Potential Molecular Mechanism for c-Src Kinase-mediated Regulation of Intestinal Cell Migration

The ubiquitously expressed Src tyrosine kinases (c-Src, c-Yes, and c-Fyn) regulate intestinal cell growth and differentiation. Src activity is also elevated in the majority of malignant and premalignant tumors of the colon. The development of fibroblasts with the three ubiquitously expressed kinases deleted (SYF cells) has identified the role of Src proteins in the regulation of actin dynamics associated with increased cell migration and invasion. Despite this, unexpectedly nothing is known about the role of the individual Src kinases on intestinal cell cytoskeleton and/or cell migration. We have previously reported that villin, an epithelial cell-specific actin-modifying protein that regulates actin reorganization, cell morphology, cell migration, cell invasion, and apoptosis, is tyrosine-phosphorylated. In this report using the SYF cells reconstituted individually with c-Src, c-Yes, c-Fyn, and wild type or phosphorylation site mutants of villin, we demonstrate for the first time the absolute requirement for c-Src in villin-induced regulation of cell migration. The other major finding of our study is that contrary to previous reports, the nonreceptor tyrosine kinase, Jak3 (Janus kinase 3), does not regulate phosphorylation of villin or villin-induced cell migration and is, in fact, not expressed in intestinal epithelial cells. Further, we identify SHP-2 and PTP-PEST (protein-tyrosine phosphatase proline-, glutamate-, serine-, and threonine-rich sequence) as negative regulators of c-Src kinase and demonstrate a new function for these phosphatases in intestinal cell migration. Together, these data suggest that in colorectal carcinogenesis, elevation of c-Src or down-regulation of SHP-2 and/or PTP-PEST may promote cancer metastases and invasion by regulating villin-induced cell migration and cell invasion.

Src proteins play a critical role in the proliferation and differentiation of normal intestinal epithelial cells (1). In the normal intestinal mucosa, proliferative, undifferentiated cells at the base of the crypts express high c-Src activity; in contrast, differentiated cells at the tips of intestinal villi show decreased c-Src activity (1). Deregulation of Src leads to constitutive kinase activity, which also leads to cellular transformation (2, 3). Src activation is evident in 80% of human colon cancers relative to normal colonic epithelium (4) and is highly activated in colon cancers that metastasize to the liver (4, 5). The risk of colon cancer is particularly high in patients with inflammatory bowel disease, and elevated c-Src activity has also been noted in neoplastic ulcerative colitis epithelia (6). Although up-regulation of c-Yes is less frequent, it has been noted in about 50% of colorectal carcinomas (7–10). Despite the fact that there is considerable evidence associating aberrant Src activation in the development and progression of colorectal cancer, and although inhibitors of Src family kinases have entered phase I clinical trials as anticancer agents, surprisingly few investigations have analyzed the consequences of Src activation on intestinal epithelial cell function (11).

Metastasis is the most lethal property of cancer cells, and critical to this is the increased migratory state of tumor cells. One of the major functions of Src kinases is to modulate the actin cytoskeleton that controls cell migration (12). c-Src kinase activity is required for growth factor-induced migration and scattering of epithelial cells (13, 14) as well as to polarize cells to form lamellipodia (15). c-Src-deficient fibroblasts have decreased random migration (16), whereas cells deficient in all three Src kinases are unable to migrate into a wound (17). These studies imply a general role for Src proteins in actin assembly and turnover. However, the exact mechanism by which Src proteins induce remodeling of the actin cytoskeleton remains unknown.

Critical biological processes, such as cellular migration, require a coordinated and constant remodeling of the actin cytoskeleton that necessitates the concomitant action of antagonistic enzymes. Protein-tyrosine phosphatases (PTPs)3 counteract the tyrosine kinase signaling to limit inappropriate cell motility. A number of PTPs have been implicated in the regulation of cell motility; principal among them are SHP-2 and PTP-PEST (PTP proline-, glutamate-, serine-, and threonine-rich sequence) (18–21). Both SHP-2 and PTP-PEST have also

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3 The abbreviations used are: PTP, protein-tyrosine phosphatase; DN c-Src, dominant negative c-Src; VIL/FL, full-length human villin; VIL/ANFM, NH2-terminal tyrosine phosphorylation site mutant of villin; VIL/AYFM, tyrosine phosphorylation site mutant of villin lacking all 10 identified tyrosine residues; GST, glutathione S-transferase; SH2 and SH3, Src homology 2 and 3, respectively; Ad-EGFP, adenovirus-enhanced green fluorescent protein; EGF, epidermal growth factor; MDCK, Madin-Darby canine kidney; RT, reverse transcription.
been shown to reduce carcinoma invasiveness-related properties of cells in vitro (22, 23). Both SHP-2- and PTP-PEST-regulated cell signaling have been noted in intestinal tissue (24). Overexpression of SHP-2 has been shown to inhibit epithelial cell migration and morphogenesis in collagen gels (25), whereas defects in PTP-PEST have been noted in some colon cancers (26, 27).

Villin is an actin-polymerizing and -depolymerizing protein that is expressed in most significant amounts in intestinal and renal epithelial cells (reviewed in Refs. 28 and 29). Studies done with the villin−/− mice have demonstrated the importance of villin in cytoskeletal remodeling, cellular plasticity, apoptosis, and cell migration (30–34). All of these functions of villin require tyrosine phosphorylation of villin (30, 31, 33, 35, 36). We have identified ten tyrosine residues in villin that are required for its function in cell migration (31, 36). In vitro recombinant villin can be phosphorylated by c-Src kinase (37), whereas in cells, inactivation of Src proteins by pharmacological inhibitors prevents villin-induced changes in actin reorganization, cell morphology, and cell migration (32, 38). Because of structural and functional redundancy among the Src proteins, the role of the individual Src proteins in the function of villin have not been determined unambiguously.

The major finding of this study is the identification of the molecular mechanism, namely the stimulatory and inhibitory mechanisms, for the regulation of cell migration by c-Src kinase activation. Our findings provide the first evidence that active c-Src kinase and not c-Yes or c-Fyn directs the phosphorylation of an epithelial cell-specific actin-binding protein, villin, thus guiding cell migration. Our study also demonstrates that the phosphorylation status of villin, which is determined by c-Src kinase activation, can be regulated by inactivation of c-Src catalytic activity by the tyrosine phosphatases, SHP-2 and PTP-PEST. In addition, our study provides detailed analysis of the expression of Jak3 in intestinal epithelial cell lines and tissue and disproves previous descriptions of Jak3 expression in intestinal tumor cell lines. Data presented in this report provide a biochemical and cellular model that may help to explain these and other cases of changes in actin dynamics and cell motility by Src activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—SYF, SYF/c-Src, and Caco-2 cells (clone C2BB1e) were purchased from ATCC. HT29/19A clone was a kind gift from Dr. A. P. Naren (University of Tennessee, Memphis, TN). Jurkat cells were a kind gift from Dr. M. Radic (University of Tennessee, Memphis, TN). Antibodies were purchased from the following sources: villin, c-Fyn and SHP-2 (BD Transduction Laboratories); villin (Immunotech); glutathione S-transferase (GST) and enolase (Sigma); c-Src (c-Src-GD11; Upstate Biotechnology); c-Yes (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); Y416 to phospho-c-Src (Cell Signaling); PTP-PEST (Chemicon); phosphotyrosine antibody (PY-20; ICN); Jak3 (Santa Cruz Biotechnology and Upstate Biotechnology). Human Jak3 plasmid and polyclonal antibodies to Jak3 were a kind gift from Dr. John O’Shea (National Institutes of Health, Bethesda, MD). Human Syk2 plasmid and antibodies were a kind gift from Dr. S. C. Mueller (Georgetown University Medical School, Washington D. C.). c-Yes plasmid was a kind gift from Dr. Marius Súdol (Geisinger Health System, Danville, PA). Recombinant adeno-viral c-Fyn was a kind gift from Dr. Cynthia Mastick (University of Nevada School of Medicine). Recombinant adeno-virus expressing constitutively active PTP-PEST and SHP-2, the recombinant EGFP adenovirus, and dominant negative c-Src kinase were a kind gift from Dr. Aviv Hes-sid (University of Tennessee, Memphis, TN). GST yc was a kind gift from Dr. R. A. Kirken (University of Texas, El Paso, TX). DIFF Quick was purchased from IMEB Inc. An SH2 domain array was purchased from Panomics. Recombinant c-Src kinase, recombinant Jak3, PP2, Jak3 inhibitor VI (3′-pyridyl oxindole), and the Jak3 inhibitor negative control WHI-P258 (4-phenylamino-6,7-dimethoxyquinazoline) were purchased from Calbiochem. p-Nitrophenyl phosphate was purchased from Upstate Biotechnology.

**Expression of c-Yes, c-Fyn, Jak3, Syk2, SHP-2, and PTP-PEST in SYF Cells**—SYF cells were stably transected with c-Yes (SYF/c-Yes), Jak3 (SYF/Jak3), or Syk2 (SYF/Syk2) using Lipofectamine (39). To express c-Fyn (SYF/c-Fyn), SHP-2, and PTP-PEST, SYF cells were either mock-infected with Ad-EGFP (vector) or infected with these specific recombinant adenoviruses at a multiplicity of infection of 5 by using standard protocol (40). Cell lysates were analyzed by Western blotting for the expression of these specific proteins. Clones expressing comparable levels of these proteins were used in this study.

**Expression of Wild-type or Mutant Villin**—Full-length human villin (VIL/FL) or mutant villin (VIL/ANFM and VIL/AYFM) were cloned and stably transected in MDCK Tet-Off or HeLa Tet-Off cells as described previously (31, 32). Alternatively, SYF and SYF/c-Src cells were transiently transsected with superenhanced yellow fluorescent protein (SEYFP)-tagged full-length villin (SEYFP/VIL) or SEYFP-tagged mutant villin protein lacking the N-terminus (Y46F, Y60F, Y81F, and Y256F), SEYFP-VIL/ANFM, essentially as described before (30). Full-length human villin (VIL/FL) and tyrosine phosphorylation site mutant (lacking all 10 phosphorylation sites) of villin (VIL/AYFM) were cloned in the adenoviral vector system as described previously (30). To express full-length human villin (VIL/FL) or mutant villin (VIL/AYFM), SYF cells were either mock-infected with Ad-EGFP (MOI of 5) or infected with these specific recombinant adenoviruses at a multiplicity of infection of 25 by using a standard protocol (40). To examine the expression of endogenous villin in Caco-2 cells, cells were infected with adenovirus to express Ad-EGFP (control) or dominant negative c-Src kinase (DN c-Src), as described previously (32).

**Cell Motility Assay**—Cell motility was measured as described previously (32). Confluent monolayers were scraped with a sharp blade across the diameter of the well, and images were obtained at the initial time of wounding and at various times postwounding by using a Nikon Eclipse TE2000-U inverted microscope equipped with a CoolSnap ES charge-coupled device camera, an Optiscan motorized stage system, and an Intel Pentium IV computer with Metamorph image analysis software. Images were collected by programming the x, y, and z coordinates of each wound location, allowing the stage to return to the precise location of the original wound. Cell migra-
tion was measured by determining the distance moved (in μm) from the original wound margin 12 or 24 h postwounding, depending on the cell type used. Data were normalized to control values (set as 100) and expressed as percentage change in migration compared with control. For Caco-2 cell migration, control refers to cell migration 24 h post-wounding in the absence of any inhibitors and infected with Ad-EGFP. For all other cell types used in this study, control refers to cell migration 12 h postwounding in cells transfected/infected with vector in the absence of inhibitors. Caco-2 cell migration was measured in the presence of mitomycin C (50 μg/ml).

Cell Invasion Assay—HeLa Tet-Off cells stably transfected with villin as described before (32) were plated in 6-well invasion chambers coated with Matrigel (1 × 10⁵ cells/well). Hepatocyte growth factor (50% conditioned media from MRC-5 cell culture supernatant) was added to the lower chamber, and cells were incubated overnight. Cells that had transmigrated to the lower surface of the membrane were stained using Diff-Quick, and a total of 20 fields per filter were examined for each cell type. Cell invasion was measured in the absence or presence of the Src kinase inhibitor PP2 (100 nM). In addition, cell invasion was measured in HeLa cells that were infected with adenovirus to overexpress either DN c-Src or vector alone, essentially as described before (32).

Co-immunoprecipitation Analysis—Tyrosine-phosphorylated villin redistributes to the Triton X-100-soluble fraction of the plasma membrane (31, 41). Hence, SYF or SYF cells expressing full-length or mutant villin protein and/or the specific tyrosine kinase/phosphatase were extracted in a buffer containing 1% Triton X-100 and 150 mM NaCl. Tyrosine-phosphorylated villin, c-Src, c-Yes, c-Fyn, Jak3, and Syk2, were immunoprecipitated from SYF/c-Src, SYF/c-Yes, and SYF/c-Fyn cells, respectively (37).

In Vitro Kinase/Phosphatase Assay—To determine if the transfected kinases were catalytically active, the kinases were immunoprecipitated from SYF/c-Src, SYF/c-Yes, SYF/c-Fyn, and SYF/Jak3 cells, respectively, and an in vitro kinase assay was performed as described previously in the absence or presence of the appropriate substrate, enolase (0.125 mg/ml) for the Src kinases and recombinant GST-yc (2 μg) for Jak3 (42, 43). In addition, recombinant villin was tyrosine-phosphorylated in vitro using recombinant c-Src kinase, recombinant Jak3, or by immunoprecipitating the specific kinase from transfected/infected SYF cells, as described previously (37). To measure dephosphorylation of villin, recombinant tyrosine-phosphorylated villin was prepared as described before (37). Phosphorylated villin was incubated with either immunoprecipitated SHP-2 or PTP-PEST in buffer containing 30 mM HEPES, pH 7.5, 120 mM NaCl, and 5 mM dithiothreitol. Samples were incubated for 60 min at 25 °C. The reaction was stopped by boiling the sample with Laemmli buffer. Control experiments were done by incubating recombinant tyrosine-phosphorylated villin with alkaline phosphatase (2.5 units). Tyrosine phosphorylation of the substrates was determined by Western analysis using a phosphotyrosine antibody (PY-20). To measure the phosphatase activity in SYF cells infected with SHP-2 or PTP-PEST, both phosphatasers were immunoprecipitated from these cells, and the activity was measured in phosphate buffer using 10 mM p-nitrophenyl phosphate as the substrate. Samples were incubated for 60 min at 25 °C. Phosphatase activity was terminated by the addition of 0.2 N NaOH. The enzyme activity was determined by measuring the absorbance at 405 nm. A molar absorption constant of 18,330 M⁻¹ cm⁻¹ was used to determine the amount of p-nitrophenol released.

Immunohistochemistry—C57BL/6 mice were sacrificed, and the duodenum, jejunum, ileum, and colon were removed and fixed in 3.7% buffered formalin. Paraffin-embedded sections of 5-μm thickness were prepared and immunostained for Jak3. A negative control lacking the primary antibody was included in each assay.

Total RNA and RT-PCR Amplification—Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer’s instruction. The DNase-treated RNA (2.0 μg) was used as a template for cDNA synthesis with oligo(dT) as primer. For PCR amplification, Jak3 (NM_000215)-specific primers were designed to amplify the conserved region from −14 to +745: 5′-GGCAAGTTGCACTCATGG-3′ (forward) and 5′-GGCT-GGATCCAGCAGGCTC-3′ (reverse). PCR was performed using the Advantage cDNA PCR kit. RT-PCR was carried out on PTC 200 (MJ Research). The PCR product was analyzed on a 1.7% agarose gel and stained with ethidium bromide.

SH2 Domain Array—Tyrosine-phosphorylated recombinant villin protein was generated as described previously (37). In vitro binding of tyrosine-phosphorylated villin with SH2 domain of proteins was checked using an SH2 domain array according to the manufacturer’s instructions.

RESULTS

In Vitro Villin Can Be Tyrosine-phosphorylated by c-Src, c-Yes, and c-Fyn—We have previously reported that villin is tyrosine-phosphorylated both in vitro and in vivo (37, 41) and that phosphorylation of villin is required for its function in cell migration (30, 32). In this report, we characterize the significance of the three ubiquitously expressed Src kinases in the regulation of villin function by expressing c-Src (SYF/c-Src cells), c-Yes (SYF/c-Yes cells), and c-Fyn (SYF/c-Fyn cells) individually in the c-Src, c-Yes, and c-Fyn null (SYF) cells. To examine if the transfected/infected Src kinases in SYF cells were catalytically active, an in vitro kinase assay was performed using enolase as a substrate with c-Src, c-Yes, and c-Fyn immunoprecipitated from SYF/c-Src, SYF/c-Yes, and SYF/c-Fyn cells, respectively. As shown in Fig. 1A, all three Src kinases phosphorylate enolase in vitro. To examine the tyrosine phosphorylation of villin, recombinant full-length human villin protein was phosphorylated in vitro using Src kinases immunoprecipitated from SYF/c-Src, SYF/c-Yes and SYF/c-Fyn cells, respectively (37). In vitro, all three tyrosine kinases phosphorylated recombinant villin protein (Fig. 1B). Similar data were obtained when c-Src, c-Yes, and c-Fyn were immunoprecipitated from the colon adenocarcinoma cell line, Caco-2 (data not shown). Cell lysates immunoprecipitated in the absence of primary antibody (Fig. 1B, from left, lanes 3) and GST protein were used as negative controls (last panel) in this assay. It may be noted that intestinal brush border membranes express all three kinases of the Src family (44). Likewise, most colon adenocarcinoma cell lines,
c-Src, SHP-2, and PTP-PEST Regulate Epithelial Cell Migration

![Figure 1](image-url)

**A**
- IB: PY-20
- IB: c-Src
- IB: c-Yes
- IB: c-Fyn
- Enolase

**B**
- IB: PY-20
- IB: c-Src
- IB: c-Yes
- IB: c-Fyn
- IB: GST

**C**
- SYF
- SYF/c-Src
- SYF/c-Yes
- SYF/c-Fyn

**D**
- Control
- DN c-Src

**Figure 1.** c-Src kinase is required for the intracellular localization of villin at the cell margin. A, Src tyrosine kinase activity was monitored in vitro by measuring phosphorylation of enolase. Phosphorylation of enolase was detected by Western analysis using phosphotyrosine antibody (PY-20). These data are representative of two experiments with similar results. B, recombinant villin protein was phosphorylated in vitro with Src kinases immunoprecipitated from SYF/c-Src, SYF/c-Yes, and SYF/c-Fyn cells, respectively. Immunoprecipitates in the absence of primary antibodies to c-Src, c-Yes, and c-Fyn were run as negative controls (from left, lanes 3). These data are representative of three other experiments with similar results. C, SYF, SYF/c-Src, SYF/c-Yes, and SYF/c-Fyn cells were transiently transfected with SEYFP-VIL/FL. In addition, SYF/c-Src cells were transiently transfected with villin mutant, SEYFP-VIL/ANFM. Bar, 10 μm. *, p < 0.01, n = 20. D, Caco-2 cells were infected with recombinant adenovirus to express either DN c-Src or Ad-EGFP (control). Bar, 10 μm. IB, immunoblot.

including Caco-2 and HT-29, express all three nonreceptor Src tyrosine kinases at significantly high levels (45, 46).

c-Src but Not c-Yes or c-Fyn Regulates Intracellular Distribution of Villin in Cells—Overexpression of villin in epithelial and nonepithelial cell lines results in the localization of villin at or near the cell surface, accompanied by changes in the microfila-

ment structure and cell morphology (32, 47, 48). This intracellular distribution of villin at the cell periphery is required for villin function in cell migration (30–32). We have previously reported that three tyrosine residues in the NH2-terminal domain of human villin (Tyr-60, -81, and -256) regulate this function of villin (32). We elected to identify the specific Src family tyrosine kinase that regulates the intracellular distribution of villin. For these studies, the SYF, SYF/c-Src, SYF/c-Yes, and SYF/c-Fyn cells were transiently transfected with superenhanced yellow fluorescent protein SEYFP-tagged full-length villin (SEYFP-VIL/FL) (49). As shown in Fig. 1C, in the absence of c-Src, c-Yes, and c-Fyn, the majority of villin was distributed intracellularly in the cytoplasm. These data confirm our previous observation that tyrosine phosphorylation of villin is required for its intracellular localization at the cell surface (32). Expression of c-Src kinase in SYF cells resulted in the redistribution of villin to the cell margin. In addition, the cells appeared more rounded less flat. We and others have previously reported that overexpression of villin in fibroblasts and other nonepithelial cells induces drastic changes in the cell morphology and actin reorganization (32, 47, 48), consistent with data shown in Fig. 1C. To confirm these data, we overexpressed the NH2-terminal phosphorylation site mutant (VIL/ANFM) of villin in SYF/c-Src cells. Expression of VIL/ANFM in SYF/c-Src cells revealed largely intracellular distribution of villin (Fig. 1C). Expression of villin in both SYF/c-Yes and SYF/c-Fyn cells showed some cell surface expression of villin, but the majority of the villin protein was distributed intracellularly. Most of the villin protein expressed at or near the cell surface in SYF/c-Yes and SYF/c-Fyn cells appeared in punctate aggregates and was not uniformly expressed as seen in SYF/c-Src cells. Similar punctate distribution of villin was seen intracellularly in both SYF/c-Yes and SYF/c-Fyn cells. A quantitative analysis of these data was obtained by scoring of multiple cells for each cell type, demonstrating that most SYF/c-Src cells expressed villin at the cell
c-Src, SHP-2, and PTP-PEST Regulate Epithelial Cell Migration

To characterize the role of the three Src kinases in villin-induced cell migration, we used SYF, SYF/c-Src, SYF/c-Yes, and SYF/c-Fyn cells infected with adenoviruses to express either Ad-EGFP (VIL/Null), full-length human villin (VIL/FL), or a phosphorylation site mutant of villin that lacked all 10 tyrosine phosphorylation sites in villin (VIL/AYFM) (31). As shown in Fig. 2A, cells expressing comparable levels of villin in all four cell lines were used for these studies. Likewise, VIL/Null and VIL/FL SYF cells were checked for expression of c-Src, c-Yes, and c-Fyn, and clones expressing comparable levels of these Src kinases were used for these studies (Fig. 2B). For quantitative analysis, a loading control of actin was run with each cell lysate, as shown in Fig. 2, A and B. An in vitro wound-healing assay was employed to measure cell migration essentially as described before (31). SYF/c-Src cells migrated significantly faster than SYF cells (2-fold increase, \( p < 0.01, n = 12 \)), consistent with previous reports, underscoring the significance of c-Src kinase in the regulation of cell motility (17) (Fig. 2C). Expression of villin in SYF cells had no effect on cell migration, suggesting that tyrosine phosphorylation of villin is essential for its intracellular distribution at the cell surface and further demonstrate that c-Src but not c-Yes or c-Fyn regulate villin distribution in cells. To demonstrate a similar regulation of endogenous villin in intestinal epithelial cells, the colon adenocarcinoma cells, Caco-2, were infected with recombinant adenovirus to express either vector alone (Ad-EGFP) or dominant negative c-Src kinase essentially as described before (30). As shown in Fig. 1D, over-expression of DN c-Src resulted in the redistribution of villin from the cell margin to the cytoplasm.

**c-Src but Not c-Yes or c-Fyn Is Required for Villin-induced Increase in Cell Migration**—To characterize the role of the three Src kinases in villin-induced cell migration, we used SYF, SYF/c-Src, SYF/c-Yes, and SYF/c-Fyn cells infected with adenoviruses to express either Ad-EGFP (VIL/Null), full-length human villin (VIL/FL), or a phosphorylation site mutant of villin that lacked all 10 tyrosine phosphorylation sites in villin (VIL/AYFM) (31). As shown in Fig. 2A, cells expressing comparable levels of villin in all four cell lines were used for these studies. Likewise, VIL/Null and VIL/FL SYF cells were checked for expression of c-Src, c-Yes, and c-Fyn, and clones expressing comparable levels of these Src kinases were used for these studies (Fig. 2B). For quantitative analysis, a loading control of actin was run with each cell lysate, as shown in Fig. 2, A and B. An in vitro wound-healing assay was employed to measure cell migration essentially as described before (31). SYF/c-Src cells migrated significantly faster than SYF cells (2-fold increase, \( p < 0.01, n = 12 \)), consistent with previous reports, underscoring the significance of c-Src kinase in the regulation of cell motility (17) (Fig. 2C). Expression of villin in SYF cells had no effect on cell migration, suggesting that tyrosine phosphorylation of villin is essential for its intracellular distribution at the cell surface and further demonstrate that c-Src but not c-Yes or c-Fyn regulate villin distribution in cells.

**c-Src kinase is required for villin-induced increase in cell migration.** A, cells were infected with recombinant adenovirus to express vector alone (−VIL/FL) or full-length villin protein (+VIL/FL). Western blots are representative of three other experiments with similar results. B, this Western blot shows comparable levels of c-Src, c-Yes, and c-Fyn in all three cell lines used for this study. C, cell migration was recorded in cells expressing recombinant adenoviral vector (VIL/Null) or full-length villin protein (VIL/FL). Values are means ± S.E. (n = 12); *, \( p < 0.01 \), statistically significant compared with control cells. \( \dagger \), over-expression of DN c-Src resulted in the redistribution of villin from the cell margin to the cytoplasm.

**FIGURE 2.** c-Src kinase is required for villin-induced increase in cell migration. A, cells were infected with recombinant adenovirus to express vector alone (−VIL/FL) or full-length villin protein (+VIL/FL). Western blots are representative of three other experiments with similar results. B, this Western blot shows comparable levels of c-Src, c-Yes, and c-Fyn in all three cell lines used for this study. C, cell migration was recorded in cells expressing recombinant adenoviral vector (VIL/Null) or full-length villin protein (VIL/FL). Values are means ± S.E. (n = 12), *, \( p < 0.01 \), statistically significant compared with control cells. \( \dagger \), over-expression of DN c-Src resulted in the redistribution of villin from the cell margin to the cytoplasm.

surface (\( p < 0.001, n = 20 \); compared with the number of cells that expressed some villin intracellularly; Fig. 1C). These data substantiate our previous observations that tyrosine phosphorylation of villin is required for the function of villin in cell migration, concurrent with our previous data (31, 32). In contrast, expression of villin in SYF/c-Src significantly enhanced cell
migration (40% increase compared with SYF/c-Src cells in the absence of villin, \( p < 0.01, n = 12 \)). These data demonstrate that whereas c-Src increased SYF cell migration, the presence of villin further enhanced SYF/c-Src cell migration. We have previously reported that the villin-induced increase in cell migration is not cell type-specific (30). Consistent with these data, treatment of SYF/c-Src cells expressing SEYFP-tagged VIL/AYFM with EGF (100 ng/ml) resulted in the rapid formation of lamellipodia and membrane ruffles and redistribution of villin to these developing cell surface structures (Fig. 2D). A cell surface marker, wheat germ agglutinin Alexa 555, was also used as an independent marker of cell surface changes (supplemental Fig. 1A). Consistent with data shown in Fig. 2D, cells labeled with wheat germ agglutinin Alexa 555 also showed large membrane ruffles and lamellipodia in SYF/c-Src cells transfected with full-length villin as well as redistribution of villin to these developing lamellipodia. During cell locomotion, development of a polarized phenotype and the formation of a cell surface protrusion in the direction of cell movement is a critical step. A role for c-Src in this event has been demonstrated previously (15). In addition, we have previously reported that villin participates in this rapid actin polymerization during the initial steps of cell migration, thus enhancing the rate of cell migration (30, 31). To validate these data, we over-expressed the villin phosphorylation site mutant, VIL/AYFM, in SYF and SYF/c-Src cells (Fig. 2E). Expression of VIL/AYFM in SYF/c-Src cells had no effect on cell migration \(( p < 0.01, n = 12 \) compared with SYF/c-Src cells transfected with VIL/FL; Fig. 2F). Consistent with these data, treatment of SYF/c-Src cells expressing SEYFP-tagged VIL/AYFM with EGF (100 ng/ml) resulted in no cell surface lamellipodia or membrane ruffle formation (Fig. 2G). Wheat germ agglutinin Alexa 555 was also used as a marker of cell surface changes and likewise showed no significant changes in cell surface morphology.

Expression of c-Yes in SYF cells had no effect on basal SYF or villin-induced cell migration (Fig. 2C). Expression of c-Fyn in SYF cells likewise had no effect on either basal or villin-induced cell migration (Fig. 2C). Together with data presented in Fig. 1, these data demonstrate that whereas villin is a substrate of all three Src kinases in vitro, the function of villin in cell migration specifically requires c-Src kinase activation. These data also substantiate our hypothesis that by examining a specific substrate of the Src kinases, in this case villin, the biological functions of the three Src kinases can be distinguished unambiguously.

Villin-induced Increase in Cell Invasion Is Regulated by c-Src Kinase—Since increased cell migration does not always correlate with increased cell invasiveness (50, 51), we elected to examine the role of c-Src kinase on HeLa cell invasion in the absence or presence of villin by using a Matrigel-based invasion assay. To determine if this function of villin could be regulated by c-Src kinase, which has been associated with increased invasiveness and metastases of tumor cells, we examined the effect of down-regulation of c-Src kinase using the pharmacological inhibitor, PP2. As shown in Fig. 3A, expression of wild-type villin significantly \(( p < 0.001 \text{ compared with VIL/Null cells})\) enhanced cell invasion, whereas in VIL/FL cells pretreated with PP2, there was a significant inhibition of villin-induced cell invasion \(( p < 0.001 \text{ compared with VIL/FL cells in the absence of PP2})\). To validate these observations, we also expressed DN c-Src or vector alone (control) in VIL/Null and VIL/FL cells using recombinant adenovirus, essentially as described before (32). Similar inhibition of villin-induced cell invasion was seen in VIL/Null cells expressing DN c-Src \(( p < 0.05 \text{ compared with VIL/Null cells in the absence of DN c-Src; Fig. 3B})\) and in VIL/FL cells expressing DN c-Src \(( p < 0.001 \text{ compared with VIL/FL cells in the absence of DN c-Src})\). These
data demonstrate that c-Src kinase regulates both villin-induced cell motility and villin-induced increase in cell invasion.

JAK3 Does Not Regulate Tyrosine Phosphorylation of Villin or Villin-Induced Cell Migration, and Jak3 Is, in Fact, Not Relevant of Jak3 in Cell Locomotion. Expression of villin had no effect on either SYF or SYF/Jak3 cell migration, suggesting that phosphorylation of villin by Jak3 cannot regulate villin-induced cell migration in the absence of Src kinases. Therefore, to determine the role of Jak3 in villin-induced cell migration in the absence of Src proteins, we stably transfected SYF cells with full-length human Jak3 (SYF/Jak3 cells) and expressed full-length human villin in SYF and SYF/Jak3 cells (Fig. 4A). To demonstrate that the transfected Jak3 kinase was catalytically active in SYF cells, an in vitro kinase assay was performed using recombinant yc protein as substrate, as described previously (43). For these studies, Jak3 was immunoprecipitated from SYF/Jak3 cells. As shown in Fig. 4B, Jak3 expressed in SYF cells maintains its kinase activity. GST was used as a negative control in this assay. Using the in vitro wound-healing assay, we determined that in the absence of Src proteins, Jak3 had no effect on SYF cell migration, suggesting that Jak3 cannot substitute for c-Src in the regulation of cell motility (Fig. 4C). These data undermine the relevance of Jak3 in cell locomotion. Expression of villin had no effect on either SYF or SYF/Jak3 cell migration, suggesting that phosphorylation of villin by Jak3 cannot regulate villin-induced cell migration in the absence of Src kinases (Fig. 4C). However,
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since villin-induced cell migration has been shown to be regulated by Jak3 in colon cancer cell lines, including Caco-2 (53), we elected to examine the effect of the specific Jak3 inhibitor, V1, on Caco-2 and MDCK cell migration. Caco-2 are human colon adenocarcinoma cells that endogenously express villin and represent the most well characterized “enterocyte-like” intestinal epithelial cell line. MDCK are renal epithelial cells that are not transformed and also function as “enterocyte-like” cells. MDCK cells do not express villin, and we have stably transfected them with human villin, thus allowing us to examine the specific effect of villin and of Jak3 on villin, in the regulation of epithelial cell migration (31, 49). Using these two cell lines, we determined that the Jak3 inhibitor had no effect on either Caco-2 or MDCK cell migration (Fig. 4D). MDCK cells expressing villin in the absence or presence of the Jak3 inhibitor migrated significantly faster than villin-null cells. Unlike previous studies done with HT-29 and Caco-2, both of which endogenously express villin (53), VIL/Null and VIL/FL MDCK cells allow us to definitively conclude that Jak3 does not regulate villin-induced cell migration even in the presence of Src kinases. The Jak3 negative control, WHI-P258, likewise had no effect on cell migration (data not shown). It may be noted that in both studies that have described Jak3 protein in colon cancer cell lines, functional studies were done with the pharmacological inhibitor AG-490 (52), which is now described as a Jak2 inhibitor or with WHI-P131 (53), which has more recently been shown to have effects unrelated to Jak3 (55, 56). Based on these facts, we reasoned that one explanation for the disparate results could be that villin may not be a substrate of Jak3 in cells. Immunoprecipitation of villin from SYF/Jak3 cells confirmed that villin is not phosphorylated in SYF/Jak3 cells (data not shown). To validate these observations, we immunoprecipitated Jak3 from SYF/Jak3 cells to phosphorylate recombinant villin in vitro, since in vitro phosphorylation of villin by Jak3 has been reported (53). As shown in Fig. 4E, our studies demonstrate that Jak3 cannot tyrosine-phosphorylate villin either in cells or in vitro. In vitro phosphorylation of recombinant villin by c-Src kinase was run as a positive control with this assay. Using recombinant Jak3 to phosphorylate recombinant villin in vitro also failed to tyrosine-phosphorylate villin (Fig. 4F). For these studies, untagged recombinant villin was phosphorylated in vitro by recombinant c-Src kinase (positive control) or Jak3. Note that recombinant c-Src kinase was purchased as a GST-tagged protein (~90 kDa in size), and untagged recombinant villin (~95 kDa in size) was phosphorylated in vitro by GST-c-Src kinase. Data shown in Fig. 4F (from left, lane 4) shows a phosphorylated band, which represents autophosphorylated c-Src. In the presence of recombinant villin and c-Src kinase, two tyrosine-phosphorylated proteins were identified. The lower band corresponds to autophosphorylated c-Src, and the upper band corresponds to phosphorylated recombinant villin (from left, lane 5). This was confirmed by Western analysis with villin and c-Src antibodies. No phosphorylation of recombinant villin by recombinant Jak3 was noted by us (Fig. 4F, from left, lane 3). It may be noted that tyrosine phosphorylation of recombinant villin with recombinant Jak3 has been reported by Kumar et al. (53). Our data thus far contradict data presented in this previous study.

In contrast to the relatively ubiquitous expression of Jak1, Jak2, and Tyk2, Jak3 has been reported to have a more restricted and regulated expression, being limited to NK cells and thymocytes, and can be induced in T, B, and myeloid cells. Both studies that have reported the expression of Jak3 in colon adenocarcinoma cell lines lacked adequate controls, which made it difficult to judge whether Jak3 was indeed expressed in intestinal epithelial cells. Hence, we chose to investigate the relative expression of Jak3 in intestinal and renal epithelial cell lines by Western analysis of cell lysates (Fig. 4G) or by immunoprecipitation of Jak3 from these cell lysates (Fig. 4H). SYF cells stably transfected with Jak3 were used as positive controls. Consistent with previously published reports, neither of these approaches detected Jak3 protein in intestinal epithelial cell lines, including HT29/19A cells, which have been shown, specifically, to express Jak3 by Western analysis, immunoprecipitation assay, and confocal microscopy (53). To definitively confirm our observations, we also looked for Jak3 expression in mouse intestinal tissue (duodenum, jejunum, ileum, and colon both in the crypt and villus epithelial cells). Consistent with other data presented in this report, we detected no Jak3 expression in mouse intestinal epithelial cells either in the crypt or in the villi in the small (Fig. 4I) and large intestine (data not shown). Some Jak3-positive cells were noted below the crypts and in the lamina propria that are very likely hematopoietic cells in the submucosa (Fig. 4I (a)). Since villin is only expressed in surface epithelial cells, these data show no Jak3 expression in cells that express villin. Also shown in this figure is the negative control, which was stained in the absence of Jak3 antibody (Fig. 4I (b)). To confirm these data further, we also looked for Jak3 mRNA in the two intestinal epithelial cell lines Caco-2 and HT29/19A, both of which have been reported to express Jak3 (53). RT-PCR was done to detect Jak3 mRNA. RNA-free template was run as a negative control, and Jurkat cells were used as a positive control. S15G was used as a reference control. Jak3 genomic DNA contamination was ruled out by PCR. As shown in Fig. 4I, unlike Jurkat cells, the intestinal cell lines Caco-2 and HT29/19A had no detectable levels of Jak3 mRNA. We believe that our study provides the first detailed analysis of Jak3 expression and function in intestinal epithelial cells and demonstrates convincingly the absence of both Jak3 mRNA as well as protein in intestinal epithelial cells and cell lines.

Using recombinant tyrosine-phosphorylated villin protein and an SH2 domain array, we also identified in vitro the association of tyrosine-phosphorylated villin with the SH2 domain of the tyrosine kinase, Syk2 (data not shown). To test the significance of this interaction in cell migration, we stably transfected SYF cells with Syk2 (SYF/Syk2 cells; Fig. 5A). As shown in Fig. 5B, SYF cells stably transfected with Syk2 migrated like Syk2-null SYF cells. Likewise, SYF/Syk2 cells expressing villin could not recapitulate the villin-induced increase in cell migration seen in SYF/c-Src cells. To confirm these data, we also examined the tyrosine phosphorylation of villin in vitro by Syk2. For these studies, Syk2 was immunoprecipitated from SYF/Syk2, and recombinant villin protein was phosphorylated in vitro. As shown in Fig. 5C, villin is not a substrate of Syk2. In vitro phosphorylation of recombinant villin by c-Src kinase (immunoprecipitated from SYF/c-Src cells) was used as a positive control
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Since SHP-2 and PTP-PEST both inhibited c-Src kinase-regulated cell migration in SYF/c-Src cells, we hypothesized that these PTPs could prevent the catalytic activation of c-Src kinase itself. Hence, we elected to examine the dephosphorylation of c-Src kinase by SHP-2 and PTP-PEST. Src tyrosine kinase activity is principally augmented by phosphorylation of a tyrosine (tyrosine 416) within the kinase domain. c-Src activity has been demonstrated to be negatively regulated by dephosphorylation of this catalytic site through the action of several tyrosine phosphatases (57, 58). For these studies, SYF/c-Src cells expressing either SHP-2 or PTP-PEST were immunoprecipitated with phospho-c-Src antibody (tyrosine 416). The expression of catalytically active c-Src kinase was examined by Western blot using c-Src antibody. A nonspecific band was detected in all of the samples by this antibody (upper band). Only SYF/c-Src cells had detectable levels of activated c-Src kinase (phospho-c-Src (tyrosine 416) lower band indicated with an arrow). The identity of the phospho-c-Src protein was confirmed by Western blot analysis using c-Src antibodies. As shown in Fig. 6H (right) in SYF/c-Src expressing either PTP-PEST (from left, lane 2) or SHP-2 (from left, lane 3), we detected no phospho-c-Src, demonstrating that expression of these phosphatases in SYF/c-Src cells resulted in the dephosphorylation and inactivation of c-Src kinase. Together, these data demonstrate that SHP-2 and

Dephosphorylation of Catalytically Active c-Src Kinase by SHP-2 and PTP-PEST Regulates Villin-induced Increase in Cell Migration—SHP-2 and PTP-PEST are two PTPs that have been shown to regulate both Src activation as well as cytoskeletal reorganization in cells. To get a better understanding of c-Src mediated regulation of villin function, we elected to examine the role of these PTPs in villin-induced cell function. For these studies, we infected SYF/c-Src cells with recombinant adenovirus to overexpress constitutively active SHP-2 or PTP-PEST (Fig. 6A). Expression of catalytically activated SHP-2 and PTP-PEST was confirmed by measuring phosphatase activity using p-nitrophenyl phosphate as the substrate (Fig. 6B). SYF/c-Src cells expressing SHP-2 or PTP-PEST were transiently transfected with SEYFP-tagged full-length villin. As shown in Fig. 6C, overexpression of either SHP-2 or PTP-PEST prevented the cell surface distribution of villin in SYF/c-Src cells. These data suggested that both of these PTPs could modify villin-induced cell migration. To characterize this further, we examined SYF and SYF/c-Src cell migration in the absence or presence of these PTPs. As shown in Fig. 6D, in the absence of c-Src neither of these phosphatases had any effect on either basal (VIL/Null) or villin-induced (VIL/FL) cell motility. However, expression of these PTPs in SYF/c-Src cells inhibited both c-Src-regulated cell migration as well as villin-induced cell migration in SYF/c-Src cells (Fig. 6E). Likewise, the addition of both phosphatases together inhibited SYF/c-Src as well as villin-induced SYF/c-Src cell migration (Fig. 6F). These data were confirmed in the colon cancer cell line, Caco-2, which endogenously expresses villin (Fig. 6G).

The expression of catalytically active c-Src kinase was examined by Western blot using c-Src antibody. A nonspecific band was detected in all of the samples by this antibody (upper band). Only SYF/c-Src cells had detectable levels of activated c-Src kinase (phospho-c-Src (tyrosine 416) lower band indicated with an arrow). The identity of the phospho-c-Src protein was confirmed by Western blot analysis using c-Src antibodies. As shown in Fig. 6H (right) in SYF/c-Src expressing either PTP-PEST (from left, lane 2) or SHP-2 (from left, lane 3), we detected no phospho-c-Src, demonstrating that expression of these phosphatases in SYF/c-Src cells resulted in the dephosphorylation and inactivation of c-Src kinase. Together, these data demonstrate that SHP-2 and
PTP-PEST inhibit SYF/c-Src and villin-induced cell motility in SYF/c-Src cells by inactivating c-Src kinase itself. Other tyrosine phosphatases that were examined but did not contribute to dephosphorylation of villin or phosphorylation of villin by c-Src include DEP1, LAR, and PTP-1B (data not shown).

DISCUSSION

Villin expression is maintained in neoplastic tissue and has been widely used as a marker for primary tumors and tumor metastases from tissue that normally express villin (28). Villin expression has also been noted in all intestinal metaplasia, suggesting that induction of villin expression may play a role in the development of precancerous lesions (28). Studies done with the villin \( ^{-/-} \) mice have clearly established the role of villin in regulating epithelial cell actin dynamics, cell morphology, apoptosis, and epithelial cell plasticity and remodeling during injury and repair (33, 34, 59, 60). It may be noted that the majority of metastatic cancers are carcinomas, and further, most metastatic epithelial cells, such as those derived from pancreatic ductal cells, biliary ductal cells, colon epithelial cells, and esophageal and stomach epithelial cells as well as osteocytes, express villin (28). Thus, understanding the regulation of villin-induced cell motility and cell invasion is imperative to our understanding of metastatic carcinomas.

Src kinases are the most ubiquitously expressed and highly homologous nonreceptor tyrosine kinases. c-Src and c-Yes, for instance, share over 80% homology (61). Because of the high homology and their widely overlapping tissue distribution, there is strong evidence for functional overlap between the three Src kinases (62). Further evidence for this functional redundancy comes from studies done with the c-Src and c-Yes knock-out mice (63). Despite this, a few studies have also indicated specificity between the Src kinases, particularly in their subcellular localization, intermolec-

![Diagram of protein tyrosine phosphatases, SHP-2 and PTP-PEST, regulating villin-induced cell migration by regulating the catalytic inactivation of c-Src kinase.](image)
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FIGURE 7. A schematic representation of c-Src-, SHP-2-, and PTP-PEST-mediated regulation of intestinal cell migration. Our study demonstrates that catalytically active c-Src kinase is required for tyrosine phosphorylation of villin and villin-induced increase in cell migration. Inactivation of catalytically active c-Src kinase by the phosphatases SHP-2 or PTP-PEST by dephosphorylation of the tyrosine residue Tyr-416 within the c-Src kinase domain prevents the phosphorylation of villin, thus inhibiting villin-induced increase in intestinal cell migration.

ular binding partners, and ability to mediate downstream signaling (45, 64–66). The functional role of the individual Src protein-tyrosine kinases c-Src, c-Yes, and c-Fyn has been difficult to establish, primarily because (i) these closely related kinases appear to play redundant roles (e.g. activation of all three kinases by platelet-derived growth factor); (ii) expression of kinase-inactive or dominant interfering variants of individual Src family members interferes with the function of multiple Src tyrosine kinases due to the high degree of homology of the SH2 and SH3 domains; (iii) Src kinases can activate other families of protein tyrosine kinases; and (iv) small molecule inhibitors that block the activity of specific tyrosine kinases have not been developed. However, several cellular events regulated by Src protein-tyrosine kinases can be examined by identifying substrates specifically phosphorylated by these enzymes. In this report, we have used this approach to correlate changes in cell behavior and signaling by examining the phosphorylation of villin and regulation of villin-induced cell migration by c-Src, c-Yes, and c-Fyn.

Most defects associated with the absence of Src kinases have been associated with deficiencies in cellular processes that are dependent on dynamic regulation of the actin cytoskeleton (67, 68). Since cytoskeletal rearrangements are a hallmark of cell transformation, it is likely that c-Src, c-Yes, and c-Fyn may play divergent roles in the onset and/or progression of the transformed phenotype. We hypothesized that functional domain differences between c-Src, c-Yes, and c-Fyn may prevent one or more of these kinases from compensating in signaling pathways that regulate the function of villin in cell migration. Studies reported here were undertaken to identify the signaling speci-ficity between these kinases in villin-induced cell migration. From these studies, we determined that although all three Src kinases can tyrosine-phosphorylate villin in vitro, only c-Src kinase could regulate the villin-induced increase in cell migration. c-Src kinase was required for intracellular distribution of villin, villin-induced changes in cell morphology, and villin-induced increase in cell migration and cell invasion. We have previously reported that all of these properties of villin require tyrosine phosphorylation of villin (31, 32). Genetic evidence implies that Src signaling stimulates cell migration by promoting spreading of the leading edge and turnover of focal adhesions at the trailing edge (67, 69). We have previously reported that the villin-induced increase in cell migration is regulated by the rapid increase in actin polymerization at the leading edge, which is enhanced by tyrosine phosphorylation of villin and by the catalytic activation of PLC-γ1 by phosphovillin (30, 31). Together, with data presented in this study, we can suggest that tyrosine phosphorylation of villin by c-Src kinase promotes cell spreading, thus enhancing cell migration (Fig. 7). The major findings of this study then are as follows: (i) we demonstrate for the first time that c-Src but none of the other ubiquitously expressed Src kinases regulates villin-induced cell migration; (ii) we demonstrate that c-Src kinase regulates the phosphorylation of an actin-binding protein, resulting in changes in cell morphology, cell migration, and cell invasion, physiological functions that have been attributed to Src kinases, thus providing a potential molecular mechanism for Src function in intestinal cells; and (iii) we provide a likely molecular mechanism for c-Src-induced changes in intestinal epithelial cell migration and cell invasion by demonstrating that c-Src regulates the redistribution of an actin binding protein, thus regulating changes in the cellular microfilament structure and cell morphology, both of which are required for cell migration and cell invasion.

A study by Murata and colleagues has demonstrated that Jak3-/-/ mice develop spontaneous inflammatory bowel disease, thus implicating a role for this kinase in intestinal injury and repair (70). It may be noted that several studies have demonstrated that Jak3-/-/ mice exhibit severe combined immunodeficiency but no nonimmune defects (71–73). Consistent with this, Murata et al. (70) determined that the abnormal immune function in the Jak3-/-/ mice was the cause of inflammatory bowel disease-like symptoms in these mice. This report is consistent with the restricted expression of Jak3 in hematopoietic cells. However, one previous report has shown the expression of Jak3 in colon carcinoma tumors and cell lines, suggesting a show a statistically significant (*, \( p < 0.001 \), \( n = 6 \)) increase in phosphatase activity in cells infected with constitutively active SHP-2 and PTP-PEST compared with their respective control cells expressing vector alone. C, SYF/c-Src cells expressing SHP-2 or PTP-PEST transfected with SEYFP-VIL/FL. Bar, 10 \( \mu \)m. D, SYF cells were infected with recombinant adenovirus to express vector alone or VIL/FL, and cell migration rates were determined. E, cell migration rates were determined in SYF/c-Src cells infected with recombinant adenovirus to express vector alone (VIL/Null) or VIL/FL without or with SHP-2 or PTP-PEST. Values are means \( \pm \) S.E. (\( n = 12 \)). †, \( p < 0.01 \), statistically significant compared with control cells; ‡, \( p < 0.01 \), statistically significant compared with SYF/c-Src cells expressing full-length villin. F, cell migration rates were recorded in SYF and SYF/c-Src cells infected with adenovirus to express vector (VIL/Null) or VIL/FL without or with SHP-2 and PTP-PEST. Values are means \( \pm \) S.E. (\( n = 12 \)). *, \( p < 0.01 \), statistically significant compared with cells expressing vector alone; †, \( p < 0.01 \) statistically significant compared with SYF/c-Src cells expressing vector alone. G, cell migration was measured in Caco-2 cells overexpressing SHP-2 and/or PTP-PEST. Values are means \( \pm \) S.E. (\( n = 12 \)). †, \( p < 0.01 \), statistically significant compared with control cells. H (left), dephosphorylation of recombinant tyrosine-phosphorylated villin by PTP-PEST or SHP-2. Alkaline phosphatase (2.5 units) was used as a positive control (from left, lanes 5 and 6). These Western blots are representative of three experiments with similar results. Night, dephosphorylation of catalytically active c-Src kinase by SHP-2 and PTP-PEST measured by immunoprecipitating phospho-c-Src (Tyr-416). From the left, lane 1 shows phospho-c-Src in SYF/c-Src cells (lower band denoted by arrow); lanes 2 and 3 show no phospho-c-Src expression in SYF/c-Src cells expressing either PTP-PEST or SHP-2, respectively. IB, Immunoblot; IP, immunoprecipitation.
role for Jak3 activation in oncogenesis of colon cancer (52). A more recent study has reported the expression of Jak3 in two colon cancer cell lines (Caco-2 and HT29/19A), both of which endogenously express villin, and, further, reported the tyrosine phosphorylation of villin and regulation of villin-induced cell migration by Jak3 in these two cell lines (53). In addition, the authors of this study have reasoned that tyrosine phosphorylation of villin by Jak3 and regulation of villin-induced cell migration by Jak3 could also play a significant role in the pathogenesis of inflammatory bowel disease, often a precursor of colon cancer (53). Since colon cancer cell lines express very high levels of Src proteins and since Src proteins very often activate other tyrosine kinases, we elected to examine the effect of Jak3 on villin-induced cell migration in the absence of Src kinases. Contrary to these published reports, our studies show no effect of Jak3 on either SYF or villin-induced cell migration in SYF cells. Further, we determined that villin cannot be tyrosine-phosphorylated either in cells or in vitro by Jak3. More importantly, we found no expression of Jak3 protein in colon cancer cell lines, including in Caco-2 and HT29/19A cells that have previously been reported to express Jak3 (53). Likewise, we found no expression of Jak3 in intestinal epithelial cells from mice (crypt or villus; duodenum to colon were examined) (53). In addition, RT-PCR allowed us to confirm that in fact colon cancer cell lines used in these published reports (namely Caco-2 and HT29/19A) do not have any detectable levels of Jak3 mRNA. We believe our study is the first that has examined both the expression and potential function of Jak3 in intestinal epithelial cells and tissue in such detail, and our findings demonstrate that Jak3 message and protein are not expressed in intestinal epithelial cells, thus demonstrating that Jak3 is unlikely to have any physiologic function in intestinal epithelial cells and/or pathophysiologic function in colon carcinogenesis. Further, we note that since HT29/19A cells are only available from one source and were obtained by us from the same source, the differences between our findings and these previously published reports cannot be attributed to clonal differences in cells. It may be noted that although our data contradict data published by Lin et al. (52) and Kumar et al. (53), they are consistent with at least three previous studies that have demonstrated the absence of Jak3 protein in HT29 cells (although these were not clone 19A) (74–76). In at least one of these previous reports, the absence of Jak3 in HT29 was also demonstrated by Northern analysis.

Src protein-tyrosine kinases are ordinarily in equilibrium between inactive and primed states by a balance of regulatory kinases and its counteracting tyrosine phosphatase(s). PTP-PEST has been shown to regulate both the dephosphorylation and inactivation of actin-regulatory proteins, such as WASp and filamin, as well as dephosphorylation and inactivation of c-Src kinase itself (77–79). Although PTP-PEST did not regulate the dephosphorylation of phosphovillin, concurrent with these published reports, we determined that overexpression of PTP-PEST does inactivate c-Src kinase activity by dephosphorylating a positive regulatory tyrosine, Tyr-416, within the c-Src kinase domain (Fig. 7). Likewise, SHP-2 has been shown to inhibit c-Src catalytic activity by dephosphorylating tyrosine 416 within the Src kinase domain (78), consistent with our findings in this report. Thus, villin-induced morphological changes and villin-induced cell migration, both of which depend on c-Src kinase activation are inhibited in cells that overexpress either SHP-2 or PTP-PEST (Fig. 7). We speculate that this balance between c-Src kinase and these PTPs could be important for the normal control of epithelial cell motility. Thus, in this report we demonstrate for the first time (i) the dephosphorylation of c-Src kinase itself (Tyr-416) by SHP-2 or PTP-PEST, thus providing a molecular mechanism for c-Src down-regulation that could be extended to other cell types; (ii) the role of SHP-2 and PTP-PEST in the regulation of villin, an actin-binding protein that shares structural and functional homology with a large family of actin-binding proteins; and (iii) the possible role of both SHP-2 and PTP-PEST in the regulation of intestinal cell function, namely cell migration and cell invasion. SYF cells are nonpolarized cells, whereas intestinal epithelial cells that express villin are polarized. However, all our previous studies as well as those reported by other investigators demonstrate that the function of villin in either actin reorganization, regulation of cell morphology, cell migration, or apoptosis is independent of the cell type (32, 33, 47, 48, 60). Based on these observations, we feel confident that although our studies were done in SYF cells, they very likely identify the molecular mechanism for c-Src kinase-mediated regulation of intestinal cell migration. The aberrant regulation of Src family kinases and/or SHP-2 and PTP-PEST has been shown to associate with the initiation and progression of various cancers. Whether villin-induced increase in cell migration and cell invasion is aberrantly regulated by c-Src kinase and/or SHP2/PTP-PEST during colon cancer progression and whether it contributes to colon tumor invasion and metastasis remain to be investigated in the future. However, data presented here indicate that modulation of villin tyrosine phosphorylation by c-Src, SHP-2, and PTP-PEST is critical for the induction of the actin-regulatory functions of villin in these cells, contributing to both the migratory and invasive property of intestinal epithelial cells, including colon cancer cell lines. Data shown in this report together with our previous studies demonstrate that attenuation of c-Src activity as well as inhibition of villin phosphorylation can inhibit migration of human colon cancer cells. We have previously shown that overexpression of the villin phosphorylation site mutant, VIL/AYFM, in the human adenocarcinoma cells, Caco-2, inhibits cell migration (30, 38). Likewise, we have shown that treatment with PP2 or dominant negative c-Src inhibits Caco-2 cell migration (30, 38). In this report, we demonstrate that overexpression of SHP-2 and PTP-PEST inhibits Caco-2 cell migration. The data are the first, to our knowledge, to demonstrate a role for a specific Src kinase in the regulation of intestinal cell migration and specifically, colon cancer cell migration. Elucidation of the contribution of the specific tyrosine kinases to intestinal cell migration may lead to the design of rational drugs capable of specifically blocking the unique signaling pathways utilized by these kinases and would be ideal for studying the biological pathways affected by individual Src family members. This approach could also prove beneficial in the treatment of colon cancer in which Src kinase is abnormally activated, since they could avoid the potentially deleterious effects of inhibiting the function of multiple Src family members. Our data may help...
guide the choice of drugs to be used in the treatment of colon cancer.

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