels of Yes during mitosis, we measured these rates in unsynchronized and mitotic HT-29 cells. To examine the rate of synthesis, unsynchronized cells or those treated with nocodazole for 20 h were pulse-labeled with 100 μCi of PRO-MIX[^{35}S] per ml for 15 min to 8 h. Yes was immunoprecipitated from lysate containing 200 μg of total cellular protein and resolved by SDS-PAGE (Fig. 4A). The rate of synthesis of Yes in mitotic cells was significantly lower than that in unsynchronized cells (compare lanes 1 to 5 with lanes 6 to 10).

To examine the degradation rate of Yes, HT-29 cells were pulse-labeled with 50 μCi of PRO-MIX[^{35}S] per ml for 1 h, washed, and maintained in the presence or absence of nocoda-

TABLE 1. Relative tyrosine kinase activities of Src and Yes during mitosis of fibroblast and colon carcinoma cells[a]

Cell line	Src		Yes	
	Autophosphorylation	Enolase phosphorylation	Autophosphorylation	Enolase phosphorylation
HT-29	2.6	2.5	0.1	0.3
WiDr	1.5	2.0	0.1	0.3
SW 480	0.5	0.6	0.4	1.0
FLOW 2000	ND	1.9	ND	0.2
208F	1.1	1.3	1.2	1.0
NIH 3T3	1.2	1.3	ND	0.9

[a] Lysates containing 100 μg of total cellular protein were prepared from unsynchronized or nocodazole-treated cells. Proteins were immunoprecipitated with MAb 327 or Yes 3 antibody and incubated with acid-denatured rabbit muscle enolase in an in vitro kinase assay. Radiolabeled proteins were detected by autoradiography and quantified by counting excised gel bands and by scanning densitometry. The two methods gave similar results. Values from two to six independent measurements were averaged for each cell line. Values are those for mitotic cells and are expressed relative to those for unsynchronized controls. ND, not detected.

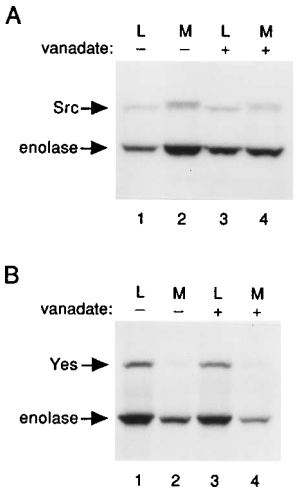

FIG. 5. Effect of the tyrosine phosphatase inhibitor vanadate on mitotic Src and Yes activities in HT-29 cells. Logarithmically growing (L) or nocodazole-treated (M) cells were incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of vanadate for 30 min prior to cell lysis. Src (A) or Yes (B) kinase activity was measured. Exposure times at −70°C with intensifying screens: (A) 15 min; (B) 25 min.

zole for up to 40 h (Fig. 4B). We found that pulse-labeling cells under these conditions delayed their entry into mitosis, possibly because of radiation damage (61). Whereas 98% of nonradiolabeled cells had entered mitosis 20 h after the addition of nocodazole (Fig. 1), only 40% of cells labeled with 50 μCi of PRO-MIX[^{35}S] per ml for 1 h prior to treatment with nocodazole had done so (data not shown). Seventy or 90% of the radiolabeled cells had entered mitosis 30 or 40 h, respectively, after addition of nocodazole. Trypan blue staining showed that 90% of the cells were viable at 40 h (data not shown). We observed that the rate of degradation of Yes in G_2/M cells (Fig. 4B; compare lane 7 with lanes 9 to 11) was significantly faster than that in unsynchronized cells (compare lane 2 with lanes 4 to 6). Thus, the decreased steady-state protein levels of Yes during mitosis appeared to be due to both decreased synthesis and increased degradation of the protein.

Mitotic changes in Src and Yes activities occur in colon carcinoma cell lines other than HT-29. To determine whether mitotic alterations in Src and Yes activities were specific to HT-29 cells, we examined activities of the kinases during mitosis of other cell lines (Table 1). In WiDr colon carcinoma cells, which, like HT-29 cells, have relatively high levels of Src activity in unsynchronized cells (10, 43), Src activity increased approximately twofold during mitosis. In contrast, in SW 480 colon carcinoma cells, which have relatively low levels of Src activity in unsynchronized cells (10, 43), Src activity decreased during mitosis. Thus, mitotic Src activation occurred in those colon carcinoma cell lines with high overall Src activity. With the exception of FLOW 2000 cells, mitotic activation of Src did not occur in fibroblast cell lines. The slower-migrating form of Src was observed in all mitotic cells (data not shown).

As measured by enolase phosphorylation, mitotic Yes activity in HT-29, WiDr, and FLOW 2000 cells was three- to fivefold lower than that in unsynchronized cells. In contrast, mitotic Yes activity in SW 480, 208F and NIH 3T3 fibroblasts was not different from that in unsynchronized cells. In summary, mitotic changes in Src and Yes activity occurred in cell lines other than HT-29.

Inhibition of tyrosine but not serine/threonine phosphatases abolishes the mitotic activation of Src. To investigate whether the mitotic activity of Src or Yes in colon carcinoma cells is affected by tyrosine phosphorylation, nocodazole-treated HT-29 cells were incubated with the tyrosine phosphatase inhibitor sodium vanadate (49, 56) for 30 min prior to lysis (Fig. 5). Following vanadate treatment, mitotic Src activity decreased to levels observed in logarithmically growing cells (Fig. 5A; compare lane 4 with lanes 2 and 3). Vanadate treatment had little effect on mitotic Yes activity, which remained lower than that in unsynchronized cells (Fig. 5B; compare lanes 3 and 4). Thus, inhibition of tyrosine phosphatases abolished the mitotic activation of Src and had little effect on the mitotic activity of Yes. To examine whether the mitotic activity of Src or Yes in colon carcinoma cells is affected by serine and/or threonine phosphorylation, mitotic HT-29 cells were treated with OA, an inhibitor of Ser/Thr phosphatases PP1 and PP2A (3, 57), for 30 min prior to lysis. While Src activity decreased in both unsynchronized and mitotic cells following OA treatment, activity in the mitotic cells was twofold higher than that in the unsynchronized cells (data not shown).

To determine whether prolonged treatment of mitotic cells with phosphatase inhibitors further affected the activity of either kinase, we treated cells with vanadate or OA for up to 150 min after replating in nocodazole-free medium (Fig. 6). Analysis of DNA content showed that >80% of cells treated with either phosphatase for 150 min remained in mitosis (data not shown). Following prolonged treatment with vanadate, mitotic Src activity decreased further, and as previously observed (2), there was a loss of the slower-migrating Src band (Fig. 6A; compare lanes 3 to 5 with lane 1). Following prolonged treatment with OA, mitotic Src activity initially decreased and then increased to levels found in untreated mitotic cells (compare lanes 6 to 8 with lane 1). In addition, the slower-migrating Src band became more evident. Mitotic Yes activity was unaffected by prolonged treatment with either phosphatase inhibitor (Fig. 6B; compare lanes 3 to 5 or 6 to 8 with lane 1).

Mitotic Src is phosphorylated at novel serine and threonine sites and dephosphorylated at Tyr-527. To determine whether mitotic Src from colon carcinoma cells is phosphorylated at novel serine and threonine sites and dephosphorylated at Tyr-527, as it is in pMc-Src cells, we compared the tryptic phosphopeptides of Src from mitotic and unsynchronized HT-29 cells (Fig. 7). Tryptic digestion of Src from unsynchronized cells generated four major phosphopeptides (Fig. 7A). Peptides 1 and 2 are known to be phosphorylated on Tyr-527 (21)

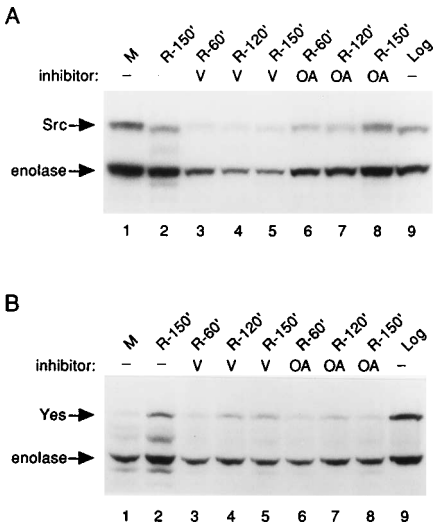

FIG. 6. Effects of prolonged treatment with vanadate or OA on mitotic Src and Yes activities in HT-29 cells. Nocodazole-treated cells were washed and replated in drug-free medium or medium containing vanadate (V) or OA for 60, 120, or 150 min (R-60′ to R-150′). Src (A) or Yes (B) kinase activity was measured. Exposure times at −70°C with intensifying screens: (A) 10 min; (B) 15 min. M, mitotic cells.

by Csk (41), peptide 4 on Ser-17 by protein kinase A (19, 44), and peptide 3 on an amino-terminal serine residue (15). Tryptic digestion of Src from mitotic cells generated six major phosphopeptides (Fig. 7B). Peptides 1 to 4 appeared similar to those from unsynchronized cells, and a mix confirmed that they comigrated (data not shown). However, Tyr-527 and Ser-17 in mitotic cells were phosphorylated at significantly lower levels than in logarithmically growing cells. Quantitative analysis showed a 45% decrease in phosphorylation of Tyr-527 on Src in mitotic cells. Phosphoamino acid analysis of the two novel phosphopeptides of mitotic Src (peptides 5 and 6) showed that peptide 5 was phosphorylated on serine and peptide 6 was phosphorylated on both serine and threonine (data not shown) (Fig. 7C). Thus, mitotic Src from HT-29 cells, similar to that from pMc-Src cells, was phosphorylated at novel serine and threonine sites and dephosphorylated at Tyr-527.

Tyrosine phosphorylation of cellular proteins is altered during mitosis of colon carcinoma cells. To examine the phosphorylated substrates of tyrosine kinases in cycling colon carcinoma cells, we performed immunoblotting with PY20 (25), an antiphosphotyrosine MAb. In unsynchronized cells (Fig. 8A), tyrosine phosphorylation of proteins was higher in a colon carcinoma cell line which has high levels of Src and Yes activity (HT-29; lane 3) than in one with low levels of activity (SW 480; lane 4) or in normal colonic cells (FHC; lane 2) or human fibroblasts (FLOW 2000; lane 1). The major tyrosine-phosphorylated proteins in HT-29 cells (p140, p125, and p60) were very similar to those detected previously (using a polyclonal antiphosphotyrosine antibody) in this colon carcinoma cell line and others which are known to contain high levels of Src and Yes activity (10, 43). Thus, colon carcinoma cell lines with elevated Src and Yes activity as measured in vitro had elevated levels of tyrosine-phosphorylated proteins as measured in vivo.

In cycling HT-29 cells (Fig. 8B), proteins phosphorylated on tyrosine during interphase were very similar to those in unsynchronized cells (compare lanes 1 to 4 with lane 6). However, a striking change in the pattern of tyrosine phosphorylation occurred during mitosis (lane 5). Here, tyrosine phosphorylation of two proteins (p160 and p180) increased considerably, whereas that of several other proteins (notably p140, 125, and p60) decreased. p60 is possibly Src, which is dephosphorylated at Tyr-527 during mitosis (Fig. 7). p125 comigrated with the focal adhesion kinase FAK (data not shown). Mechanical shake-off experiments demonstrated similar changes in tyrosine phosphorylation of proteins during mitosis (data not shown). Thus, tyrosine phosphorylation of cellular proteins was altered during mitosis of HT-29 cells.

DISCUSSION

Our results indicate that Src activity is elevated throughout the HT-29 colon carcinoma cell cycle as it is in polyomavirus mT- or F527 Src-transformed cells, and Yes activity, which is lower than that of Src in the carcinoma cells, is regulated differently during mitosis. We found several reasons why the activities of these closely related kinases are different during mitosis. First, the specific activity of Src but not Yes increased

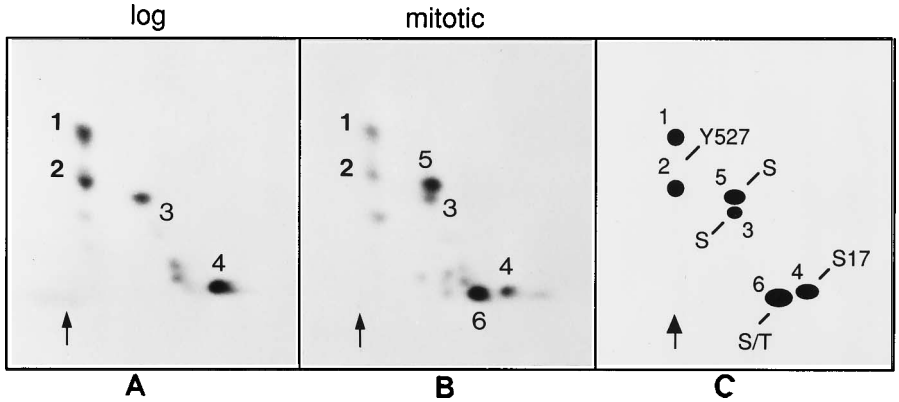

FIG. 7. Tryptic digestion of phosphorylated Src from unsynchronized or mitotic HT-29 cells. Unsynchronized or nocodazole-treated HT-29 cells were incubated with $^{32}P_i$ for 10 h, radiolabeled Src was isolated and digested with trypsin, and the phosphopeptides were resolved on TLC plates by electrophoresis (pH 1.9) in the horizontal dimension (anode at the left) and chromatography in the vertical dimension. The origin is indicated by a vertical arrow. (A) Phosphopeptides of Src from logarithmically growing cells; (B) phosphopeptides of Src from mitotic cells; (C) schematic of Src phosphopeptides and their phosphoamino acid composition. Y, tyrosine phosphorylation; S, serine phosphorylation; T, threonine phosphorylation. Each plate was loaded with 270 Cerenkov cpm. Exposure time at −70°C with intensifying screens was 3 days.

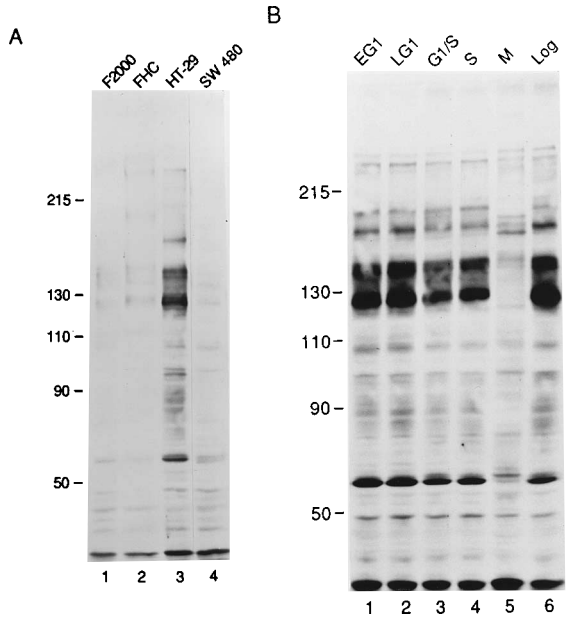

FIG. 8. Analysis of tyrosine-phosphorylated proteins during the cell cycle of colon carcinoma cells. Lysates containing 40 μg of total cellular protein were resolved by SDS-PAGE, transferred to PVDF membranes, and incubated with antiphosphotyrosine antibody PY20. Lysates were prepared from unsynchronized human fibroblasts (FLOW 2000 [F2000]), normal colonic cells (FHC), or colon carcinoma cells (HT-29 or SW 480) (A) or HT-29 cells arrested in different phases of the cell cycle (B). Positions of molecular weight markers in kilodaltons are shown at the left. M, mitotic cells.

during mitosis (Fig. 2). Src activation was likely due to dephosphorylation of Tyr-527 and possibly to phosphorylation of novel serine and threonine sites (Fig. 7). Dephosphorylation of Tyr-527 is well known to be a major upregulator of Src activity (8, 35, 46, 47). Moreover, Shalloway et al. (2, 54) and Kaech et al. (32) showed that this mechanism is required for activation of Src during mitosis of pMc-Src cells. Previously, we did not detect dephosphorylation at Tyr-527 in unsynchronized colon carcinoma cells. It is possible that tryptic phosphopeptide mapping is not sensitive enough to detect this change in the 16% of unsynchronized cells which are in mitotic phase (Fig. 1). Whether dephosphorylation of Tyr-527 during mitosis is due to activation of a tyrosine phosphatase and/or inactivation of a tyrosine kinase is unknown. The level of Csk protein is essentially the same in mitotic and unsynchronized NIH 3T3 cells (50). Overexpression of the receptor tyrosine phosphatase α in rat embryo fibroblasts results in Src activation by dephosphorylation of Tyr-527, cell transformation, and tumorigenesis (67). Recently, the Syp tyrosine phosphatase has been shown to dephosphorylate Src at Tyr-527 in vitro (45). Thus, these tyrosine phosphatases are potential regulators of Src activity during mitosis.

Phosphorylation of Ser-72, Thr-34, and Thr-46 are required for mitotic activation of chicken Src in pMc-Src cells (2, 32, 54). Mitotic Src from colon carcinoma cells had two novel tryptic phosphopeptides (Fig. 7). Peptide 5 was phosphorylated on serine, and peptide 6 was phosphorylated on serine and threonine. The mobility of peptide 5 was very similar to that of a peptide phosphorylated on Ser-72 in mitotic pMc-Src cells (54). Likewise, the mobility of peptide 6 was similar to that of a peptide phosphorylated on Thr-34 in mitotic pMc-Src cells (54) and to a peptide phosphorylated on Ser-12 by protein kinase C after stimulation of NIH 3T3 cells with serum (29) or after stimulation of the AtT20 pituitary tumor cell line with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (28). Ser-72 in chicken Src corresponds to Ser-75 in human Src. Similarly, Thr-34 in the chicken sequence corresponds to Thr-37 in the human sequence. Thus, although we lack direct proof, peptide 5 is possibly phosphorylated on Ser-75 and peptide 6 is possibly phosphorylated on Thr-37 and/or Ser-12. It is possible that altered phosphorylation of Src during mitosis of colon carcinoma cells is due to artifacts of drug treatment. However, this seems unlikely because similar alterations were observed in mitotic pMc-Src cells which had been isolated by mechanical shake-off without drug treatment (17).

Another reason why Src and Yes activities differ during mitosis of colon carcinoma cells is the decrease in abundance of the Yes protein, which fully accounts for the decrease in Yes activity. Lower levels of Yes protein were not due to poorer extraction of proteins from mitotic cells or activation of proteases during lysis, because we observed similar results when cells were lysed in boiling SDS buffer (Fig. 3A). Nor were lower levels of Yes protein due to artifacts of drug treatment, because we obtained similar results when mitotic cell populations were harvested by mechanical shake-off without drug treatment (Fig. 3A). Mechanisms which regulate Yes protein expression during mitosis are being investigated. Our initial results suggest that decreased steady-state protein levels of Yes during mitosis are due to both decreased synthesis and increased degradation of the protein (Fig. 4). Decreased synthesis could be due to decreased mRNA expression or stability. Increased degradation could be a result of activation of proteases or inactivation of their inhibitors.

Prolonged treatment of mitotic colon carcinoma cells with either vanadate or OA perturbed the cell cycle by prolonging mitosis. While the mechanisms for this are unknown, OA is known to induce a pseudo-mitotic phenotype which includes cell rounding, premature chromosome condensation, appearance of mitosis-specific antigens, dispersion of nuclear lamins, and the appearance of mitotic asters, when it is added to the culture medium of rodent fibroblasts (34, 63). Thus, OA may induce mitosis in unsynchronized cells and prolong it in cells already in mitosis. We observed that OA treatment of mitotic colon carcinoma cells caused an initial decrease in Src activity and then an increase to levels found in untreated mitotic cells (Fig. 6A). The reason for the initial decrease is unclear. OA could possibly have a toxic effect on enzyme activity, but this seems unlikely because it did not cause a similar decrease in Yes activity (Fig. 6B). With time, OA caused an increase in mitotic Src activity but not above baseline levels seen in untreated mitotic cells. Current evidence indicates that OA mediates its effect on Src activity indirectly, by upregulating a Tyr-527 phosphatase through serine/threonine phosphorylation (16). If this is true, then the Tyr-527 phosphatase may be maximally activated and/or mitotic Src may be maximally dephosphorylated at Tyr-527 in the carcinoma cells, and therefore treatment with OA would not further affect its activity. Another possibility is that treatment for longer than 150 min is required to see an effect of OA on mitotic Src activity. We observed that prolonged treatment of mitotic colon carcinoma cells with vanadate decreased mitotic Src activity below interphase levels (Fig. 6A). One explanation is that the decreased activity is due to a toxic drug effect, but again this seems unlikely because vanadate treatment did not induce a similar drop in Yes activity (Fig. 6B). Another explanation is that Src activation during interphase of colon carcinoma cells is due, in part, to dephosphorylation of Tyr-527.

Our previous studies (10, 43) and those of Bolen et al. (5) and Talamonti et al. (58) show that for both Src and Yes,

protein abundance and specific activity are higher in unsynchronized colon carcinoma cells than in fibroblasts or normal colonic cells. Here we report that Src and Yes protein levels and specific activities are also elevated during interphase in the carcinoma cells. As discussed above, the increased specific activity during interphase may be partly due to dephosphorylation of Tyr-527 on Src and Tyr-537 on Yes. We find that for any one colon carcinoma cell line, Src activity in unsynchronized cells is usually higher than that of Yes. It is possible that the overall higher Src than Yes activity in the unsynchronized cells is due to the subpopulation of mitotic cells which contain activated Src and inactivated Yes.

Several lines of evidence suggest that the function and regulation of Src in cells may differ from that of Yes. First, activation of Src, but not Yes, is required for induction of mammary tumors in transgenic mice expressing polyomavirus mT (30). Second, Yes, but not Src, expression is elevated in human malignant melanoma (38). Third, calcium-dependent activation of Src and inactivation of Yes occur in a variety of human cells, including differentiating keratinocytes, kidney tubular cells, and fibroblasts (65). One possible explanation is that Src and Yes bind, perhaps via their amino-terminal unique domains, to different regulatory proteins. For example, in differentiating keratinocytes, Yes, but not Src, complexes with two distinct cellular proteins of about 110 and 220 kDa which inhibit Yes activity in vitro (66). Another possibility is different subcellular localization of the kinases resulting, perhaps, in access to different regulatory proteins. In malignant melanoma cells, Yes localizes to the plasma membrane, perinuclear, and cytosolic compartments whereas Src predominantly associates with plasma membranes (38). Another possibility is that Src and Yes have distinct substrates and that tyrosine phosphorylation of these substrates leads, directly or indirectly, to upregulation of Src activity and downregulation of Yes activity. Unique-domain-binding proteins and subcellular localization of Src and Yes in mitotic colon carcinoma cells are being investigated.

Of interest is the 125-kDa protein whose tyrosine phosphorylation was greatly reduced during mitosis of colon carcinoma cells (Fig. 7). This protein comigrates with FAK, a tyrosine-phosphorylated focal adhesion kinase which binds to the SH2 domains of Src (18, 51, 52, 64) and Csk (50). The binding of FAK to Csk is required for Csk's suppression of Src activity. Tyrosine phosphorylation of FAK is known to be greatly reduced during mitosis of NIH 3T3 cells (50). Thus, mitotic activation of Src in the colon carcinoma cells could be due, in part, to decreased tyrosine phosphorylation on FAK, which, in turn, results in decreased binding to Csk and subsequent activation of Src.

In summary, we have shown that Src activity is elevated throughout the HT-29 colon carcinoma cell cycle as it is in polyomavirus mT- or F527 Src-transformed cells, and Yes activity, which is lower than that of Src in the carcinoma cells, is regulated differently during mitosis. Sustained activation of the kinases during interphase, together with changes in their activities during mitosis, may result in altered or inappropriate timing of substrate phosphorylation and perturbation of control mechanisms which govern intestinal cell growth.

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