# Coordination of cell polarization and migration by the Rho family GTPases requires Src tyrosine kinase activity

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**Background:** The ability of a cell to polarize and move is governed by remodeling of the cellular adhesion/cytoskeletal network that is in turn controlled by the Rho family of small GTPases. However, it is not known what signals lie downstream of Rac1 and Cdc42 during peripheral actin and adhesion remodeling that is required for directional migration.

**Results:** We show here that individual members of the Rho family, RhoA, Rac1, and Cdc42, direct the specific intracellular targeting of c-Src tyrosine kinase to focal adhesions, lamellipodia, or filopodia, respectively, and that the adaptor function of c-Src (the combined SH3/SH2 domains coupled to green fluorescent protein) is sufficient for targeting. Furthermore, Src's catalytic activity is absolutely required at these peripheral cell-matrix attachment sites for remodeling that converts RhoA-dependent focal adhesions into smaller focal complexes along Rac1-induced lamellipodia (or Cdc42-induced filopodia). Consequently, cells in which kinase-deficient c-Src occupies peripheral adhesion sites exhibit impaired polarization toward migratory stimuli and reduced motility. Furthermore, phosphorylation of FAK, an Src adhesion substrate, is suppressed under these conditions.

**Conclusions:** Our findings demonstrate that individual Rho GTPases specify Src's exact peripheral localization and that Rac1- and Cdc42-induced adhesion remodeling and directed cell migration require Src activity at peripheral adhesion sites.

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## Background

Adhesive interactions between cells and the extracellular environment, together with regulation of the intracellular cytoskeleton, control a number of key biological functions, including cell motility and invasion. Focal adhesions consist of clustered integrins and proteins that link the extracellular matrix (ECM), through the integrins, to the actin cytoskeleton and to proteins involved in adhesion-dependent signal transduction [1]. One such protein is the non-receptor tyrosine kinase c-Src that can also associate with endosomal membranes in the perinuclear region of the cell [2, 3]. Mutation of tyrosine-527 to phenylalanine in c-Src, which results in catalytic activation, as well as release of constraints on the SH3 and SH2 domains, leads to c-Src being constitutively associated with focal adhesions [4]. Similarly, temperature-sensitive variants of the viral oncoprotein v-Src localize to focal adhesions when activated by switch to permissive temperature [5]. The kinase activity of v-Src or c-Src is not required for focal adhesion localization, but mutational analysis has revealed a critical role for the SH3 domain of both proteins in their intracellular targeting [4, 6, 7]. Although the localization of Src to focal adhesions does not require Src's catalytic activity, kinase activity is required for v-Src-induced focal adhesion turnover during transformation and motility [6, 8]. Thus, in order for v-Src to induce its biological effects, it must first be translocated from its site of synthesis to its site of action at peripheral cell-matrix adhesions.

The localization of v-Src to focal adhesions requires the integrity of the actin cytoskeleton and is under the control of RhoA GTPase [5]. In fibroblasts, activation of Rho GTPases, RhoA, Rac1, and Cdc42 by different transmembrane receptors leads to distinct rearrangements of the actin cytoskeleton. Activation of RhoA stimulates actomyosin-based contractility, which leads to the assembly of actin stress fibers and focal adhesions found at the end of stress fibers [9, 10]. Rac1 activation leads to localized actin polymerization at the cell periphery, resulting in the formation of lamellipodia, while activation of Cdc42 results in the formation of fine actin-rich protrusions, known as filopodia [11–13]. Rac1 and Cdc42 stimulate the assembly of focal complexes that are associated with lamellipodia and filopodia, respectively. They contain a number of the same proteins found in Rho-induced focal adhesions, including vinculin, paxillin, and focal adhesion kinase (FAK) [13].

Here, we addressed how RhoA-, Rac1-, or Cdc42-induced changes in the actin cytoskeleton influence the intracellu-

lar localization of c-Src and Src's biological effects at these peripheral sites. As the first 251 amino acids of c-Src, comprising the site of myristylation, unique region, and SH3 and SH2 domains, are sufficient to localize c-Src to focal adhesions, we used green fluorescent protein (GFP) fused to the first 251 amino acids of c-Src (Src251-GFP) to monitor subcellular localization following stimulation of the Rho GTPases. Upon stimulation of RhoA, Rac1, or Cdc42, actin reorganization is accompanied by recruitment of c-Src to peripheral adhesion sites at stress fiber termini, lamellipodia, or filopodia, respectively. Furthermore, modulation of the actin cytoskeleton showed that RhoA activity and actin stress fibers are needed for Src's translocation from the cell interior to the membrane, with the precise peripheral localization being determined by local actin organization that is in turn controlled by RhoA, Rac1, and Cdc42 activities. In addition, Src's catalytic activity is required for the Rac1- and Cdc42-induced adhesion remodeling that is required for cell polarization and migration. We also show that impaired adhesion remodeling in the presence of Src proteins that lack kinase activity is accompanied by reduced peripheral phosphorylation events, as shown by suppression of FAK phosphorylation at tyrosine 925.

## Results

The regulation of the actin cytoskeleton by the Rho family of GTPases is well documented and readily manipulated in Swiss 3T3 cells, making them an ideal model system in which to study the role of RhoA, Rac1, and Cdc42 in the intracellular targeting of c-Src. We therefore determined whether Src251-GFP (Figure 1) was a suitable tool for studying the targeting of c-Src in these cells. We introduced Rho, Rac, and Cdc42 expression plasmids and Src251-GFP by transient transfection or microinjection (further details on the conditions used are given in the Supplementary methods section available with this article online).

### Translocation of Src251-GFP to focal adhesions is dependent on the actin cytoskeleton

When Src251-GFP was microinjected into serum-starved cells, it was located in the perinuclear region (Figure 1b, arrow). However, upon addition of serum, which resulted in reformation of stress fibers, Src251-GFP translocated to sites at the end of the actin cables (Figure 1c, arrows), where it colocalized with the focal adhesion protein vinculin (data not shown). Similar results were seen following treatment with lysophosphatidic acid (LPA) (Figure 1d), which initiates the reformation of actin stress fibers in serum-starved Swiss 3T3 cells in a RhoA-dependent manner [9]. Treatment with the actin-disrupting drug cytochalasin D prevented the reformation of the actin stress fibers in response to serum and the translocation of Src251-GFP from the perinuclear region to the peripheral focal adhesions (Figure 1e), further confirming that the translocation of Src251-GFP to focal adhesions was actin dependent. Thus, the regulation of Src251-GFP in Swiss 3T3 fibroblasts is indistinguishable from the actin-dependent translocation of v-Src to focal adhesions that we have described previously [5]. These results imply that Src251-GFP is a useful tool to monitor the control of c-Src's intracellular targeting by direct fluorescence.

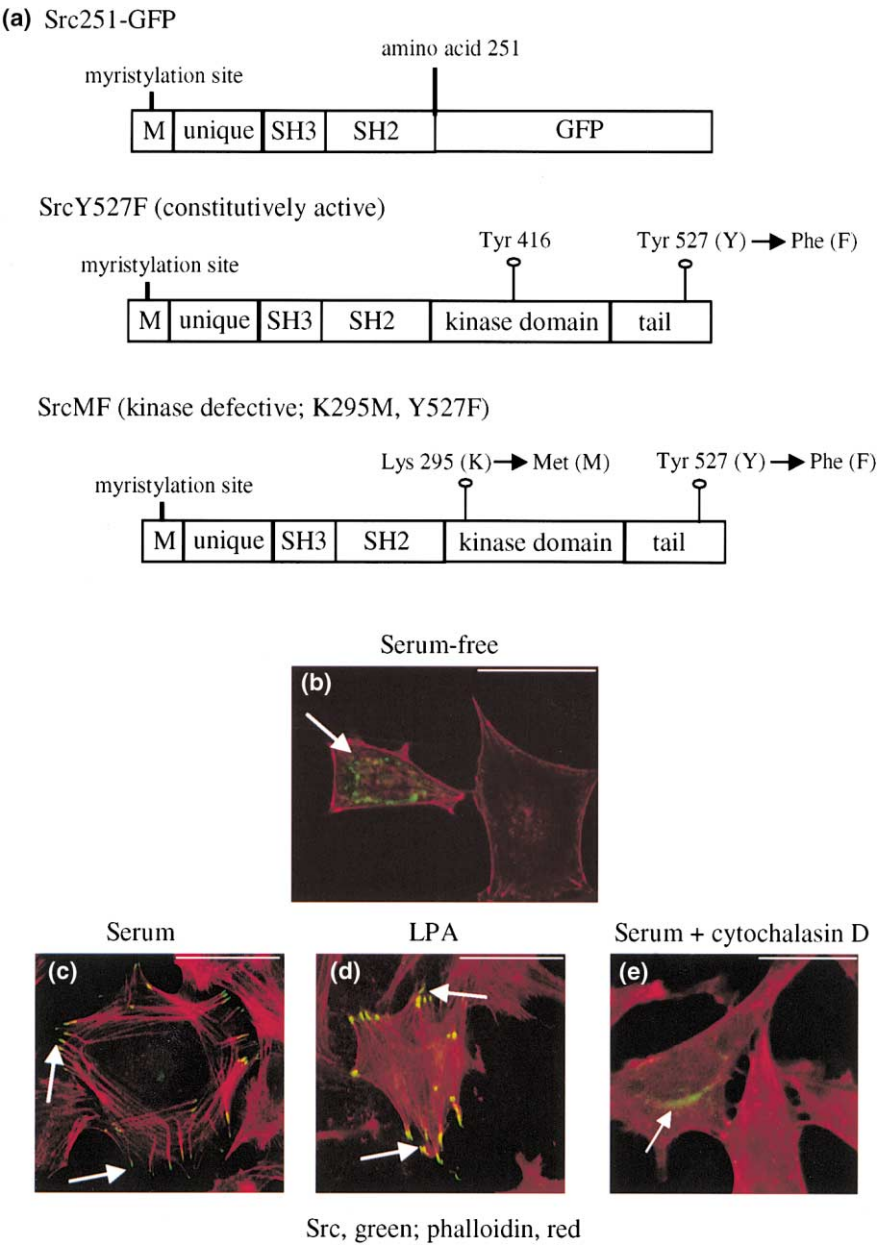
### Localization of Src251-GFP to specific peripheral sites is under the control of Rho family GTPases

As treatment with LPA, which activates RhoA, resulted in translocation of Src251-GFP to focal adhesions (Figure 1d), we set out to determine whether this was a RhoA-dependent event. Expression of a constitutively active RhoA protein (V14RhoA) in the absence of serum resulted in the formation of stress fibers (Figure 2a, broken arrow). Coexpression of V14RhoA with Src251-GFP was sufficient to localize Src to the cell periphery at the ends of stress fibers (Figure 2a, broken arrow), where it colocalized with the focal adhesion protein vinculin (data not shown).

We then addressed how activation of Rac1 or Cdc42 influenced the localization of c-Src within the cell. Activated Rac1 protein (L61Rac1) was expressed in the cells by transient transfection and resulted in the formation of lamellipodia (Figure 2b, broken arrows). Coexpression of Src251-GFP with L61Rac1 showed that Src251-GFP was localized at an actin ring around the cell periphery (Figure 2b, solid arrow) beyond which an actin ruffle had formed. Src251-GFP was also found at the tip of the ruffle that appeared to have folded back on itself (Figure 2b, broken arrows). Coexpression of a constitutively active Cdc42 protein (V12Cdc42) with Src251-GFP resulted in the formation of filopodia, and some of the expressed Src251-GFP moved from the perinuclear region to membrane regions at the base of filopodia (Figure 2c, broken arrow), and at enhanced resolution (Figure 2c, inset) it can be seen in distinct complexes spaced along the actin filopodia (broken arrows, inset).

The role of the individual Rho GTPases in the subcellular targeting of c-Src was further probed in experiments using specific inhibitors of RhoA, Rac1, and Cdc42. Expression of a dominant-negative RhoA protein (N19RhoA) prevented the reformation of stress fibers in response to serum (compare Figure 3a with 3b). The broken arrow shows a cell expressing N19RhoA, which is consequently devoid of actin stress fibers and in which Src251-GFP remained perinuclear (Figure 3b). This was in contrast to its location at peripheral focal adhesions in cells stimulated with serum (Figure 3a). Y-27632 is a chemical inhibitor of Rho kinase (ROCK) [14], which is a downstream effector of RhoA implicated in stress fiber formation [15, 16]. Treatment of cells with Y-27632 results in the loss of stress fibers, and, when cells were treated with Y-27632

**Figure 1**

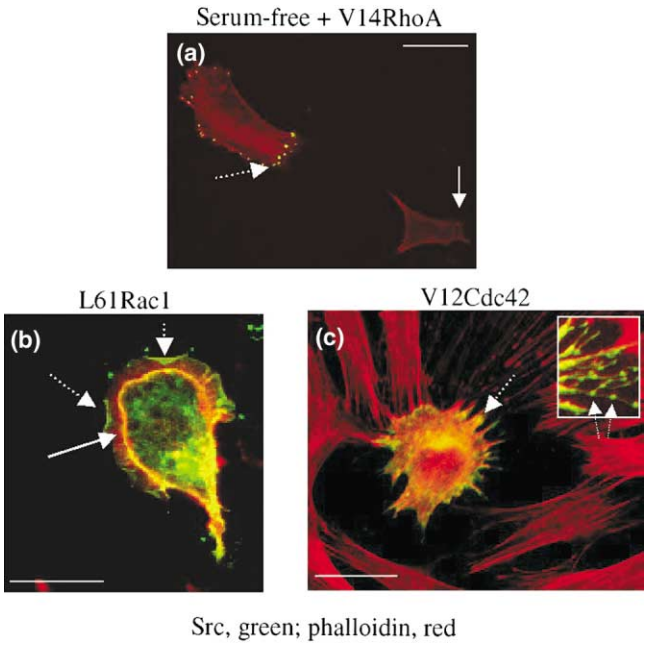



Translocation of Src251-GFP to focal adhesions is dependent on the actin cytoskeleton. **(a)** Schematic representation of the structural domains of Src251-GFP, SrcY527F, and SrcMF constructs used. **(b)** Cells were serum starved for 48 hr prior to microinjection of Src251-GFP. **(c,d)** Serum-starved cells were microinjected with Src251-GFP and, following expression of the protein, were stimulated with serum (c) or LPA (d). **(e)** Serum-starved cells were microinjected with Src251-GFP and pretreated with cytochalasin D for 2 hr before stimulation with serum. Actin was visualized with rhodamine-phalloidin, and Src251-GFP was localized by direct fluorescence. Scale bars = 25 μm.

prior to expression of Src251-GFP, the cells were devoid of stress fibers even though serum was present and translocation of Src251-GFP to peripheral focal adhesions in response to serum was blocked (Figure 3c). Y-27632 has also been reported to inhibit other kinases, and we cannot rule out the possibility that Y-27632 is inhibiting kinases other than ROCK. However, in the context of these experiments, treatment with Y-27632 results in the loss of stress fibers and taken together with the ability of N19RhoA to block translocation of Src251-GFP to focal adhesions we conclude that the actin-dependent localization of Src251-GFP to these peripheral sites is controlled by RhoA.

Platelet-derived growth factor (PDGF) stimulates the formation of lamellipodia at the cell periphery [17] via a Rac1-dependent mechanism [11]. To address the relationship between RhoA and Rac1 signaling in the localization of c-Src within the cell, we stimulated cells with PDGF while inhibiting the formation of stress fibers by treating the cells with Y-27632. As above, Y-27632 was added to the cells prior to expression of Src251-GFP, resulting in loss of stress fibers. After stimulation with PDGF, Src251-GFP remained perinuclear (Figure 3d, broken arrow) and did not translocate to the cell periphery, although the shape of the cell did change in response to

**Figure 2**




Src localization is under the control of Rho family GTPases. **(a)** Cells were serum starved for 48 hr to remove stress fibers prior to comicroinjection of V14RhoA and Src251-GFP (broken arrow). Solid arrow indicates noninjected cell. **(b)** Cells were cotransfected with L61Rac and Src251-GFP. **(c)** Cells were cotransfected with V12Cdc42 and Src251-GFP. The broken arrow indicates Src251-GFP at the base of filopodia, and the broken arrows (inset) show an enhanced resolution of Src251-GFP localizing along filopodia. Actin was visualized with rhodamine-phalloidin and Src251-GFP by direct fluorescence. Scale bars = 25 μm.

PDGF at the cell periphery (Figure 3d, solid arrows). However, if Y-27632 was washed out prior to stimulation with PDGF, Src251-GFP localized to lamellipodia (Figure 3e, solid arrows). Expression of dominant-negative N17Rac1 blocked the formation of lamellipodia in response to PDGF (Figure 3f), confirming that PDGF was working through activation of Rac1. No lamellipodia were formed in cells expressing N17Rac1 (broken arrow) as compared to the nontransfected cells (solid arrows). Although cells expressing N17Rac1 did not form lamellipodia, these cells still contained actin stress fibers, and Src251-GFP was consequently located at the ends of these stress fibers in peripheral focal adhesions (Figure 3f, broken arrow). Thus, Rac1 drives the targeting of c-Src to lamellipodia but is not involved in the initial movement of c-Src from the cell interior to the cell periphery, which is mediated by RhoA-dependent formation of stress fibers.

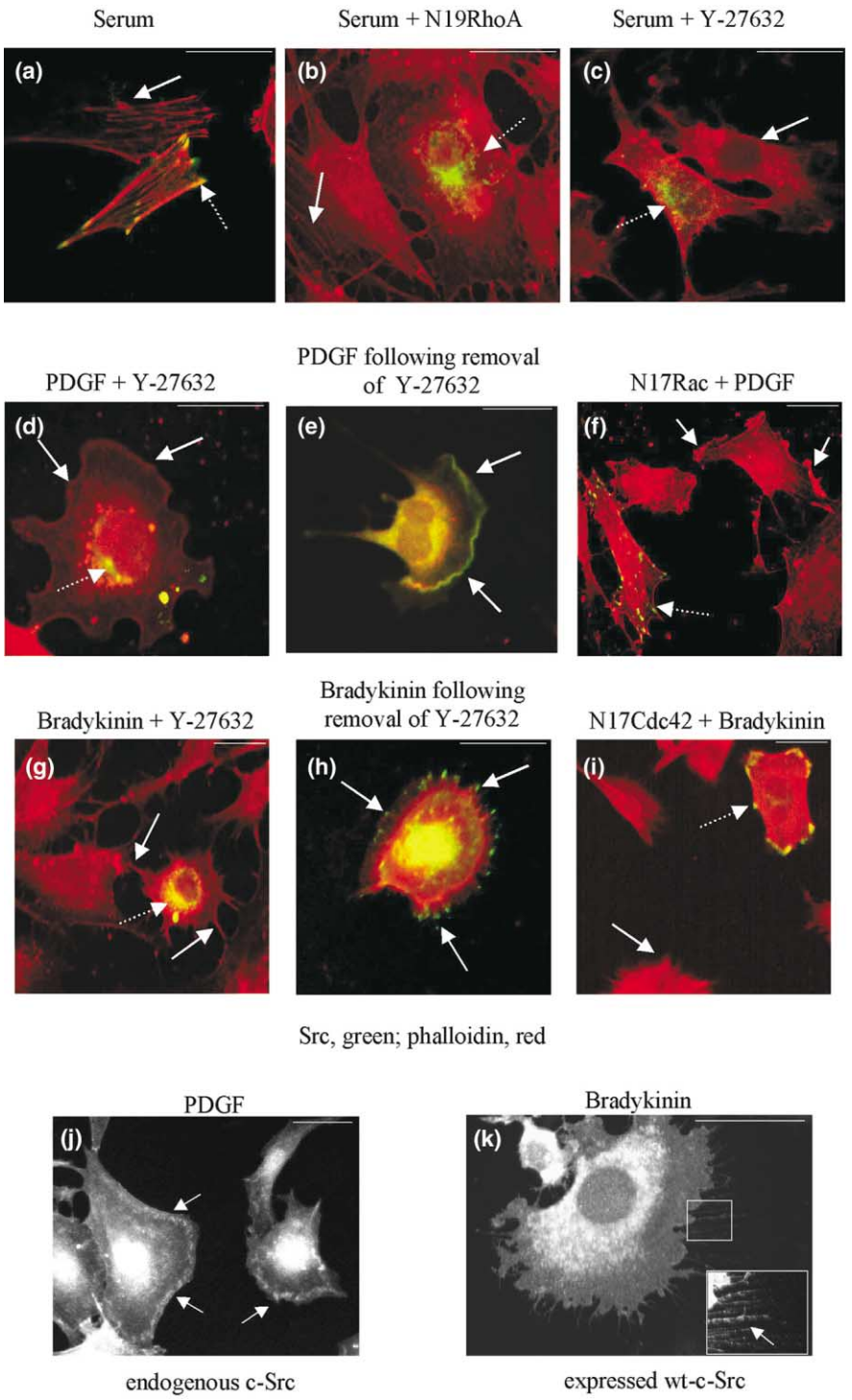
Similar experiments were carried out to define the relationship between RhoA signaling and Cdc42. Bradykinin stimulates the formation of actin microspikes, known as filopodia, at the cell periphery, by a Cdc42-mediated pathway [12, 13]. Addition of bradykinin to Y-27632-treated cells resulted in the formation of filopodia seen as small actin-containing microspikes protruding from the cell body (Figure 3g, solid arrows), although Src251-GFP remained perinuclear in the presence of Y-27632 (Figure 3g, broken arrow). However, removal of Y-27632 prior to addition of bradykinin resulted in the translocation of Src251-GFP to the peripheral microspikes in response to bradykinin (Figure 3h, solid arrows). The expression of a dominant-negative Cdc42 protein (N17Cdc42) blocked bradykinin-induced filopodia formation (Figure 3i, broken arrow) as compared to nonexpressing cells (solid arrow) that were able to induce filopodia. Coexpression of Src251-GFP with N17Cdc42, blocked bradykinin-induced translocation of Src to filopodia; however, Src251-GFP was not retained in the perinuclear region but was present in peripheral focal adhesions under these conditions (Figure 3i, broken arrow). Thus, the RhoA pathway is also required to target c-Src from the perinuclear region to the cell periphery, prior to its localization to filopodia induced by Cdc42.

To eliminate the possibility that the localization of Src251-GFP to these peripheral sites was a consequence of overexpression of available SH3 and SH2 domains, we were also able to show that endogenous Src was able to localize to lamellipodia in response to PDGF (Figure 3j). It was difficult to visualize endogenous Src in filopodia following stimulation with bradykinin, which is most likely due to problems in detection of the low levels of endogenous proteins in small structures. However, this could be overcome by expressing full-length wild-type Src protein where treatment with bradykinin resulted in Src being readily seen in filopodia (Figure 3k, inset). These data imply that the control of Src251-GFP targeting within the cell is the same as for endogenous Src or expressed wild-type Src protein.

### c-Src kinase activity is required for the efficient remodeling of cellular adhesions

It is the dynamic remodeling and interchange between focal adhesions and focal complexes associated with lamellipodia and filopodia that regulates cell motility, and we therefore addressed whether the activity of c-Src at these cellular adhesions altered their remodeling. Cells were grown in serum, where Src251-GFP is localized at focal adhesions as a consequence of RhoA activity (Figures 1 and 4c). Subsequent treatment of these cells with PDGF resulted in PDGF-induced shape changes and formation of lamellipodia. Although expression of Src251-GFP did not significantly alter the number of cells able to form lamellipodia (around 80% of cells expressing Src251-GFP underwent shape changes associated with the formation of lamellipodia), we found that the Src was localized only to discrete patches around the forming ruffle (broken arrow in Figure 4d) where it colocalized with actin (data

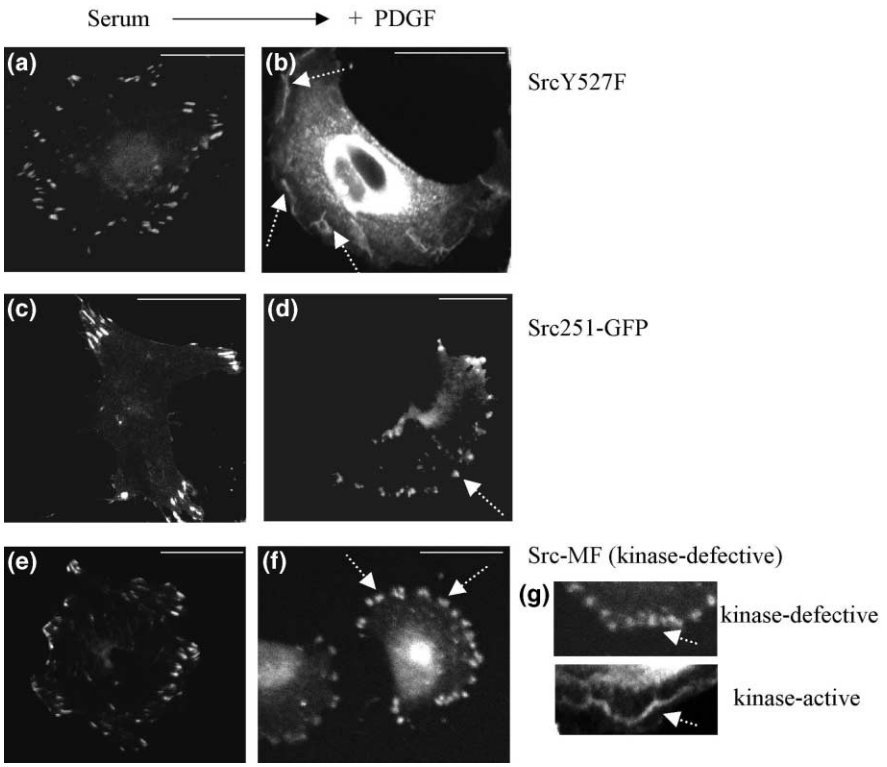
**Figure 3**




Coordinated regulation of the actin cytoskeleton by Rho family GTPases is required for Src targeting. **(a–c)** Cells were serum starved for 48 hr to remove stress fibers prior to microinjection of Src251-GFP (a,c) or comicroinjection of Src251-GFP with N19RhoA (b). Following expression of the proteins, cells were stimulated with serum (a,b) or preincubated with Y-27632 for 1 hr prior to stimulation with serum (c). Broken arrows indicate microinjected cells, while solid arrows are nonexpressing cells. **(d,e,g,h)** Cells were transfected with Src251-GFP and prior to expression of the protein were treated overnight with Y-27632 to abolish stress fibers. The cells were then stimulated with PDGF (d) or bradykinin (g) or were stimulated with PDGF or bradykinin following wash out of the Y-27632 (e,h). **(f,i)** Cells were cotransfected with Src251-GFP and N17Rac or N17Cdc42 and then placed in serum-free medium overnight to reduce the heterogeneity in the population prior to PDGF (f) or bradykinin (i) stimulation. Solid arrows show nontransfected cells, while broken arrows show cells expressing the constructs. Actin was visualized with rhodamine-phalloidin and Src251-GFP by direct fluorescence. **(j)** Cells were placed in serum-free conditions overnight, stimulated with PDGF and endogenous Src visualized using the N2-17 Src antibody. **(k)** Cells expressing wild-type Src were placed in serum-free conditions overnight and stimulated with bradykinin. Inset shows enhanced resolution of wt-Src localizing along filopodia. Exogenously expressed Src was visualized using the chicken-specific Src EC10 antibody. Scale bars = 25 μm.

not shown). This is in contrast to the uniform lamellipodia formed when Src251-GFP is held in the perinuclear region of the cell prior to stimulation with PDGF (Figure 3e), where Src251-GFP is found in small structures continuous along the lamellipodia. This suggests that it is the presence of Src251-GFP at focal adhesions prior to addition of PDGF that interferes with the actin rearrangements required for efficient formation of uniform lamellipodia. Since Src251-GFP lacks a c-Src kinase domain (Figure 1), we examined whether the presence of an activated c-Src kinase at focal adhesions facilitated the Rac1-dependent formation of uniform, continuous lamellipodia. For

**Figure 4**



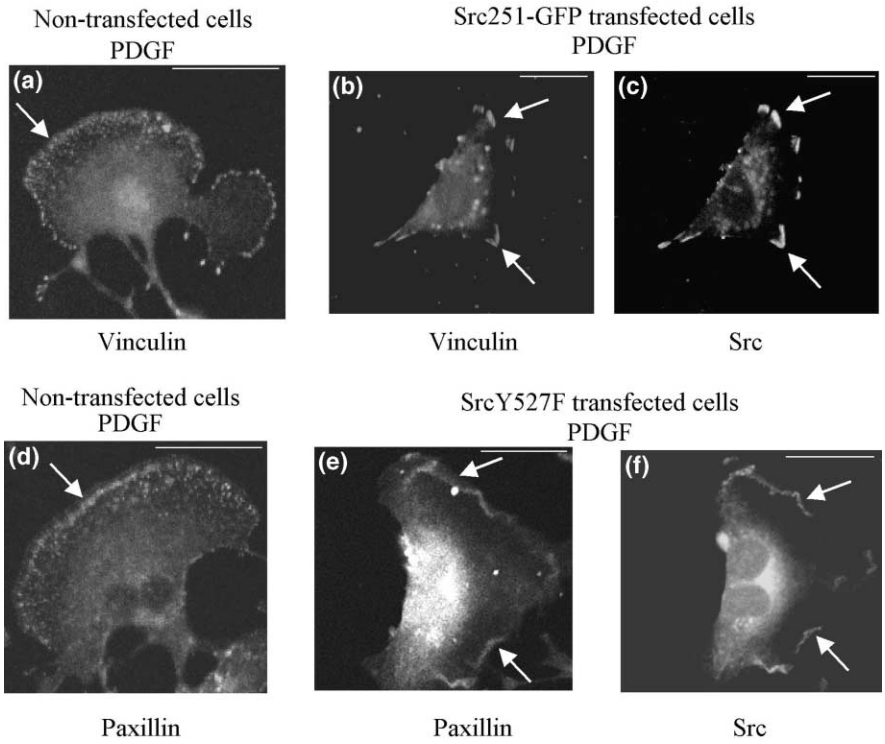

Src kinase activity is required for efficient lamellipodia formation. **(a,b)** Cells were transfected with SrcY527F and maintained in 10% serum to localize SrcY527F in peripheral focal adhesions (a). Cells were then placed in serum-free medium overnight to reduce heterogeneity in the population and treated with emetine for 30 min prior to PDGF stimulation (b). **(c,d)** Cells were transfected with Src251-GFP and maintained in 10% serum (c) or stimulated with PDGF as described above (d). **(e,f)** Cells were transfected with SrcMF and maintained in 10% serum (e) or stimulated with PDGF as described above (f). **(g)** Enhanced magnification of kinase-defective (SrcMF) (upper panel) or kinase-active Src (SrcY527F) (lower panel) localization in PDGF-induced lamellipodia. Src251-GFP was visualized by direct fluorescence, SrcY527F and SrcMF using an anti-Src antibody. Scale bars = 25 μm.

this, we expressed a c-Src protein, which has an activating tyrosine to phenylalanine mutation at position 527 in the c-Src coding sequences (SrcY527F) (reviewed in [18]) (Figure 1). SrcY527F localized to focal adhesions in the presence of serum (Figure 4a). Following treatment with PDGF, peripheral SrcY527F was located at lamellipodia that appeared continuous and relatively uniform (Figure 4b, broken arrows), in contrast to the discontinuous structures formed in cells expressing Src251-GFP (Figure 4d, broken arrow). These data imply that the efficient relocation of c-Src from RhoA-induced focal adhesions into Rac1-induced structures along lamellipodia requires Src's catalytic activity, although expression of kinase-deficient Src251-GFP did not block the initial PDGF-induced shape changes that accompany lamellipodia formation (Figure 4d). Furthermore, the protein synthesis inhibitor emetine was added prior to PDGF treatment in these experiments, implying that Rac1 activation led to relocalization of c-Src that preexisted in focal adhesions into complexes that are continuous along lamellipodia. However, we cannot rule out that some c-Src preexisted in the perinuclear region after emetine treatment and moved out into the newly forming lamellipodia upon activation of Rac1.

To further test whether the kinase activity of Src was required for efficient remodeling, we expressed a kinase-defective Src mutant, SrcMF, in the cells and examined the ability of these cells to form lamellipodia in response to PDGF. SrcMF has a point mutation in the kinase domain (K295M) rendering it kinase inactive (Figure 1). SrcMF localizes to focal adhesions in cells grown in serum (Figure 4e), and subsequent stimulation of these cells with PDGF resulted in the relocalization of SrcMF to discrete patches around the edge of the forming lamellipodia (Figure 4f) rather than to continuous lamellipodia as seen in cells expressing the activated Src protein (Figure 4b). Magnification of the edge of the lamellipodia in SrcMF and activated Src-expressing cells (Figure 4g) shows more clearly the influence of Src kinase activity on the formation of the lamellipodia. Src colocalizes with actin both in the discrete patches in the SrcMF-expressing cells and the continuous lamellipodia formed in cells expressing activated Src (data not shown). Treatment of cells with the Src kinase inhibitor PP2 also resulted in the formation of fragmented lamellipodia in response to PDGF (results shown in Supplementary materials). Taken together, these data imply a role for Src kinase activity in the efficient Rac1-induced actin remodeling associated with lamellipodia formation.

To demonstrate that the discontinuous lamellipodia found in Src251-GFP-expressing cells was associated with impaired focal adhesion remodeling into focal complexes, we compared Rac1-induced peripheral adhesion remodeling in cells expressing Src251-GFP with cells expressing

**Figure 5**




Src kinase activity is required for the remodeling of peripheral adhesions during lamellipodia formation. **(a,d)** Cells were grown in serum-free conditions overnight to reduce heterogeneity in the population, then treated with emetine for 30 min prior to stimulation with PDGF, and stained with anti-vinculin (a) or anti-paxillin antibodies (d). **(b,c)** Cells transfected with Src251-GFP or **(e,f)** SrcY527F were placed in serum-free medium overnight prior to PDGF stimulation. SrcY527F and vinculin were visualized by anti-Src and anti-vinculin antibodies, respectively. Paxillin was visualized with rhodamine-conjugated anti-paxillin and Src251-GFP by direct immunofluoresence. Scale bars = 25 μm.

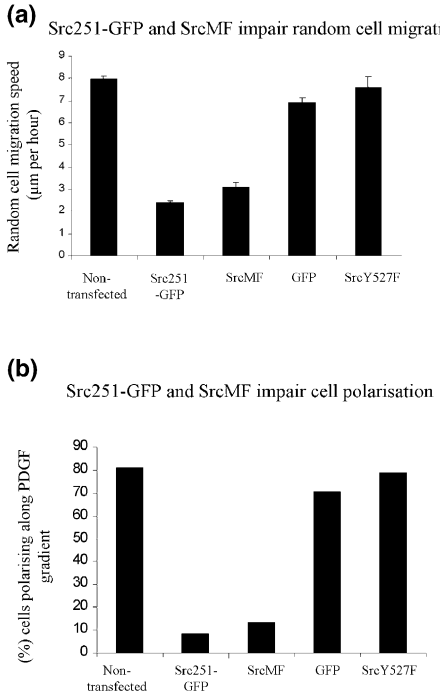
the activated c-Src mutant SrcY527F, monitoring adhesion complexes by staining with anti-vinculin or anti-paxillin. (Anti-paxillin staining was used for colocalization studies with anti-SrcY527F because anti-vinculin and anti-Src sera used were from the same species, restricting colocalization studies.) Anti-vinculin and anti-paxillin staining were indistinguishable, and, in growing cells treated with PDGF, we observed small vinculin- and paxillin-containing complexes around the base of lamellipodia (Figure 5a,d, respectively). In PDGF-treated cells expressing Src251-GFP, vinculin colocalized with Src251-GFP at the cell periphery but was located in larger discrete structures along the length of the lamellipodia (Figure 5b,c). In contrast, in activated SrcY527F-expressing cells, c-Src colocalized with paxillin along the base of uniform lamellipodia (Figure 5e,f). Paxillin localizes to focal complexes at the leading edge of lamellipodia as described by Nobes and Hall [13]; however, in cells expressing activated Src, these were difficult to visualize as discrete structures, suggesting that Src's overactivity may be further breaking down the focal complexes.

Similar to these observations with PDGF, we found that bradykinin-induced focal adhesion remodeling to produce focal complexes at filopodia was impaired in Src251-GFP-expressing cells (results shown in Supplementary materials). Thus, our data support a critical role for the catalytic activity of c-Src in the Rac1- and Cdc42-induced peripheral adhesion remodeling that results in the breakdown of larger focal adhesions and the generation of smaller focal complex adhesion structures at lamellipodia and filopodia, respectively. Further details given in Supplementary materials.

### c-Src kinase activity is required for cell motility and polarization

As the formation of lamellipodia and filopodia have been associated with the regulation of cell motility and chemotaxis [19, 20], we examined whether the impaired focal adhesion remodeling in the cells lacking kinase activity inhibited cell movement or the ability of cells to polarize in response to a chemotactic agent. We found that the random migration of cells expressing Src251-GFP or SrcMF was inhibited by around 70%, while expression of GFP alone had no effect on cell motility (Figure 6a). Expression of activated Src (SrcY527F) had no effect on the migration of the cells, suggesting that the level of endogenous Src within the cells is not limiting for migration. This has also been reported for reintroduction of activated Src into $Src^{-/-}$ cells where activated Src restored migration to a similar level as reintroduction of the wild-type protein [21]. To examine the capacity of cells to respond to a chemotactic gradient, we measured the ability of cells to polarize toward PDGF using a Dunn chamber [19]. Approximately 10% of cells expressing Src251-GFP or SrcMF were able to polarize along the PDGF

**Figure 6**




Src kinase activity is required for random cell migration and the ability of cells to remodel in response to a chemoattractant gradient. **(a)** Random cell migration of cells in 10% serum expressing Src251-GFP, GFP, SrcY527F (+ GFP), or SrcMF (+ GFP). Values are mean ± SEM taken from at least five independent experiments and represent data from between 30 and 60 cells in each case. **(b)** Polarity of cells expressing Src251-GFP, GFP, SrcY527F (+ GFP), or SrcMF (+ GFP) in response to a 10 ng/ml$^{-1}$ PGDF gradient. Values are expressed as the percentage of cells in a population able to polarize, using the criteria described in the Supplementary materials section, generated from at least five independent experiments.

gradient, while around 80% of the nontransfected cells exhibited polarity (Figure 6b). Expression of GFP or SrcY527F had no effect on the polarity of the cells in response to PDGF (Figure 6b). Thus, both cell migration and cell polarization in response to a chemotactic stimuli, biological responses that require the coordinated activities of Rho GTPases, are impaired in cells expressing either the Src251-GFP or SrcMF kinase-deficient mutants of c-Src.

### Src activity is required for phosphorylation of FAK

FAK is a known Src substrate at adhesion sites, and there is considerable evidence that FAK plays a central role in the regulation of cell motility (reviewed in [22]). We therefore addressed whether phosphorylation of FAK was suppressed in cells expressing Src kinase-defective mutants that were unable to remodel focal adhesions in response to PDGF and displayed impaired motility. Using an antibody against phosphorylated tyrosine 925 in FAK, which is a site specifically phosphorylated by Src ([23] and our unpublished data), we found that PDGF stimulated the phosphorylation of tyrosine 925, while in cells expressing SrcMF there was very little phosphorylation on this site (Figure 7a). Expression of SrcMF (Figure 7b, lower panel) did not alter the level of FAK protein within the cells (Figure 7b, upper panel). Therefore, using FAK tyrosine 925 phosphorylation as a marker of Src-dependent phosphorylation events at adhesions, we have shown that kinase-defective SrcMF expression suppresses both peripheral phosphorylation events and adhesion remodeling.

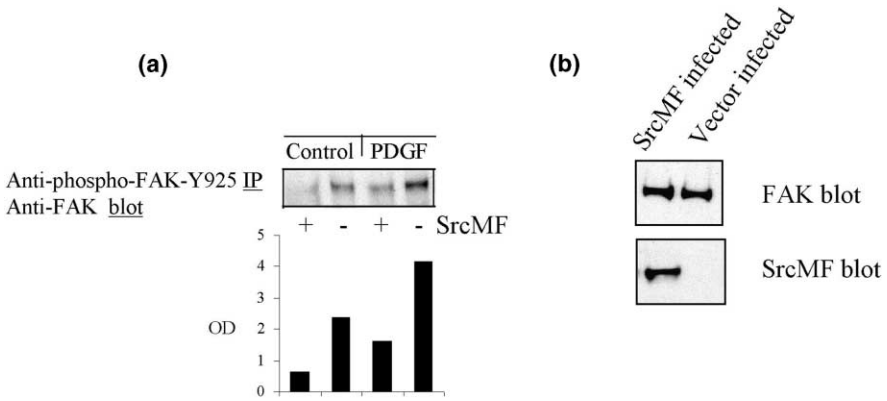
## Discussion

The ability of a cell to move depends on a cycle of adhesion, loss of attachment, and readhesion, which is governed by and coordinated with changes in the actin cytoskeleton arrangement and the microtubule system [1, 24]. The actin structures, lamellipodia, and filopodia assemble and disassemble at the leading edge of cells under the control of Rac1 and Cdc42, providing a means of polarized cell movement. Small focal complexes form at lamellipodia and filopodia, which are rapidly turned over as the cell moves forward, while the RhoA-dependent assembly of stress fibers provide contractility and propulsion required for forward cell movement. Since it is the balance between contractility and adhesion that governs the rate of cell motility, relative activities of RhoA, Rac1, and Cdc42 are critical in determining whether and how a cell moves. In this study, we examined the role of the Rho GTPases in directing the intracellular localization of the tyrosine kinase c-Src, which is known to be required for cell motility, and the role of its kinase activity in controling cell movement.

### The intracellular localization of c-Src is modulated by RhoA, Rac1, and Cdc42 activity

The localization of v-Src and c-Src to the cell periphery in fibroblasts requires an intact actin cytoskeletal network [5]. Here, we show that the peripheral localization of Src251-GFP, a fusion protein that contains Src's SH3 and SH2 domains but lacks a kinase domain, is also dependent on RhoA-mediated assembly of actin stress fibers. Although Src251-GFP was found in lamellipodia and filopodia following activation of Rac1 or Cdc42, this was also dependent on the activity of RhoA, which mediates transit from the perinuclear region to the cell periphery. This implies that Src requires actin stress fibers to incorporate into focal adhesions or into smaller focal complexes at lamellipodia or filopodia, depending on the balance of specific Rho GTPase signals within the cell. As yet, little is known of the mechanism involved in the transport of Src from the perinuclear region to the cell periphery. We and others have demonstrated that it is not dependent on the catalytic activity of c-Src but does require the Src SH3 domain [4, 6, 7]. In vitro experiments have demonstrated that the Src SH3 domain binds to a number of

**Figure 7**




Phosphorylation of FAK is suppressed in cells expressing kinase-defective Src. **(a)** Cells were placed in serum-free conditions overnight and then stimulated with PDGF. Control cells were not stimulated with PDGF. FAK phosphorylated on tyrosine 925 was immunoprecipitated (IP) from extracts of cells infected with SrcMF or the empty vector using a phospho-FAK-Y925-specific antibody. The proteins were resolved by SDS-PAGE, and the levels of phosphorylated FAK were detected following blotting with an anti-FAK antibody (blot). **(b)** Expression of FAK and SrcMF in the SrcMF and vector-infected cells was determined in whole-cell extracts using an anti-FAK antibody (top panel), and exogenously expressed SrcMF was visualized using the chicken-specific Src EC10 antibody (bottom panel).

cellular partners and that the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) is one binding partner of the v-Src SH3 domain that is important for its peripheral targeting [7]. Although the catalytic activity of Src is not required for its targeting, a number of stimuli that induce its peripheral targeting also activate Src kinase activity (e.g., PDGF and bradykinin). These stimuli can therefore release constraints on both the kinase domain and the SH3 domain of c-Src, ensuring that the activated protein is not aberrantly localized within the cell.

### Regulation of cell motility requires coordinated activity of Rho GTPases and Src

Involvement of RhoA, Rac1, and Cdc42 in cell motility is undoubtedly linked to their ability to rearrange the actin cytoskeleton. However, a secondary consequence of this actin remodeling is apparently to target other cellular proteins, such as c-Src, to these peripheral sites. We show here that once located at the cell periphery c-Src acts in concert with the Rho GTPases to regulate cell polarity and motility. Several studies have suggested that Src family kinases play a role during cell motility. For example, c-Src-deficient fibroblasts have a decreased random migration [21], and cells deficient in all three ubiquitously expressed family members, Src, Fyn, and Yes, are unable to migrate into a wound [25]. Furthermore, c-Src kinase activity is required for growth factor-induced migration and scattering in a number of cell types [26–28]. The ability of Src to influence cell motility in fibroblasts has been linked to the regulation of focal adhesion turnover. Cells lacking Src family members are able to assemble focal adhesions [25], indicating that Src is not required for their formation. However, cells in which kinase-defective mutants of Src are expressed have enlarged focal adhesions [4, 6], suggestive of a role for Src kinase activity in the turnover of these structures that is needed for cells to move [6]. The presence of functional SH2 and SH3 domains in these Src proteins suggests that, in addition to phosphorylating key substrates, Src may be acting as an adaptor protein allowing its recruitment into focal adhesion structures and/or to recruit cellular partners to these sites. One such candidate is FAK, as there is considerable evidence linking Src activity to FAK-regulated cell motility [8, 29]. It is known that in fibroblasts lacking the FAK protein there is an increased number of focal adhesions and an impaired ability to move, suggesting that FAK is required for the turnover of focal adhesions during cell motility [30], while the ability of Src to induce adhesion turnover has been linked to the phosphorylation and possibly subsequent proteolysis of FAK [6, 8]. In this study, we have shown that phosphorylation of FAK on tyrosine 925, which is a site known to be phosphorylated by Src [23], is suppressed in cells which are unable to remodel their peripheral adhesions after PDGF treatment, correlating with impaired cell motility and polarity. Thus, SrcMF expression suppresses both Src-dependent phosphorylation events at the cell periphery and stimulus-induced adhesion remodeling.

A key component of the coordinated movement of cells is their ability to polarize toward different migratory signals, and we addressed the role of c-Src in this process. The formation of Rac1-dependent lamellipodia at the leading edge is required for cell motility [19, 20, 31]. In addition, while Cdc42 is not essential for cell movement per se, it is required for directionality and the maintenance of cell polarity [19, 20, 32]. We have shown that c-Src kinase activity is required for cells to polarize and form a leading edge, and we propose that the ability of the Rho GTPases to direct the localization of Src to these peripheral sites is required for the remodeling of the more static focal adhesions into smaller more dynamic focal complexes found at the leading edge of motile cells.

The involvement of Src kinases in the regulation of integrin-ECM interactions has been reported in a number of studies [25, 33, 34]. Specifically, Src can regulate the generation of contractile force between the actin cytoskeleton and integrins, thus modulating cell motility [33]. In addition, c-Src can transiently inhibit integrin-induced activation of Rho GTPase activity [34], and oncogenic v-Src can antagonize RhoA activity via mechanisms involving activation of p190RhoGAP [35, 36]. Thus, Src may act to regulate the turnover of Rho-mediated adhesions, which is critical to the migratory process. In addition to adhesion remodeling, Src facilitates the turnover of actin filaments which stabilize focal adhesion sites [7, 36], and this may partially explain the reduction of random migration observed in our study in cells expressing kinase-defective Src mutants (Figure 6a) and also cells lacking Src and the other family members Fyn and Yes [25]. More recently, the mDia family of Rho effectors, which cooperate with ROCK in the formation of bundled stress fibers, have been shown to bind to Src, and Src kinase activity is required for the cooperative effect of mDia on stress fiber formation [37]. Thus, c-Src can have both positive and negative effects on Rho-mediated stress fiber and adhesion formation and may act at a pivotal point between their assembly and disassembly, placing it in a central position from which to coordinate the multifaceted process of cell motility.

The major challenges are now to determine (1) how motile cells integrate different signals that regulate the activity of RhoA, Rac1, and Cdc42, which in turn regulate the localization of c-Src and possibly other Src family kinases within the cell, and (2) how Src-dependent tyrosine phosphorylation regulates adhesion remodeling in response to biological stimuli that induce directed cell migration.

## Materials and methods

*Cells and plasmids*

CA10-Src251-GFP and CA10-Src were kind gifts from P. Schwartzberg (NIH, Bethesda, Maryland), and CA10-SrcY527F and CA10-SrcMF (K295M,Y527F) were a kind gift from K. Kaplan (MIT, Cambridge, Massachusetts). pEGFP (Clontech, Basingstoke, UK) was used for expression of GFP. N19- and V14RhoA, V12- and N17Cdc42, and L61- and N17Rac, all in the pRK5myc plasmid, were kindly provided by Alan Hall (UCL, London, UK). Swiss 3T3 cells were routinely grown in DMEM supplemented with 10% fetal calf serum. Plasmids were introduced into the cells by nuclear microinjection or transient transfection as indicated in the text, with further details given in the Supplementary materials.

*Immunofluorescence*

Cells were plated onto glass chamber slides 24 hr prior to transfection or onto glass coverslips 24 hr prior to microinjection. For experiments in which cells were serum starved to remove stress fibers prior to expression of the proteins, the cells were grown for 48 hr in serum-free conditions prior to microinjection of the appropriate plasmids and analysis carried out 3–5 hr later after expression of the proteins. For all other experiments, the proteins were expressed by transient transfection. Growth factor treatment of the cells were as follows: 10% serum for 30 min, PDGF, 25 ng/ml$^{-1}$ for 30 min (TCS Biologicals, Botolph Claydon, UK), bradykinin, 100 nM for 5 min or LPA 200 ng/ml$^{-1}$ for 30 min (both Sigma, Poole, UK). Y-27632 (10 μM, Welfide Corporation, Japan), cytochalasin D (0.1 μg/ml$^{-1}$), or emetine (55 μg/ml$^{-1}$, both Sigma, Poole, UK) were added as indicated. Cells were fixed in 3% paraformaldehyde, washed PBS/100 nM glycine, then permeabilized with PBS/0.1% saponin/20 mM glycine. After blocking with PBS/0.1% saponin/10% fetal calf serum, cells were incubated with anti-Src mAb EC10 to detect the exogenously expressed chicken Src proteins (Upstate Biotechnology, Lake Placid, New York), anti-Src monoclonal antibody N2-17 to detect endogenous Src protein (a kind gift from T. Hunter, Salk Institute, California), anti-vinculin mAb, rhodamine-conjugated phalloidin (both Sigma, Poole, UK), or rhodamine-conjugated paxillin antibody (Research Diagnostics, Flanders, New Jersey). Nonconjugated antibody detection was by reaction with species-specific TRITC-conjugated antibodies (Sigma, Poole, UK). Cells were visualized using a confocal microscope (MRC 600; BioRad Labs, Hercules, California).

*FAK phosphorylation*

Cells infected with pBABEpuro or pBABEpuro-SrcMF were trypsinized and plated at 2.5 × 10$^6$ onto 100 mm culture plates in growth medium for 24 hr. The cells were then serum starved for 24 hr prior to stimulation with 25 ng/ml$^{-1}$ PDGF. Cell extracts were prepared in 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1 mM EGTA, 10 mM sodium pyrophosphate, 0.5 mM sodium fluoride, 1 mM PMSF, 10 μg/ml$^{-1}$ aprotinin, and 100 μM sodium orthovanadate, and 1 mg of cell lysate incubated with 2 μg of phospho-FAK-Y925 rabbit polyclonal antibody. Generation of this antibody was as described in [38] using the peptide NDKVYENVT GLVKA(C), which corresponds to residues 921–934 in FAK, in which tyrosine 925 has been chemically phosphorylated. The immune complexes were collected with protein A sepharose and the proteins resolved by SDS-PAGE. FAK phosphorylated on tyrosine 925 was detected following incubation with anti-FAK monoclonal antibody (Transduction Laboratories, BD Biosciences, Oxford, UK) using ECL detection.

*Cell migration and polarization*

Cells were transfected with Src251-GFP or GFP or cotransfected with SrcY527F and GFP or SrcMF and GFP to allow detection of the transfected cells. To monitor polarization, cells were trypsinized 24 hr post-transfection and plated onto coverslips for 1 hr. Cells were then serum straved for 1 hr and mounted on Dunn chambers with PDGF 10 ng/ml$^{-1}$ as chemoattractant [19]. For measurement of both random migration and polarization, microscopic images of the cells were acquired from a charged couple device camera (Hamamatsu C4742) and captured by Open Lab (Improvision Software, UK) every 15 min over a 5 hr period. Details of how the mean cell speed was calculated and the criteria used to determine cell polarity are given in the Supplementary materials.

*Supplementary material*

Further experimental details on the different methods used for the introduction of plasmids into the cells and the rationale for their use can be found in the Supplementary material online at http://images.cellpress.com/supmat/supmatin.htm. More detailed descriptions are also given for the measurement of cell migration and polarity.

Additional data are provided in which we have used the kinase inhibitor PP2 as further confirmation of the requirement of Src kinase activity for efficient remodeling of cellular adhesions in response to PDGF, and data are also presented which demonstrates that bradykinin-induced focal adhesion remodeling to produce focal complexes was also impaired in Src251-GFP-expressing cells. We also provide further evidence that Src kinase activity is required for the turnover of focal adhesions in experiments where treatment of cells expressing Src251-GFP with the ROCK inhibitor results in the stabilization of focal adhesions which are normally broken down in the absence of a Rho signal.

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## References


1. Schoenwaelder SM, Burridge K: **Bidirectional signaling between the cytoskeleton and integrins.** *Curr Opin Cell Biol* 1999, **11:**274-286.
2. David-Pfeuty T, Nouvian-Dooghe Y: **Immunolocalization of the cellular src protein in interphase and mitotic NIH c-src overexpresser cells.** *J Cell Biol* 1990, **111:**3097-3116.
3. Kaplan KB, Swedlow JR, Varmus HE, Morgan DO: **Association of p60c-src with endosomal membranes in mammalian fibroblasts.** *J Cell Biol* 1992, **118:**321-333.
4. Kaplan KB, Bibbins KB, Swedlow JR, Arnaud M, Morgan DO, Varmus HE: **Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527.** *EMBO J* 1994, **13:**4745-4756.
5. Fincham VJ, Unlu M, Brunton VG, Pitts JD, Wyke JA, Frame MC: **Translocation of Src kinase to the cell periphery is mediated by the actin cytoskeleton under the control of the Rho family of small G proteins.** *J Cell Biol* 1996, **135:**1551-1564.
6. Fincham VJ, Frame MC: **The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility.** *EMBO J* 1998, **17:**81-92.
7. Fincham VJ, Brunton VG, Frame MC: **The SH3 domain directs acto-myosin-dependent targeting of v-Src to focal adhesions via phosphatidylinositol 3-kinase.** *Mol Cell Biol* 2000, **20:**6518-6536.
8. Fincham VJ, Wyke JA, Frame MC: **v-Src-induced degradation of focal adhesion kinase during morphological transformation of chicken embryo fibroblasts.** *Oncogene* 1995, **10:**2247-2252.
9. Ridley AJ, Hall A: **The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors.** *Cell* 1992, **70:**389-399.
10. Chrzanowska-Wodnicka M, Burridge K: **Rho-stimulated contractility drives the formation of stress fibers and focal adhesions.** *J Cell Biol* 1996, **133:**1403-1415.
11. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A: **The small GTP-binding protein rac regulates growth factor-induced membrane ruffling.** *Cell* 1992, **70:**401-410.
12. Kozma R, Ahmed S, Best A, Lim L: **The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia.** *Mol Cell Biol* 1995, **15:**1942-1952.
13. Nobes CD, Hall A: **Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia.** *Cell* 1995, **81:**53-62.
14. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, *et al.*: **Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension.** *Nature* 1997, **389:**990-994.
15. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, *et al.*: **Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase).** *Science* 1996, **273:**245-248.
16. Leung T, Chen XQ, Manser E, Lim L: **The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton.** *Mol Cell Biol* 1996, **16:**5313-5327.
17. Mellstrom K, Heldin C, Westermark B: **Induction of circular membrane ruffling on human fibroblasts by platelet-derived growth factor.** *Expt Cell Res* 1988, **177:**347-359.
18. Thomas SM, Brugge JS: **Cellular functions regulated by Src family kinases.** *Annu Rev Cell Dev Biol* 1997, **13:**513-609.
19. Allen WE, Zicha D, Ridley AJ, Jones GE: **A role for Cdc42 in macrophage chemotaxis.** *J Cell Biol* 1998, **141:**1147-1157.
20. Nobes CD, Hall A: **Rho GTPases control polarity, protrusion, and adhesion during cell movement.** *J Cell Biol* 1999, **144:**1235-1244.
21. Hall CL, Lange LA, Prober DA, Zhang S, Turley EA: **pp60(c-src) is required for cell locomotion regulated by the hyaluronan receptor RHAMM.** *Oncogene* 1996, **13:**2213-2224.
22. Parsons JT, Martin KH, Slack JK, Taylor JM, Weed SA: **Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement.** *Oncogene* 2000, **19:**5606-5613.
23. Schlaepfer DD, Hunter T: **Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases.** *Mol Cell Biol* 1996, **16:**5623-5633.
24. Small JV, Rottner K, Kaverina I: **Functional design in the actin cytoskeleton.** *Curr Opin Cell Biol* 1999, **11:**54-60.
25. Klinghoffer RA, Sachsenmaier C, Cooper JA, Soriano P: **Src family kinases are required for integrin but not PDGFR signal transduction.** *EMBO J* 1999, **18:**2459-2471.
26. Rodier JM, Valles AM, Denoyelle M, Thiery JP, Boyer B: **pp60$^{c\text{-}src}$ is a positive regulator of growth factor-induced cell scattering in a rat bladder carcinoma cell line.** *J Cell Biol* 1995, **131:**761-773.
27. Rahim N, Hung W, Tremblay E, Saulnier R, Elliot B: **c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells.** *J Biol Chem* 1998, **273:**33714-33721.
28. Liu J, Huang C, Zhan X: **Src is required for cell migration and shape changes induced by fibroblast growth factor 1.** *Oncogene* 1999, **18:**6700-6706.
29. Cary LA, Chang JF, Guan JL: **Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn.** *J Cell Sci* 1996, **109:**1787-1794.
30. Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, *et al.*: **Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice.** *Nature* 1995, **377:**539-544.
31. Ridley AJ, Comoglio PM, Hall A: **Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac and Rho in MDCK cells.** *Mol Cell Biol* 1995, **15:**1110-1122.
32. Stowers L, Yelon D, Berg LJ, Chant J: **Regulation of the polarization of T cells toward antigen-presenting cells by Ras-related small GTPase Cdc42.** *Proc Natl Acad Sci USA* 1995, **92:**5027-5031.
33. Felsenfeld DP, Schwartzberg PL, Venegas A, Tse R, Sheetz MP: **Selective regulation of integrin-cytoskeleton interactions by the tyrosine kinase Src.** *Nat Cell Biol* 1999, **1:**200-206.
34. Arthur WT, Petch LA, Burridge K: **Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism.** *Curr Biol* 2000, **10:**719-722.
35. Ellis C, Moran M, McCormick F, Pawson T: **Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases.** *Nature* 1990, **343:**377-381.
36. Fincham VJ, Chudleigh A, Frame MC: **Regulation of p190 Rho-GAP by v-Src is linked to cytoskeletal disruption during transformation.** *J Cell Sci* 1999, **112:**947-956.
37. Tominaga T, Sahai E, Chardin P, McCormick F, Courtneidge SA, Alberts AS: **Diaphanous-related formins bridge Rho GTPase and Src tyrosine signaling.** *Mol Cell* 2000, **5:**12-25.
38. McLean GW, Fincham VJ, Frame MC: **v-Src induces tyrosine phosphorylation of focal adhesion kinase independently of tyrosine 397 and formation of a complex with Src.** *J Biol Chem* 2000, **275:**23333-23339.