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

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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).

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; Manour et al., 1994). Additionally, it is reported that MLCK kinase (MLCK) is a 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 (Zhang 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 caderhins 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 J. F. Fidler, University of Texas M.D. Anderson Cancer 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, Houston, TX) and KM12C cells expressing active Src (KM12C/Src527F; Avizienyte et al., 2002) were cultured in poly-HEMA (10 mg/ml; Sigma)-coated dishes for 24 h in MEM. Cells were transfected with vectors as described, except that 200 ng/ml pBabe Puro vector was used to mock-transfect these cells.

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 MgCl2, 300 mM sucrose, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) containing protease and phosphatase inhibitors (2 mM phenylmethylsulfonyl 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, Invitrogen, 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 monoclonal anti-phospho-STAT3 (Upstate Ltd), 1:1000 polyclonal anti-Akt (Sal et al., 1993) or 50 μg/ml polyclonal anti-STAT3 (Upstate Ltd) conjugated to agarose beads (CN Biosciences, Nottingham, UK). The immune complexes were washed and then resolved by 10% SDS-PAGE. Proteins were probed with 1:3000 monoclonal anti-Src (clone 327; CN Biosciences).

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 3B6, BD Biosciences, Oxford, UK) and 2.5 μg/ml monoclonal 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; Biorse, Biosource, Nivelles, Belgium) antibodies. Cells were washed and incubated with 1:1000 polyclonal anti-rabbit IgG (Rockland, Exeter, UK) conjugated to Alexa fluor 488 or 568, 1:500 polyclonal anti-mouse IgG (Rockland, Exeter, UK) conjugated to Alexa fluor 488, 568 or 647 (Thermo Scientific, Exeter, UK) or 500 g/ml monoclonal anti-STAT3 (Upstate Ltd) conjugated to Alexa fluor 488, 568 or 647 (Thermo Scientific, Exeter, UK). Cells were washed one last time in TBS and mounted in 1:1 PBS, DAPI, and 60% glycerol. Images were acquired using a confocal microscope (MRC600; Bio-Rad, Hercules, CA).

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 1% sodium deoxycholate) containing protease and phosphatase inhibitors (2 mM phenylmethylsulfonyl 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, Invitrogen, 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 monoclonal anti-phospho-STAT3 (Upstate Ltd), 1:1000 polyclonal anti-Akt (Sal et al., 1993) or 50 μg/ml polyclonal anti-STAT3 (Upstate Ltd) conjugated to agarose beads (CN Biosciences, Nottingham, UK). The immune complexes were washed and then resolved by 10% SDS-PAGE. Proteins were probed with 1:3000 monoclonal anti-Src (clone 327; CN Biosciences).

RESULTS

Inhibitors of MEK/ERK and MLCK Suppress Src- and Scaffold-Integrated 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 MLCK phosphorylation in vector- or Src527F-expressing KM12C cells. We found that phosphorylation of MLCK 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/
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 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.
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, was 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, b). 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 1C, 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-
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
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 (b), KM12C/Src527F/R175L (c) or KM12C/Src527F/W118A cells to medium containing high calcium (Figure 4A, 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 localizes 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 e 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 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 remained. 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
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 mesenchymal 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 protrusions.

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.

Figure 6. Inhibition of integrin signal transduction restores E-cadherin localization at cell-cell contact sites. KM12C/Src527F cells were cultured in high-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.
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.
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. Releasing 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-like 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.

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REFERENCES


