Molecular Biology of the Cell
Vol. 15, 2794–2803, June 2004

Src SH3/2 Domain-mediated Peripheral Accumulation of Src and Phospho-myosin Is Linked to Deregulation of E-cadherin and the Epithelial-Mesenchymal Transition

Egle Avizienyte,*† Valerie J. Fincham,* Valerie G. Brunton,* and Margaret C. Frame*†‡

*Beatson Institute for Cancer Research, Cancer Research UK Beatson Laboratories, Bearsden, Glasgow G61 1BD, United Kingdom; and ‡Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

Submitted December 10, 2003; Revised March 30, 2004; Accepted March 30, 2004
Monitoring Editor: Carl-Henrik Heldin

Elevated Src kinase in epithelial cancer cells induces adhesion changes that are associated with a mesenchymal-like state. We recently showed that Src induces dynamic integrin adhesions in KM12C colon cancer cells, whereas E-cadherin–dependent cell-cell contacts become disorganized. This promotes a fibroblastic-like morphology and expression of the mesenchymal marker vimentin. Furthermore, Src-induced deregulation of E-cadherin, and the associated mesenchymal transition, is dependent on integrin signaling (Avizienyte *et al.*, Nat. Cell Biol. 2002, *4,* 632–638), although the nature of downstream signals that mediate these Src- and integrin-dependent effects are unknown. Here we show that the SH2 and SH3 domains of Src mediate peripheral accumulation of phospho-myosin, leading to integrin adhesion complex assembly, whereas loss of SH2 or SH3 function restores normal regulation of E-cadherin and inhibits vimentin expression. Inhibitors of MEK, ROCK, or MLCK also suppress peripheral accumulation of phospho-myosin and Src-induced formation of integrin-dependent adhesions, whereas at the same time restoring E-cadherin redistribution to regions of cell-cell contact. Our data therefore implicate peripheral phospho-myosin activity as a point of convergence for upstream signals that regulate integrin- and E-cadherin–mediated adhesions. This further implicates spatially regulated contractile force as a determinant of epithelial cell plasticity, particularly in cancer cells that can switch between epithelial and mesenchymal-like states.

INTRODUCTION

The switch between epithelial- and mesenchymal-like phenotypes occurs during embryonic development and during the later stages of epithelial cancer progression. Cells that undergo the epithelial to mesenchymal transition (EMT) become morphologically altered, losing many of their epithelial characteristics. Specifically, loss of E-cadherin function induced by multiple mechanisms is often associated with EMT in carcinoma cells, whereas elevated or activated tyrosine kinases are often linked to gain of the mesenchymal phenotype (reviewed in Thiery, 2002). In general, epithelial cancer cells that have undergone EMT are regarded as potentially more migratory, and, in turn, may be more invasive or metastatic. In this regard, it is known that elevation or activation of oncogenic signal transduction proteins, including Src and Ras, may contribute to tumor spread via promotion of the mesenchymal phenotype (reviewed in Thiery, 2002). For example, in the case of the rat bladder carcinoma cell model, both oncoproteins induce a mesenchymal state but use different mechanisms (Boyer *et al.*, 1997).

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03–12–0879. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03–12–0879.

† Corresponding authors. E-mail addresses: m.frame@beatson.gla.ac.uk and e.avizienyte@beatson.gla.oc.uk.

Our work in KM12C colon cancer cells derived from the Fidler model of colorectal metastasis (Morikawa *et al.*, 1988) showed that expression of activated c-Src kinase induces an EMT with assembly of integrin adhesion structures and deregulation of E-cadherin (Avizienyte *et al.*, 2002; Jones *et al.*, 2002). We further showed that Src cooperates with integrin-dependent signals to induce a mesenchymal-like state and to suppress E-cadherin function. However, the specific signals downstream of Src and integrin engagement that mediate the transition between epithelial- and mesenchymal-like states are not known.

Breast epithelial cells (MCF10A) that express exogenous activated H-Ras also acquire a mesenchymal/fibroblastic morphology with decreased cell-cell junctions and increased focal adhesions and associated stress fibers (Zhong *et al.*, 1997). Because of activation of RhoA, the oncogene-induced EMT in these cells is accompanied by elevated phosphorylation of myosin light chain (MLC) when compared with untransformed cells, resulting in increased contractility. However, although inhibition of RhoA partially restores normal morphology, cell-cell junctions do not reform. These findings imply that multiple events triggered by oncogenic Ras are required for the fully transformed phenotype of these epithelial cells (Zhong *et al.*, 1997). Because oncogenic Ras in breast epithelial cells produces a similar phenotype to elevated c-Src activity in KM12C colon cancer cells, we addressed whether one of the primary signaling pathways

downstream of Ras, the mitogen-activated protein kinase cascade involving MEK and ERK/MAP kinase, is required for Src-induced EMT. Interestingly, ERK/MAP kinase is known to be constitutively active in Src-transformed cells and is required for transformation (Gupta *et al.*, 1992; Mansour *et al.*, 1994). Additionally, it is reported that MLC kinase (MLCK) is a direct substrate of ERK/MAP kinase during cell migration (Klemke *et al.*, 1997). This, together with the proposed role for myosin-dependent contractility in Ras-induced mesenchymal transition in breast epithelial cells (Zhong *et al.*, 1997), raises the possibility that ERK/MAP kinase and MLCK/myosin activities may function downstream of c-Src to elicit its effects in KM12C colon cancer cells.

Here, we demonstrate that ERK/MAP kinase and MLCK activities are critical mediators when Src induces assembly of peripheral adhesion complexes with concomitant deregulation of E-cadherin. We show that modulating Src's localization and activity, and SH2- and SH3-dependent accumulation of phospho-myosin at the cell periphery, determines whether or not cadherins localize properly to sites of intercellular contact and whether the cells acquire, and maintain, a mesenchymal- or epithelial-like morphology.

MATERIALS AND METHODS

Cell Culture and Cell Lines

Colon carcinoma cells (KM12C) from the Fidler model of colon cancer metastasis (a gift from I. J. Fidler, University of Texas M.D. Anderson Center, Houston, TX) and KM12C cells expressing active Src (KM12C/Src527F; Avizienyte *et al.*, 2002) were cultured in Eagle's minimal essential medium (MEM) supplemented with MEM vitamins (X2), nonessential amino acids, L-glutamine (2 mM), and sodium pyruvate (1 mM; all from Life Technologies, Paisley, UK) in the presence of 10% fetal bovine serum (FBS). When required, cells were serum-starved in serum-free MEM for 20 h and stimulated with MEM containing 10% FBS for 10 min. Src527F containing mutations that disrupt either the Src SH2 or SH3 domain binding capacity were generated with the QuickChange mutagenesis kit (Stratagene, Amsterdam, The Netherlands) using the mutant sense oligonucleotide 5′-CCT TCT TGG TCC TGG AGA GCG AGA CG-3′ to create the SH2 domain mutant (Arg to Leu at position 175 of chicken Src; Mayer *et al.*, 1992 and Bibbins *et al.*, 1993) or 5′-GAA GGT GAC GCG TGG CTG GCT CA-3′ to create the SH3 domain mutant (Trp to Ala at position 118 of chicken Src; Erpel *et al.*, 1995). The resulting Src527F/R175L and Src527F/W118A were cloned into the retroviral pBabe Puro vector. Kinase-defective Src527F mutant (Src527F/K295M; a gift from K.B. Kaplan, Massachusetts Institute of Technology, Cambridge, MA) was cloned into *Bam*HI and *Sal*I sites of pBabe Puro vector. KM12C cells were infected with Src mutants using the Phoenix-Ampho packaging cell line. Cells expressing mutant Src proteins were selected with MEM containing 3 μg/ml puromycin. For calcium manipulation experiments cells were maintained in keratinocyte growth medium (KGM; Clonetics, San Diego, CA) containing 0.4% bovine pituitary extract, 0.1 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 50 ng/ml amphotericin, 50 μg/ml gentamicin (Clonetics), and 0.03 mM $CaCl_2$ (low calcium) for 18–24 h. Cells were switched to KGM containing 1 mM $CaCl_2$ (high calcium) for the indicated periods of time. Where cells were plated on fibronectin or poly-L-lysine, chamber slides were incubated with 10 μg/ml fibronectin (BD Biosciences, Oxford, UK) or 10 μg/ml poly-L-lysine (Sigma, Irvine, UK) overnight at 4°C, washed with phosphate-buffered saline (PBS) and the cells were plated in serum-free medium (MEM). When required, the MEK inhibitor UO126 (25 μM; Promega, Southampton, UK), MLCK inhibitors ML7 (5 μM) or ML-9 (7.6 μM; both from Calbiochem, Nottingham, UK), Src kinase inhibitor PP2 (20 μM; Calbiochem, Kansai, Japan), Rho kinase (ROCK) inhibitor Y27632 (10 μM; Welfide Corporation, Japan), PI 3-kinase inhibitor LY294002 (50 μM), cell-permeable ERK activation inhibitory peptide II (100 μM), or cell-permeable STAT3 inhibitory peptide (100 μM; all from Calbiochem) were added to the medium. To prevent KM12C/Src527F cells from adhesion to extracellular matrix, cells were cultured in poly-HEMA (10 mg/ml; Sigma)-coated dishes for 24 h in MEM.

Confocal Immunofluorescence Microscopy

Cells were fixed with 3.7% paraformaldehyde in Tris-buffered saline (TBS), permeabilized with TBS containing 0.5% Triton X-100 and 1% bovine serum albumin (BSA) for 15 min, and blocked with 5% fetal bovine serum (FBS) in TBS for an hour. Cells were incubated with 2.5 μg/ml monoclonal anti–E-cadherin (clone 36; BD Biosciences, Oxford, UK), 2.5 μg/ml monoclonal anti-paxillin (clone 349; BD Biosciences), 1:100 polyclonal anti-phospho-MLC (Ser19; Cell Signaling, Lexington, KY), 1:500 polyclonal anti-phospho-Erk (Thr202/Tyr204; Cell Signaling) or 1:500 anti-phospho-Src (Tyr416; Biosource, Nivelles, Belgium) antibodies. Cells were washed and incubated with fluorescein (FITC)- or Texas Red–conjugated anti-mouse or anti-rabbit IgG antibody (Jackson Laboratories, Bar Harbor, ME) diluted 1:100. Cells were examined using a confocal microscope (MRC600; Bio-Rad, Hercules, CA).

Quantitation of percentage of cells that localize E-cadherin to cell-cell contacts after switch to high-calcium medium was performed as follows. Cells in which E-cadherin stained strongly cell-cell contacts were counted as cells that formed cadherin-mediated cell-cell contacts. Only cells in small colonies or around the edges of the larger colonies were included in the study. Approximately 200 cells in different fields were analyzed.

Immunoblotting and Immunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors (2 mM phenylmetanesulfonyl fluoride, 10 μg/ml aprotinin, 1 mM EGTA, 10 μg/ml leupeptin, 0.5 mM benzamidine, 1.5 mM sodium fluoride, 300 μM sodium vanadate, 10 mM sodium pyrophosphate) and clarified by centrifugation at 4°C. Ten to 20 μg of total protein was analyzed under reducing conditions using NuPAGE 4–12% Bis-Tris precast gels (Invitrogen, Inchinnan, UK). Proteins were transferred to nitrocellulose, blocked, and probed with 1:1000 polyclonal anti-phospho-myosin (Ser19; Cell Signaling), 1:1000 monoclonal anti-MLC (clone MY-21; Sigma), 1 μg/ml monoclonal anti-phospho-STAT3 (Tyr705; clone 9E12; Upstate Ltd, Buckingham, UK), 2 μg/ml polyclonal anti-STAT3 (Upstate Ltd), 1:1000 polyclonal anti-phospho-Akt (Ser473; Cell Signaling), or 1:1000 polyclonal anti-Akt (Cell Signaling). Src proteins were immunoprecipitated from cell extracts that were prepared in 10 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100 containing protease and phosphatase inhibitors as above and clarified by centrifugation after sonication (2× 5 s, 10 μm). One milligram of clarified cell lysate was precleared with protein A-agarose and then incubated with 10 μl Src antibody (clone 327) conjugated to agarose beads (CN Biosciences, Nottingham, UK). The immune complexes were washed and then resolved by 10% SDS-PAGE. Proteins were probed with 1:1000 monoclonal anti-Src (clone 327; CN Bioscience).

Detergent Extractions

To determine expression levels of vimentin intermediate filament, adherent cells were initially extracted with buffer 1 (10 mM Pipes, pH 6.8, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, 1% Triton X-100, 1 μg/ml phalloidin) containing protease and phoshatase inhibitors (described above) at room temperature for 5 min (membrane/soluble fraction). Supernatants were collected and cells were rinsed three times with the buffer 1 without Triton X-100 followed by incubation in buffer 2 (buffer 1 supplemented with 2% SDS) for 5 min at room temperature. Lysates were collected and sonicated (2× 5 s, 10 μm; cytoskeletal/cytokeratin fraction). Ten micrograms of total protein was resolved by 10% SDS-PAGE. Proteins were probed with 1:500 monoclonal antivimentin (clone 65E; Affiniti, Exeter, UK) or 1:5000 monoclonal anti–β-actin (clone AC-15; Abcam, Cambridge, UK) antibody.

RESULTS

Inhibitors of MEK/ERK and MLCK Suppress Src- and Serum-induced Integrin Adhesion Complexes in KM12C Colon Cancer Cells

Because elevated Src expression and/or activity is often associated with the development of human colon cancer, we previously addressed the effects of raising the intracellular activity of c-Src in a nonmetastatic human colon cancer cell line (Avizienyte *et al.*, 2002). Src induced an "adhesion switch" phenotype that was associated with enhanced assembly of integrin adhesions and deregulation of calcium-induced E-cadherin localization to cell-cell contacts (Avizienyte *et al.*, 2002). To examine involvement of phosphorylation of myosin, which is a downstream readout of MLCK activity, in the Src-induced "adhesion switch" phenotype, we first examined the adhesion dependence of MLC phosphorylation in vector- or Src527F-expressing KM12C cells. We found that phosphorylation of MLC required cells to be adhered to the substratum (Figure 1A). Under basal conditions in low serum, phospho-MLC was increased as a consequence of Src527F expression, and levels equivalent to those in KM12C parental cells were restored by the MLCK inhibitors ML7 and ML9 (shown for ML9 in Figure 1B). As we reported previously (Jones *et al.*, 2002), plating KM12C cells expressing active Src527F (KM12C/

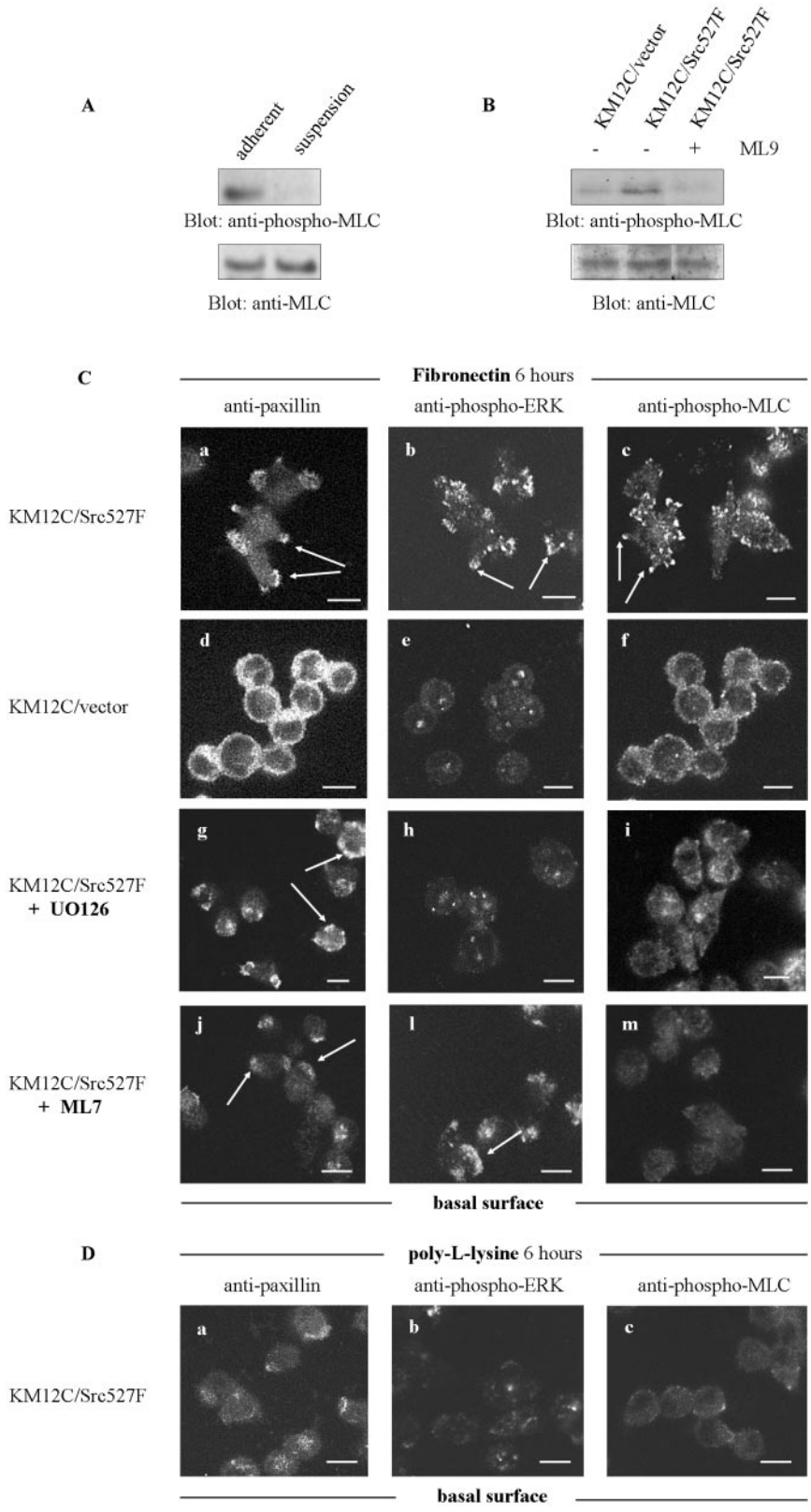

Figure 1. ERK/MLCK activities are required for Src-induced integrin adhesion assembly in KM12C colon cancer cells. (A) KM12C cells transfected with Src527F (KM12C/Src527F) were cultured in uncoated plastic dishes (adherent) or in poly-HEMA–coated dishes (suspension). Phospho-ERK or phospho-MLC levels were detected by probing total lysates with the anti-phospho-ERK (Thr202/Tyr204) or anti-phospho-MLC (Ser19) antibodies (top panels). The filters were reprobed with anti-ERK or anti-MLC antibodies (lower panels). (B) Increased level of total cellular phospho-MLC in KM12C/Src527F cells was blocked by MLCK inhibitor ML9 (7.6 μM). (C) (a–f) KM12C/Src527F (a–c) or KM12C/vector (d–f) cells were plated on fibronectin-coated substratum for 6 h, fixed, and stained with anti-paxillin (a and d), anti-phospho-ERK (Thr202/Tyr204; b and e) or anti-phospho-MLC (Ser19; c and f) antibodies. Arrows show paxillin, phospho-ERK and phospho-MLC localized at cell-matrix adhesion complexes at the ends of protrusive structures in KM12C/Src527F cells. (g–m) MEK inhibitor UO126 (25 μM; g–i) or MLCK inhibitor ML7 (5 μM; j–m) blocked the formation of protrusive integrin-mediated adhesions in KM12C/Src527F cells. Arrows show localization of paxillin or phospho-ERK at nonprotrusive cell-matrix adhesion structures at the cell periphery. Scale bars, 10 μm. (D) KM12C/Src527F cells were plated on poly-L-lysine–coated substratum, fixed, and stained with anti-paxillin (a), anti-phospho-ERK (Thr202/Tyr204; b) or anti-phospho-MLC (Ser19; c) antibodies. Scale bars, 10 μm.

Src527F) onto fibronectin resulted in formation of paxillin-containing structures that did not readily form in vector-expressing cells (Figure 1C, a and d). These structures are at the tips of cell protrusions and actin staining revealed that these are most likely distinct from Src-induced podosomes that form on the basal surface of Src-expressing cells (our unpublished results). In addition, immunostaining and visualization of the basal surface of cells interacting with fibronectin-coated sub-

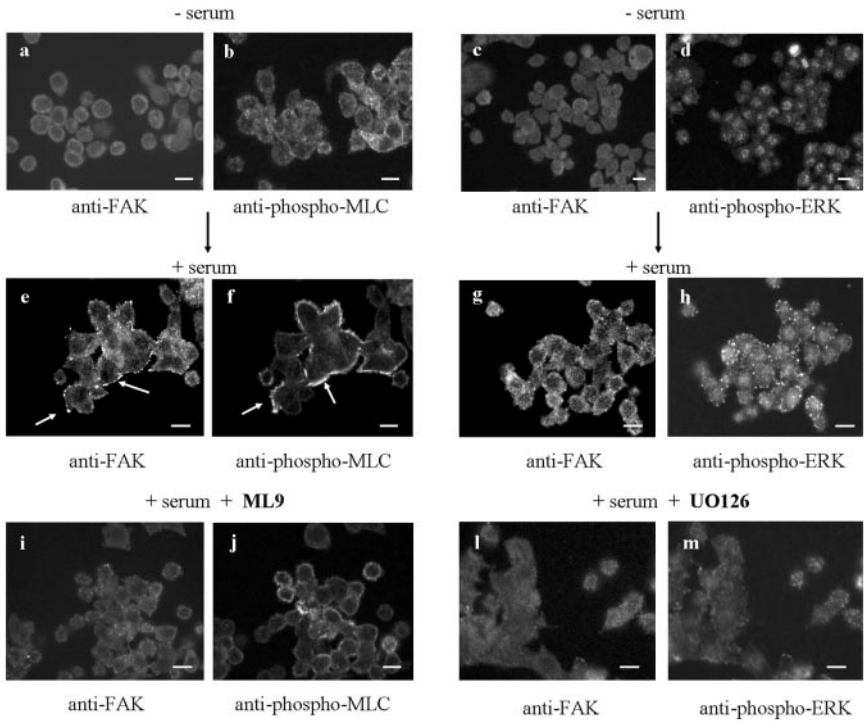

Figure 2. Serum induces peripheral integrin adhesion structures in KM12C cells via MEK/ERK and MLCK activities. (a--d) KM12C/vector cells were switched to serum-free low-calcium medium (KGM) for 24 h, fixed, and costained with anti-FAK and anti-phospho-MLC (Ser19; a and b) or anti-FAK and anti-phospho-ERK (Thr202/Tyr204) (c and d) antibodies. Scale bars, 10 μm. (e–h) Serum-starved KM12C/vector cells were stimulated with MEM containing 10% FBS for 10 min, fixed, and costained with anti-FAK and anti-phospho-MLC (Ser19) (e and f) or anti-FAK and anti-phospho-ERK (Thr202/Tyr204) antibodies (g and h). Arrows in e indicate the formation of peripheral focal adhesion complexes after stimulation with serum. Arrows in f show serum-induced accumulation of phospho-MLC at the cell periphery. Scale bars, 10 μm.(i–m) KM12C/vector cells were treated as described above except that stimulation with serum was carried out in the presence of ML9 (7.6 μM; i and j) or UO126 (25 μM; l and m). Cells were fixed and costained with anti-FAK and anti-phospho-MLC (Ser19) (i and j) or anti-FAK and anti-phospho-ERK (Thr202/Tyr204) antibodies (l and m). Scale bars, 10 μm.

stratum demonstrated that newly formed protrusive integrin adhesions contained both phospho-ERK and phospho-MLC (Figure 1C, b and c). Next, we used pharmacological agents that inhibit upstream activating kinases during the process of integrin adhesion assembly. Treating cells with inhibitors of MEK/ERK signaling (UO126) or MLCK (ML7 and ML9; shown for ML7) inhibited the formation of the c-Src–induced protrusions with paxillin-containing structures at their ends. In treated cells, paxillin localized to peripheral membranes, but the protrusive structures seen in untreated cells did not form (Figure 1C, g and j). Treating cells with the MEK inhibitor UO126 blocked accumulation of both phospho-ERK and phospho-MLC at these paxillin-containing clusters (Figure 1C, h and i), whereas MLCK inhibitor ML7 did not affect localization of phosphorylated ERK/MAP kinase at these structures (Figure 1C, l and m). This is in agreement with published data showing that MLCK is a direct downstream substrate of MAP kinase (Klemke *et al.*, 1997). Plating KM12C/Src527F cells on poly-L-lysine did not support assembly of paxillin-containing protrusions, suggesting that their formation was both c-Src– and integrin-dependent (Figure 1D). Although plating on poly-L-lysine does not prove integrin dependence, this interpretation is consistent with our previous finding that formation of these structures is inhibited by antibodies blocking α_v or β_1 integrin subunits (Avizienyte *et al.*, 2002).

Although the nonmetastatic KM12C cells did not readily form robust integrin adhesions (Figure 2, a–d; Jones *et al.*, 2002), we noticed that after serum stimulation (10 min), phospho-MLC, phospho-ERK, and integrin-linked adhesion components, such as FAK, were either concentrated around the cell membrane (in the case of phospho-MLC) or in discrete peripheral structures (Figure 2, e–h). Furthermore, treatment of cells with inhibitors of either MEK/ERK signaling (UO126) or MLCK (ML7 or ML9; shown for ML9) interfered with assembly of these structures (Figure 2, i–m). Our results suggest that MEK/ERK and MLCK activities are required for c-Src– and integrin-induced peripheral adhesion assembly in KM12C colon cancer cells.

The Src Homology Domains Are Required for Accumulation of Phospho-MLC at the Cell Periphery

Because we detected phosphorylated forms of ERK and MLC in newly forming cell-matrix adhesions in Src-expressing cells, but not in control cells (Figure 1C), we asked whether the Src SH2 or SH3 domains were involved in the induction of cell-matrix adhesions in colon cancer cells. We made single point mutations in either SH2 (Src527F/R175L) or SH3 (Src527F/W118A) domains, the latter which abolishes the proper peripheral targeting and function of v-Src (Fincham *et al.*, 2000). The mutant Src proteins were all expressed at high levels compared with signal in the vector-transfected cells (shown in Figure 3A). Furthermore, active Src, detected by anti-phospho-Src (phospho-Tyr416) staining, localized to focal contacts at the tips of cellular protrusions when KM12C/Src527F cells were plated on fibronectin for 6 h (Figure 3B, a). Similarly, active Src527F was localized to large peripheral focal complexes in growing adherent cells (Figure 3B, d). In contrast, the SH2 or SH3 domain mutants (Src527F/R175L or Src527F/W118A, respectively) were unable to localize properly to the cell periphery, as judged by diffuse anti-phospho-Src (Tyr416) staining of cells (Figure 3B, b, c and e, f). Next we examined total cellular levels of phospho-MLC in cells expressing active Src527F, or the Src527F/R175L or Src527F/W118A mutants. Phosphorylation of MLC was markedly increased in all three cell lines when compared with vector-transfected control cells, indicating that proper localization of active Src was not required for induction of MLC phosphorylation (Figure 3C). Because phospho-MLC was present in newly assembled focal contacts when KM12C/Src527F cells were plated on fibronectin (Figure 1 C, c), we investigated localization of phospho-MLC in adherent KM12C cells expressing Src527F, Src527F/R175L, or Src527F/W118A. In adherent Src-expressing KM12C cells, phospho-MLC staining was concentrated either at the leading edge of protrusive adhesion structures or around the membrane (Figure 3D, a). We found that muta-

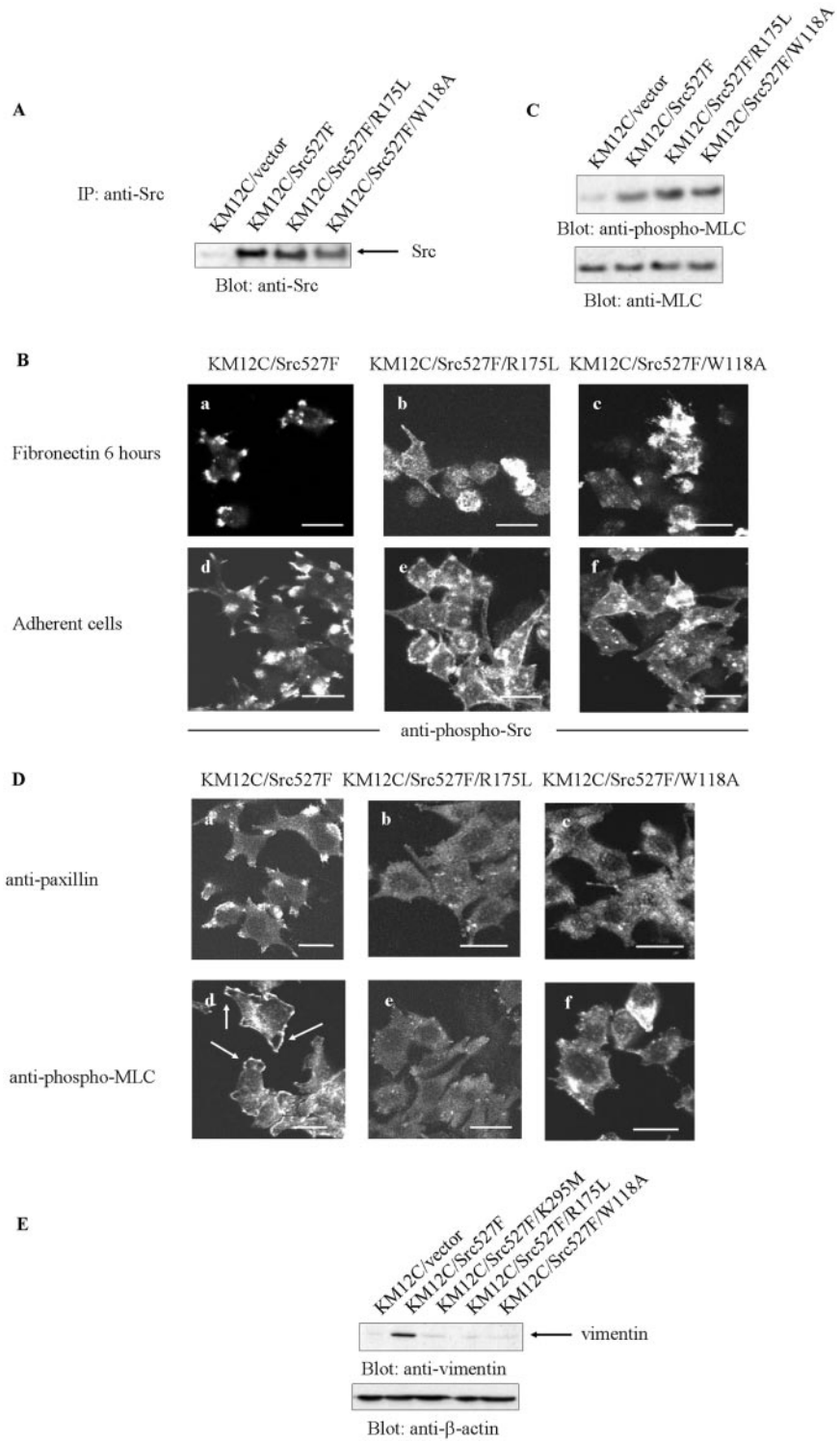

Figure 3. SH2 and SH3 domains of Src are required for accumulation of phospho-MLC at the cell periphery. (A) Expression levels of mutant Src kinase proteins in KM12C cells. (B) KM12C/Src527F, KM12C/Src527F/R175L, or KM12C/Src527F/W118A cells were plated on fibronectin for 6 h (a–c) or cultured in MEM and switched to low-calcium medium (d–f). Cells were fixed and stained with anti-phospho-Src (Tyr416) antibody. Scale bars, 20 μm. (C) Phospho-MLC levels in KM12C/vector, KM12C/Src527F, KM12C/Src527F/R175L, or KM12C/Src527F/W118A cells were detected by probing total lysates with the anti-phospho-MLC (Ser19) antibody (top panel). The filters were reprobed with anti-MLC antibody (bottom panel). (D) KM12C/Src527F, KM12C/Src527F/R175L, or KM12C/Src527F/W118A cells were switched to low-calcium medium, fixed, and stained with anti-paxillin (a–c) or anti-phospho-MLC (Ser19) (d–f) antibody. Arrows in d show accumulation of phospho-MLC at the cell periphery. Scale bars, 20 μm. (E) Vimentin expression levels in KM12C/vector, KM12C/Src527F, KM12C/Src527F/K295M, KM12C/Src527F/R175L, or KM12C/Src527F/W118A cells.

tions in either the Src SH2 or SH3 domain impaired the ability of cells to form prominent peripheral adhesion complexes as judged by paxillin staining (Figure 3D, b and c). In addition, phospho-MLC did not accumulate at the cell periphery (Figure 3D, e and f). These findings indicate that the SH2 and SH3 domains of Src are critically required for accumulation of phospho-MLC at the cell periphery, and this localization is linked to formation of integrin adhesion complexes in mesenchymal-like cells. We also found that expression of active Src527F in KM12C cells induced vimentin expression (Figure 3E) that is a marker of the mesenchymal phenotype (Kirschmann *et al.*, 1999; Boyer *et al.*, 2002). By contrast, kinase-defective Src (Src527F/K295M) or active Src with mutated SH2 (Src527F/R175L) or SH3 (Src527F/W118A) domains failed to induce expression of vimentin (Figure 3E). This indicates that Src catalytic activity and

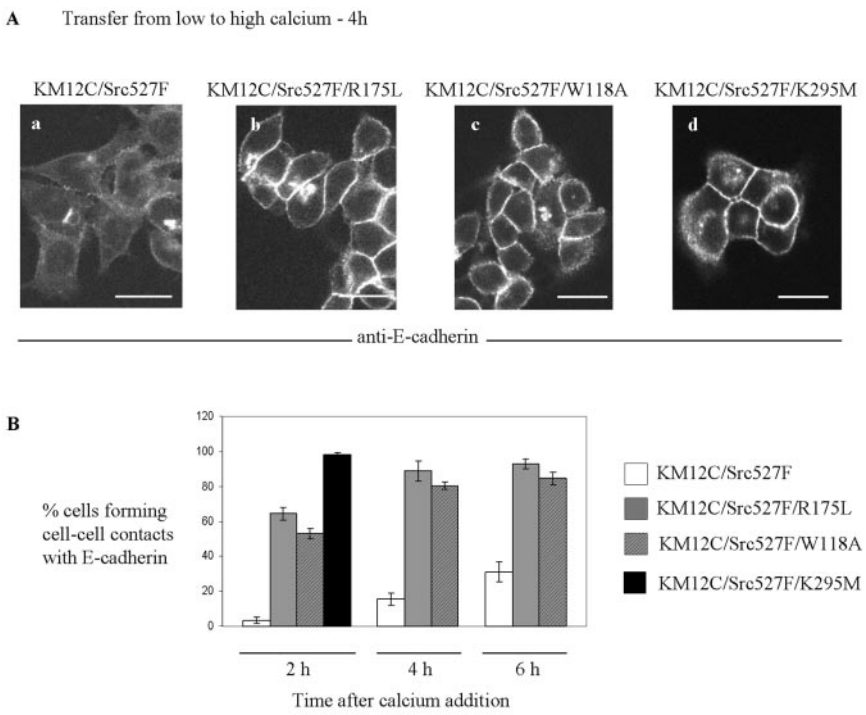

Figure 4. Src kinase activity at peripheral integrin-mediated adhesion complexes is required to impair E-cadherin localization. (A) KM12C cells expressing Src527F (a), Src527F/R175L (b), Src527F/W118A (c) or kinase-defective Src527F/K295M) (d) were switched from low- to high-calcium medium for 4 h. Cells were fixed and stained with anti-E-cadherin. Scale bars, 20 μm. (B) Quantitation of percentage of cells which localize E-cadherin to cell-cell contacts after switch to high-calcium medium. The mean and range of two independent experiments is shown.

SH3/SH2 domain functions are required to convert KM12C cells to a mesenchymal-like state, and this is tightly linked to peripheral accumulation of phospho-myosin.

Src Kinase Activity at Protrusive Cell-Matrix Adhesion Complexes Is Required to Disrupt Cadherin-mediated Cell-Cell Contacts

Because Src-induced formation of integrin-mediated adhesions resulted in deregulation of cadherin-mediated cell-cell junctions in active Src-expressing cells (Avizienyte *et al.*, 2002), we investigated whether expression of either active Src with mutated SH2 (Src527F/R175L) or SH3 (Src527F/W118A) domains, and kinase-defective Src (Src527F/K295M), influenced E-cadherin localization to cell-cell contacts. As we showed before, cells expressing active Src displayed impaired ability to form cell-cell contacts when the cells were switched to high calcium as judged by anti–E-cadherin staining (Figure 4A, a; for control compare staining of E-cadherin between KM12C-vector control cells; see Figure 6A, a). In contrast, E-cadherin accumulated at cell-cell contacts after switching KM12C/Src527F/R175L or KM12C/Src527F/W118A cells to medium containing high calcium (Figure 4, b and c). Similarly, rendering Src527F kinase-defective (Src527F/K295M) inhibited Src-induced E-cadherin deregulation, and E-cadherin was now localized to sites of intercellular contact (Figure 4A, d). Quantitation of the number of cells with contact-associated E-cadherin after switch to high calcium showed that almost 100% of cells expressing Src527F/K295M localized E-cadherin to cell-cell contacts by 2 h (Figure 4B). Similarly, E-cadherin accumulated between most cells expressing Src527F/R175L or Src527F/W118A by 4 h after addition of high calcium (Figure 4B). These results demonstrate that active Src kinase has to be localized to peripheral integrin-associated adhesion complexes in order to impair E-cadherin recruitment to sites of cell-cell contact. Proper localization of E-cadherin is restored when the SH2 or SH3 domains of Src are rendered nonfunctional and cause loss of phospho-myosin accumulation to the cell periphery.

MEK/ERK, ROCK, and MLCK Activities Are Involved in Src-mediated Suppression of E-cadherin–associated Contact Assembly

Because proper Src localization, specified by the SH2 and SH3 domains, deregulates E-cadherin function during promotion of the "adhesion switch" phenotype, we next asked whether MEK/ERK, ROCK, or MLCK activities were also required for loss of E-cadherin function. Because peripheral accumulation of phospho-MLC correlated strongly with formation of Src-induced protrusive adhesion structures and disassembly of cadherin-mediated cell-cell contacts in active Src-expressing KM12C/Src527F cells (Figures 3 and 4), we first examined localization of phospho-MLC in KM12C/Src527F cells treated with pharmacological agents that inhibit the Src family kinases themselves (PP2) or inhibitors of either MEK, ROCK, or MLCK activity. PP2 (20 μM) treatment caused visible loss of phospho-MLC staining at the cell periphery (Figure 5, compare a and b). Peripheral phospho-MLC was also lost upon treatment with a MEK inhibitor (UO126) or ROCK inhibitor (Y27632), although, in each case, some cytoplasmic phospho-MLC remained. This implies that a membrane-localized pool of phospho-MLC was specifically affected by inhibitors of these upstream kinases (Figure 5, compare a with c and d). Similarly, treatment of KM12C/Src527F cells with MLCK inhibitors ML7 and ML9 (shown for ML7) also blocked phospho-MLC staining at the cell periphery, although again some cytoplasmic phospho-MLC staining remained (Figure 5C, e). This data suggest that peripheral accumulation of phosphorylated MLC in KM12C/Src527F cells is dependent on MEK, MLCK, and ROCK signaling pathways. Importantly, these data also imply that MEK, MLCK, and ROCK activities are all required for the observed peripheral accumulation of phospho-myosin in active Src expressing KM12C cells and that their

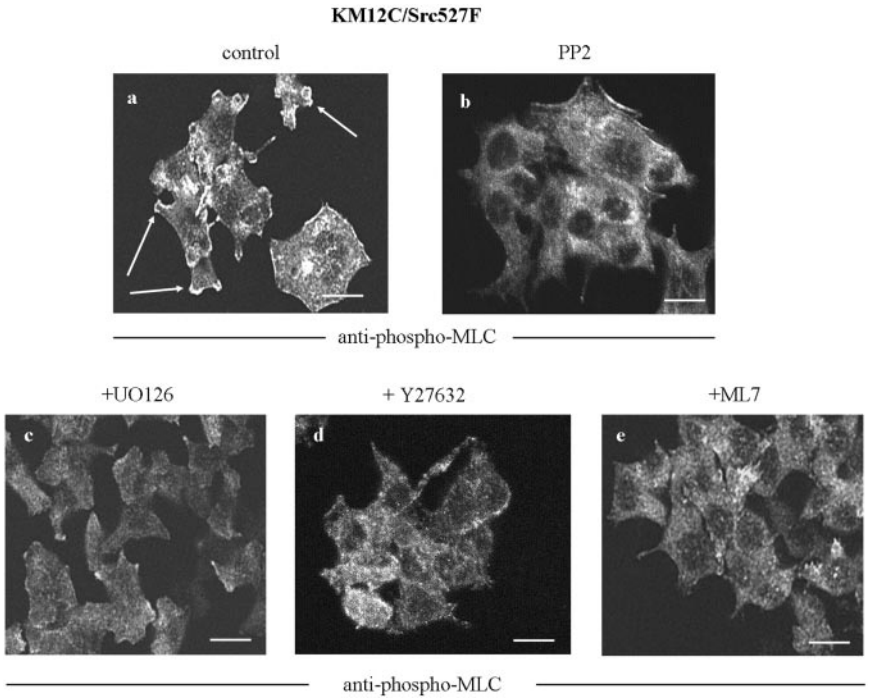

Figure 5. Inhibition of Src kinase, MEK/ERK, ROCK, or MLCK activity impairs localization of phospho-MLC at the cell periphery. KM12C/Src527F cells were cultured in high-calcium medium (MEM), further maintained in low-calcium medium and treated with either PP2 (20 μM; b), UO126 (25 μM; c), Y27632 (10 μM; d), or ML7 (5 μM; e) for 4 h. Cells were fixed and stained with anti-phospho-MLC (Ser19) antibody. Arrows show phospho-MLC localization at the leading edge of protrusive adhesion structures. Scale bars, 10 μm.

activities critically converge on the membrane-associated pool of phospho-myosin that is tightly linked to the "adhesion switch" phenotype.

KM12C cells that fail to accumulate phospho-MLC at the cell periphery do not form protrusive integrin adhesion complexes, but can recruit E-cadherin to the membrane and assemble E-cadherin–mediated contacts. Thus, we examined calcium-induced translocation of E-cadherin to newly formed contacts between adherent Src-expressing cells treated with MEK, ROCK, or MLCK inhibitors. We found that E-cadherin localized to continuous regions of membrane between KM12C/vector cells (Avizienyte *et al.*, 2002; Figure 6A, a). However, expression of active c-Src in KM12C/Src527F cells impaired E-cadherin localization between cells, although occasionally E-cadherin appeared to localize to fragmented structures between two cells (Figure 6A, b). In contrast, when KM12C/Src527F cells were treated with the MEK inhibitor (UO126), an ERK activation inhibitor peptide II (Kelemen *et al.*, 2002) or the MLCK inhibitor (ML7), E-cadherin localization was restored between most cells (Figure 6A, c, d, and f). Treatment with the ROCK inhibitor (Y27632) also caused distribution of E-cadherin to cell-cell contacts when the cells were switched to high calcium (Figure 6A, e). Quantification of KM12C/Src527F cells in which E-cadherin translocation to regions of cell-cell contact was restored by the inhibitors of MEK or MLCK indicates that after 6 h in high calcium, up to 90% of cells can form continuous E-cadherin–containing structures (Figure 6B). We also found that treatment with MEK or MLCK inhibitors caused E-cadherin to concentrate at regions of cell-cell contact in KM12C/Src527F cells that were attached to fibronectin-coated substrata after plating (Figure 6C, b, c, e, and f), whereas E-cadherin was normally localized uniformly around the membrane of KM12C/Src527F cells that were untreated (Figure 6C, a and d). In addition, plating on poly-L-lysine instead of fibronectin also led to some constitutive localization of E-cadherin to cell-cell contact sites (Figure 6D, a), suggesting that suppressing integrin signaling can rescue Src-induced mis-localization of E-cadherin as cells attach and spread. The concentration of E-cadherin to cell-cell contacts by the MEK/MLCK inhibitors, or by blocking integrin signaling on poly-L-lysine, was reminiscent of the concentration of E-cadherin at contact sites in vector-transfected KM12C cells plated on fibronectin (Figure 6D, b).

Because multiple other signaling proteins contribute to Src transformation in addition to the ERK/MAP kinase pathway, including PI 3-kinase and STAT3 (Penuel and Martin, 1999; Bowman *et al.*, 2001; Garcia *et al.*, 2001), we considered whether or not these might also contribute to the Src-induced mesencymal transition of KM12C colon cancer cells. We used the PI 3-kinase selective inhibitor LY294002, which blocks Akt phosphorylation downstream of PI 3-kinase (Figure 7A, left panels) and the STAT3 inhibitor peptide (Turkson *et al.*, 2001). In neither case was there restoration of E-cadherin–dependent cell-cell contacts (Figure 7B). In regard of the STAT3 inhibitory peptide, this was not surprising because we could detect no phosphorylated STAT3 in KM12C cells expressing Src527F (Figure 7A, right panels).

DISCUSSION

Here, we identified signaling downstream of Src that is responsible for induction of the "adhesion switch" phenotype we described previously in KM12C colon cancer cells (Avizienyte *et al.*, 2002). MEK/ERK and MLCK/myosin activities, which others have shown to be directly linked in a biochemical pathway needed for cell migration (Klemke *et al.*, 1997), are necessary for the changes that lead to enhanced integrin adhesion assembly and reduced cell-cell contact formation associated with loss of proper E-cadherin regulation (previously reported in Avizienyte *et al.*, 2002). We show that phospho-ERK and phospho-MLC, downstream readouts of MEK and MLCK activities, respectively, accumulate at newly forming integrin-dependent adhesions, whereas inhibitors of MEK or MLCK activities block the Src- or serum-induced formation of integrin-associated protru-

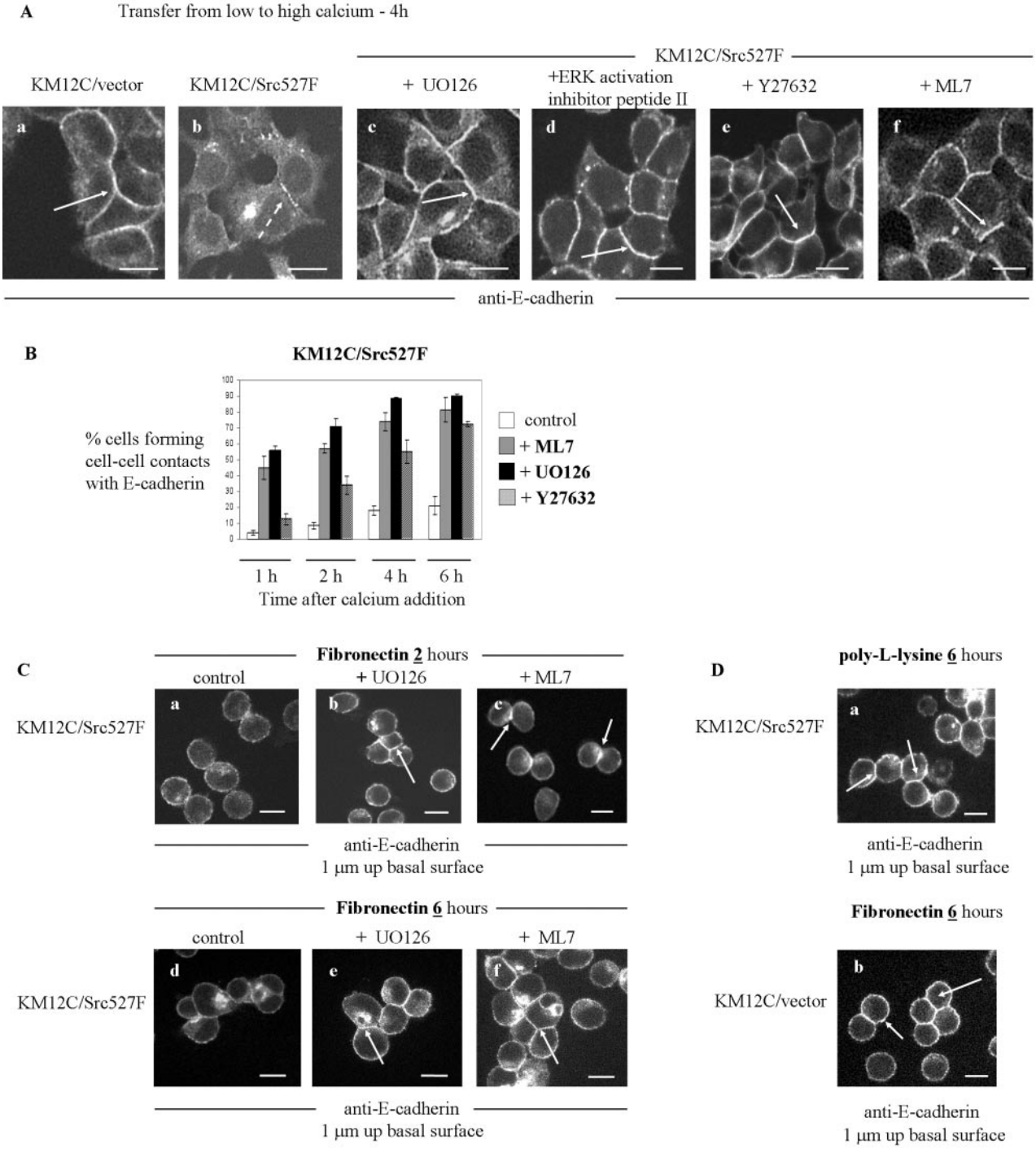

Figure 6. Inhibition of MEK/ERK, ROCK, and MLCK activities reverses Src-induced deregulation of E-cadherin–associated cell-cell contacts. (A) KM12C/vector or KM12C/Src527F cells were switched from low- to high-calcium medium for 4 h (a and b). MEK inhibitor UO126 (25 μM; c), ERK activation inhibitor peptide II (100 μM; d), ROCK inhibitor Y27632 (10 μm; e), or MLCK inhibitor ML7 (5 μM; f) was added to high-calcium medium and KM12C/Src527F cells were maintained for 4 h in such medium. Cells were fixed and stained with anti–E-cadherin antibody. Solid arrows show accumulation of E-cadherin at cell-cell contacts in KM12C/vector cells and KM12C/Src527F cells treated with either UO126, ERK activation inhibitor peptide II, Y27632, or ML7 (a, c, and d–f). Broken arrow in b points to disrupted E-cadherin staining in KM12C/Src527F cells after the switch to high-calcium medium. Scale bars, 10 μm. (B) Quantitation of percentage of KM12C/Src527F cells that are forming cadherin-mediated cell-cell contacts when cells are switched to high-calcium medium containing ML7, UO126, or Y27632. (C) KM12C/Src527F were plated on fibronectin-coated substratum for 2 or 6 h (a–f) without inhibitors (a and d) or with the MEK inhibitor UO126 (25 μM; b and e) or MLCK inhibitor ML7 (5 μM; c and f). Cells were fixed and stained with anti–E-cadherin antibody. Arrows show accumulation of E-cadherin at cell-cell contacts in KM12C/Src527F cells plated on fibronectin when MEK/ERK or MLCK activity is inhibited. Scale bars, 10 μm. (D) (a) KM12C/Src527F were plated on poly-L-lysine–coated substratum for 6 h and E-cadherin was visualized by staining with anti–E-cadherin antibody. Arrows indicate accumulation of E-cadherin between active Src527F expressing cells when integrin signaling is suppressed. (b) KM12C/vector cells were plated on fibronectin for 6 h, fixed, and stained with anti–E-cadherin antibody. Arrows show accumulation of E-cadherin at cell-cell contacts. Scale bars, 10 μm.

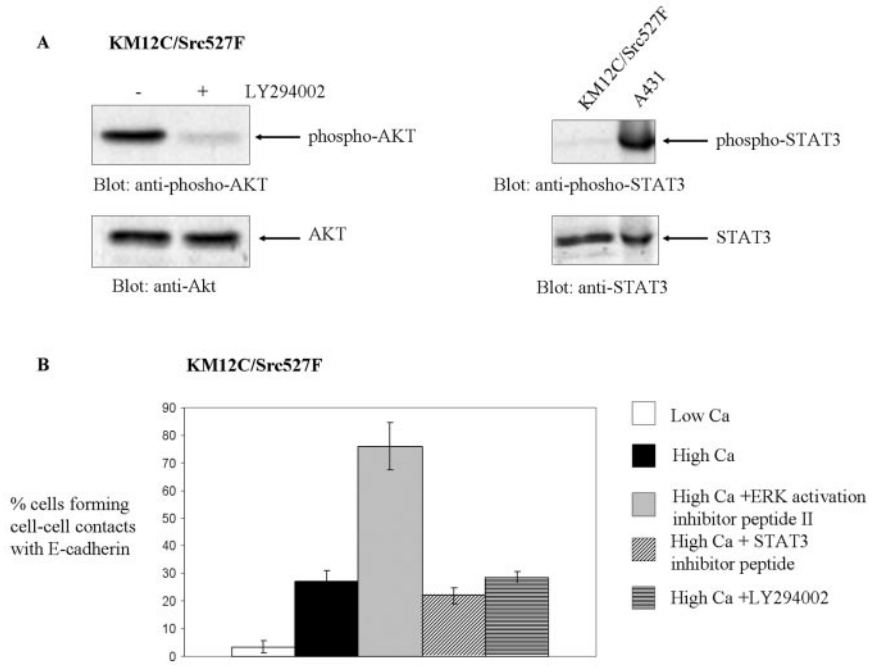

Figure 7. Inhibition of PI 3-kinase or STAT3 activity does not induce cell-cell contact formation in active Src-expressing cells. (A) KM12C cells expressing active Src527F were treated with the PI 3-kinase inhibitor LY294002 (50 μM). Cell lysates were probed with anti-phospho-Akt (Ser473) or anti-Akt antibody (left panels). Levels of active STAT3 (phospho-Tyr705) and total STAT3 in KM12C/Src527F cells or A431 cells stimulated with EGF are shown in the right panels. (B) Percentage of KM12C/Src527F cells that are forming cadherin-mediated cell-cell contacts after the cells are switched to high-calcium medium without inhibitors or with ERK activation inhibitory peptide II (100 μM), STAT3 inhibitory peptide (100 μM), or PI 3-kinase inhibitor LY294002 (50 μM).

sive adhesion structures (Figures 1 and 2). Recent data showed strong and persistent activation of ERK and MLCK in extending cell pseudopodia, whereas inhibition of MEK or MLCK activities prevented pseudopodia extension, although not retraction (Brahmbhatt and Klemke, 2003). More recently, it has been reported that Src and FAK signal through ERK and MLCK to induce focal adhesion turnover during cell migration, although how exactly increased contractility leads to dissociation of these structures is still unclear (Webb *et al.*, 2004). Our data suggest that generation of contractility at newly forming adhesion structures in epithelial cancer cells is required for extension of cellular protrusions. At the same time, E-cadherin localization to the cell membrane, is restored by preventing active Src from causing peripheral accumulation of phospho-myosin, and presumably from generating contractility there, as judged by loss of membrane-associated phospho-myosin staining when MEK/ERK, ROCK, or MLCK activities are inhibited (Figures 3–5). Our results indicate that the Src SH3 and SH2 domains are required for the peripheral accumulation of phospho-myosin. This may reflect the fact that these Src domains are required to target Src to the periphery (Figure 3B) and to localize Src kinase activity for the local activation of ERK, ROCK, and MLCK. Alternatively, or in addition, the Src SH2 and SH3 domains may recruit, either directly or indirectly, protein complexes that may cause localized contractile force generation at integrin adhesion sites, resulting in suppression of E-cadherin-mediated cell-cell contact formation. Although we have been able to detect a biochemical complex between endogenous Src and MLC in KM12C cells (our unpublished results), we have not established whether Src directly interacts with myosin and where this complex is localized in the cell. Nevertheless, the Src-induced epithelial to mesenchymal switch, which is associated with increased vimentin expression, is tightly linked to peripheral phospho-myosin activity.

The normal balance of integrin- and cadherin-mediated adhesion requires precise and dynamic regulation of the peripheral actin cytoskeleton. Therefore, it is likely that this is perturbed by Src-induced peripheral accumulation of phospho-MLC and enhanced acto-myosin contractility. Relieving contractility by inhibitors of MEK or MLCK may restore normal cytoskeletal remodeling, enabling E-cadherin–mediated adhesions to form in the presence of calcium.

Previous studies have reported that RhoA-stimulated contractility is required for the Ras-induced mesenchymal phenotype of MCF10A breast cancer cells (Zhong *et al.*, 1997). However, in these cells, blocking RhoA-induced contractility with C3 exotransferase or a dominant-inhibitory RhoA protein, suppresses focal adhesion organization but fails to restore normal cell-cell contacts (Zhong *et al.*, 1997). As RhoA activity is reported to be necessary for cadherin-dependent contacts to form between epithelial cells (Braga *et al.*, 1997; Takaishi *et al.*, 1997; Jou and Nelson, 1998; reviewed in Fukata and Kaibuchi, 2001), the findings that increased contractility is associated with oncogene-induced E-cadherin deregulation, in some cases mediated by RhoA activation, indicates that the role of RhoA in epithelial cell-cell contact dynamics is complex. In keeping with this, different RhoA effectors can contribute in opposing ways to cell-cell junctions. Specifically, the RhoA effector kinase ROCK and acto-myosin contractility disrupts junctions between a variety of tumorigenic and nontumorigenic epithelial cells, whereas signaling through Dia1 is linked to stabilization of adherens junction complexes (Sahai and Marshall, 2002). Thus, the balance of signaling through these two RhoA effector pathways in a particular cellular context is likely to determine the net balance of cell-cell contact assembly and disassembly and may explain the apparently paradoxical positive and negative effects of RhoA. Our results show that ROCK activity is involved in accumulation of phosphorylated MLC at the cell periphery in active Src-expressing KM12C cells and that interfering with ROCK activity in these cells can restore E-cadherin–mediated cell-cell contacts.

Recent work examined the status of cellular RhoA activity upon induction of contact between epithelial cells either at high density or after plating Chinese hamster ovary cells expressing C-cadherin onto the extracellular domain of C-

cadherin (Noren *et al.*, 2001). In both cases, substantial reduction of GTP-loading on RhoA was observed, leading Burridge and colleagues to propose that it may be necessary to keep cellular contractility low to avoid tension being applied to the newly formed cell-cell junctions (Noren *et al.*, 2001, 2003). Indeed, during the process of chicken embryo fibroblast spreading, the rate of spreading is inversely related to myosin activity (Wakatsuki *et al.*, 2003). It therefore appears that relaxation of contractile forces may be a common feature of the early stages of actin remodeling events that accompany formation of a number of adhesion types. We show here that suppressing peripheral accumulation of phospho-myosin, which normally occurs as a result of integrin engagement, promotes E-cadherin–associated contacts to form between KM12C colon cancer cells.

In conclusion, the Src SH3 and SH2 domains cooperate with MEK/ERK, MLCK and ROCK signaling to promote peripheral accumulation of phospho-myosin and to maintain a mesenchymal phenotype. When peripheral accumulation of phospho-myosin is blocked, E-cadherin can relocalize to membrane contact sites between cells and KM12C cells adopt an epithelial-like phenotype. Taken together, our data indicate that there is reciprocal, and interdependent, regulation of integrin- and cadherin-associated adhesions and that signals which regulate both adhesion types converge on a peripherally targeted pool of cellular phospho-myosin, presumably controlling localized contractility. This implicates spatially regulated contractile force as a critical determinant of epithelial cell plasticity, particularly in cells that can switch between epithelial and mesenchymal-like states.

ACKNOWLEDGMENTS

We are grateful to Isaiah Fidler for providing KM12C cells. This work was supported by Cancer Research UK.

REFERENCES

Avizienyte, E., Wyke, A.W., Jones, R.J., McLean, G.W., Westhoff, M.A., Brunton, V.G., and Frame, M.C. (2002). Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. Nat. Cell Biol. *4,* 632–638.

Bibbins, K.B., Boeuf, H., and Varmus, H.E. (1993). Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides. Mol. Cell. Biol. *13,* 7278–7287.

Bowman, T. *et al.* (2001). Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. Proc. Natl. Acad. Sci. USA *98,* 7319–7324.

Boyer, B., Roche, S., Denoyelle, M., and Thiery, J.P. (1997). Src and Ras are involved in separate pathways in epithelial cell scattering. EMBO J. *16,* 5904–5913.

Boyer, B., Bourgeois, Y., and Poupon, M.F. (2002). Src kinase contributes to the metastatic spread of carcinoma cells. Oncogene *21,* 2347–2356.

Braga, V.M., Machesky, L.M., Hall, A., and Hotchin, N.A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. J. Cell Biol. *137,* 1421–1431.

Brahmbhatt, A.A., and Klemke, R.L. (2003). ERK and RhoA differentially regulate pseudopodia growth and retraction during chemotaxis. J. Biol. Chem. *278,* 13016–13025.

Erpel, T., Superti-Furga, G., and Courtneidge, S.A. (1995). Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. EMBO J. *14,* 963–975.

Fincham, V.J., Brunton, V.G., and Frame, M.C. (2000). The SH3 domain directs acto-myosin-dependenttargetingofv-Srctofocaladhesionsviaphosphatidylinositol 3-kinase. Mol. Cell. Biol. *20,* 6518–6536.

Fukata, M., and Kaibuchi, K. (2001). Rho-family GTPases in cadherin-mediated cell-cell adhesion. Nat. Rev. Mol. Cell. Biol. *2,* 887–897.

Garcia, R. *et al.* (2001). Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene *20,* 2499–2513.

Gupta, S.K., Gallego, C., Johnson, G.L., and Heasley, L.E. (1992). MAP kinase is constitutively activated in gip2 and src transformed rat 1a fibroblasts. J. Biol. Chem. *267,* 7987–7990.

Jones, R.J., Avizienyte, E., Wyke, A.W., Owens, D.W., Brunton, V.B., and Frame, M.C. (2002). Elevated c-Src is linked to altered cell-matrix adhesion rather than proliferation in KM12C human colorectal cancer cells. Br. J. Cancer *87,* 1128–1135.

Jou, T.S., and Nelson, W.J. (1998). Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. J. Cell Biol. *142,* 85–100.

Kelemen, B.R., Hsiao, K., and Goueli, S.A. (2002). Selective in vivo inhibition of mitogen-activated protein kinase activation using cell-permeable peptides. J. Biol. Chem. *277,* 8741–8748.

Kirschmann, D.A., Seftor, E.A., Nieva, D.R., Mariano, E.A., and Hendrix, M.J. (1999). Differentially expressed genes associated with the metastatic phenotype in breast cancer. Breast Cancer Res. Treat. *55,* 127–136.

Klemke, R.L., Cai, S., Giannini, A.L., Gallagher, P.J., de Lanerolle, P., and Cheresh, D.A. (1997). Regulation of cell motility by mitogen-activated protein kinase. J. Cell Biol. *137,* 481–492.

Mansour, S.J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande Woude, G.F., and Ahn, N.G. (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. Science *265,* 966–970.

Mayer, B.J., Jackson, P.K., Van Etten, R.A., and Baltimore, D. (1992). Point mutations in the abl SH2 domain coordinately impair phosphotyrosine binding in vitro and transforming activity in vivo. Mol. Cell. Biol. *12,* 609–618.

Morikawa, K., Walker, S.M., Nakajima, M., Pathak, S., Jessup, J.M., and Fidler, I.J. (1988). Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. Cancer Res. *48,* 6863–6871.

Noren, N.K., Niessen, C.M., Gumbiner, B.M., and Burridge, K. (2001). Cadherin engagement regulates Rho family GTPases. J. Biol. Chem. *276,* 33305–33308.

Noren, N.K., Arthur, W.T., and Burridge, K. (2003). Cadherin engagement inhibits RhoA via p190RhoGAP. J. Biol. Chem. *278,* 13615–13618.

Penuel, E., and Martin, G.S. (1999). Transformation by v-Src: Ras-MAPK and PI3K-mTOR mediate parallel pathways. Mol. Biol. Cell *10,* 1693–1703.

Sahai, E., and Marshall, C.J. (2002). ROCK and Dia have opposing effects on adherens junctions downstream of Rho. Nat. Cell Biol. *4,* 408–415.

Takaishi, K., Sasaki, T., Kotani, H. Nishioka, H., and Takai, Y. (1997). Regulation of cell-cell adhesion by rac and rho small G proteins in MDCK cells. J. Cell Biol. *139,* 1047–1059.

Thiery, J.P. (2002). Epithelial-mesenchymal transitions in tumour progression. Nat. Rev. Cancer *2,* 442–454.

Turkson, J. *et al.* (2001). Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. J. Biol. Chem. *276,* 45443–45455.

Webb, D.J., Donais, K., Whitmore, L.A., Thomas, S.M., Turner, C.E., Parsons, J.T., and Horwitz, A.F. (2004). FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat. Cell Biol. *6,* 154–161.

Wakatsuki, T., Wysolmerski, R.B., and Elson, E.L. (2003). Mechanics of cell spreading: role of myosin II. J. Cell Sci. *116,* 1617–1625.

Zhong, C., Kinch, M.S., and Burridge, K. (1997). Rho-stimulated contractility contributes to the fibroblastic phenotype of Ras-transformed epithelial cells. Mol. Biol. Cell *8,* 2329–2344.