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RhoB and Actin Polymerization Coordinate Src Activation with Endosome-Mediated Delivery to the Membrane

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Summary

We have used a c-Src-GFP fusion protein to address the spatial control of Src activation and the nature of Src-associated intracellular structures during stimulus-induced transit to the membrane. Src is activated during transit, particularly in RhoB-containing cytoplasmic endosomes associated with the perinuclear recycling compartment. Knocking out RhoB or expressing a dominant-interfering Rab11 mutant suppresses both catalytic activation of Src and translocation of active kinase to peripheral membrane structures. In addition, the Src- and RhoB-containing endosomes harbor proteins involved in actin polymerization and filament assembly, for example Scar1, and newly polymerized actin can associate with these endosomes in a Src-dependent manner. This implies that Src may regulate an endosome-associated actin nucleation activity. In keeping with this, Src controls the actin dependence of RhoB endosome movement toward the plasma membrane. This work identifies RhoB as a component of "outside-in" signaling pathways that coordinate Src activation with translocation to transmembrane receptors.

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Introduction

We previously studied assembly of complexes that contain the v-Src oncoprotein, or its c-Src cellular counterpart, and identified a critical role for the actin cytoskeleton (Fincham et al., 1996, 2000). Membrane translocation of v-Src is kinase independent (Fincham and Frame, 1998) but requires binding of the Src SH3 domain to proteins like the regulatory subunit of PI3-kinase (Fincham et al., 2000). In the case of c-Src too, kinase activity is dispensable for membrane targeting, and stimulation of actin rearrangements by the activities of RhoA, Rac1, or Cdc42 cause recruitment of Src to adhesion complexes at stress fiber termini, lamellipodia, or filopodia, respectively (Timpson et al., 2001). RhoA activity is particularly needed to generate actin filaments that mediate translocation of Src from the perinuclear region to adhesion sites at the plasma membrane (Timpson et al., 2001), although it remains unknown how Src functionally interacts with the actin assembly machinery or how it moves through the cytoplasm.

Although much of the data on the mechanism of Src intracellular targeting has been obtained using v-Src mutants, there is no evidence that c-Src and v-Src use distinct mechanisms. In fact, there are many obvious similarities. Earlier studies demonstrated that a substantial proportion of exogenously expressed c-Src is localized in the perinuclear region, colocalizing with endosome markers and the trans-Golgi network (Kaplan et al., 1992). Furthermore, constitutively active c-Src is targeted to fibroblast focal adhesions, mediated by the amino-terminal half of Src that contains the SH3 and SH2 domains (Kaplan et al., 1994). A protein consisting of green fluorescent protein (GFP) fused to the kinase-deficient amino-terminal half of c-Src containing the SH3/2 domains (Src251-GFP) is constitutively targeted to the cell periphery, although this is still dependent on the activity of Rho GTPases (Timpson et al., 2001). Thus, translocation of both c-Src and v-Src is initiated in the perinuclear region, requires the SH3/SH2 domains in both cases (even although the SH3 domains of c-Src and v-Src have distinct binding specificities [Hauck et al., 2001]), and is directed by the coordinated activities of Rho GTPases.

Important questions remain about regulation of Src's subcellular distribution, particularly in response to extracellular stimuli. It is not known how activation of the kinase is spatially regulated. In addition, the significance of Src's association with endosomes, and the possible roles of Rho GTPases other than RhoA, Rac1, and Cdc42, such as RhoB or RhoD that associate with endosomes, are not known. Recent findings suggest that Src can link endosome and actin regulation and that a RhoD-dependent signaling pathway may control movement of associated endosomes along actin filaments or microtubules (Gasman et al., 2003). RhoB also localizes to endosomes (Adamson et al., 1992), and recent work has identified a role for RhoB in the endosomal recruitment of the serine/threonine kinase PRK1 and in controlling EGF

receptor trafficking (Gampel et al., 1999; Mellor et al., 1998). In addition, RhoB and PRK1 cause recruitment of the PI3-kinase effector PDK1 to endosomes (Flynn et al., 2000), and RhoB controls the intracellular trafficking of Akt (Adini et al., 2003), a PDK1 effector protein that is often linked to cell survival. Thus, evidence to date strongly implicates RhoB as a mediator of vesicle trafficking that contributes to regulation of at least one signaling cascade, the PI3-kinase/PDK1/Akt pathway. Whether this represents a more general role for RhoB in the membrane trafficking of signaling proteins, such as Src, is not known.

In the present study, we have characterized a c-Src-GFP fusion protein (Src-WT-GFP) that is regulated normally in cells. Together with use of an antibody that specifically recognizes autophosphorylated Src, this allowed us to address where Src was active in cells and the nature of intracellular structures with which it associated during transit to the plasma membrane. We found that the actin cytoskeleton was needed for catalytic activation as well as for peripheral membrane targeting of Src, and established an important role for RhoB, the Rab11-dependent endosome compartment and endosome-associated actin assembly.

Results

Src-WT-GFP Is Similarly Regulated to Src-WT

The direct fusion of GFP to Src is problematic. Addition at the amino terminus interferes with myristylation-dependent events, while addition at the carboxy terminus interferes with the regulation of Src folding and hence tight control of its catalytic activity. To circumvent this problem, we fused GFP sequences to the carboxy terminus of Src by engineering a glycine/serine-rich flexible linker peptide that distanced the GFP moiety from the rest of the Src protein (depicted in Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/7/6/855/DC1/). However, it was important to test whether the Src-WT-GFP created was regulated normally within the cell. Using autophosphorylation of Src-Y416 as a measure of Src activity, we show that, like the nontagged proteins, Src-Y527F-GFP is considerably more active than Src-WT-GFP (Figure 1A, bottom left). This is consistent with a substantial proportion of Src-WT-GFP being maintained in an inactive state when compared to Src-Y527F-GFP. To complement use of the phospho-Y416-Src-specific antibody, we confirmed by in vitro kinase assays that Src-WT and Src-WT-GFP were much less active against enolase substrate than Src-Y527F or Src-Y527F-GFP (Figure 1A, right). In addition, we showed that Src-WT and Src-WT-GFP were both phosphorylated at the negative regulatory Src-Y527 residue, implying that addition of the GFP moiety did not affect the ability of c-Src to act as a substrate for Csk (Figure 1B).

Another measure of Src activation state is availability of its SH3 and SH2 domains to bind cellular partners, such as focal adhesion kinase (FAK). We found that in unstimulated cells, FAK coimmunoprecipitated with Src-Y527F-GFP (which is "open" and has its SH3/2 domains available for binding; Figure 1C, left). By comparison, only a very small amount of FAK was complexed with Src-WT-GFP (Figure 1C, bottom left). We also showed that in unstimulated cells, addition of GFP did not increase FAK binding (Figure 1C, right).

Src-WT-GFP Is Activated Modestly by PDGF and More Strongly by Cell Adhesion

PDGF is a serum mitogen that requires Src for its biological activity (Broome and Hunter, 1996; Erpel et al., 1996; Roche et al., 1995; Twamley-Stein et al., 1993). Although there was some variability in the actual degree of activation from experiment to experiment, we found that Src-WT-GFP and Src-WT were both consistently, but relatively modestly (typically about 1.5- to 2-fold), more active in PDGF-treated cells (Figure 2A, top). Moreover, autophosphorylated Src-WT or Src-WT-GFP was already detectable in adherent, serum-deprived control cells (Figure 2A, top, left two lanes), showing that mitogen stimulation was not a prerequisite for Src activity. We also tested whether cell adhesion was a major activator of Src-WT (as reported, for example, in Kaplan et al., 1995). We found that in Src/Yes/Fyn$^{-/-}$ (SYF) cells, exogenous Src activity was not readily detected (although some could be detected in very long exposures in suspended cells [not shown]) but was strongly induced after plating cells on fibronectin (Figure 2B, top).

Src Activation and Peripheral Targeting Are Tightly Linked

We compared the spatial distribution and activation of Src-WT-GFP with Src-WT (Figure 2C, top and bottom, respectively) with that of Src-Y527F (Supplemental Figure S2A) in response to stimuli that activate specific Rho GTPases. Active Src-WT and active Src-WT-GFP similarly translocated to LPA-induced (RhoA-mediated) focal adhesions, PDGF-induced (Rac1-mediated) membrane ruffles, and bradykinin-induced (Cdc42-mediated) filopodia (active Src was visualized as red in Figure 2C). Only a small proportion of Src (relative to the total Src present after transfection) was ever targeted to these membrane sites, and most of the Src remained inactive in the perinuclear region (broken arrows). In contrast, Src-Y527F was almost entirely located in peripheral adhesion structures and was active at these sites (judged by yellow staining of merged red/green in Supplemental Figure S2A). Src-Y527F was not usually visible in the perinuclear region, indicating that most of it had translocated to membrane sites, even under conditions of serum deprivation (Figure 2D, right). Thus, the GFP-fusions of Src-WT responded as their nontagged counterparts, i.e., Src-WT-GFP was tightly controlled with respect to activity and membrane targeting, with most of the protein remaining inactive and cytoplasmic. Inactive Src-WT, like inactive *ts* mutants of v-Src we described previously, was retained in the perinuclear region of cells (Fincham et al., 1996). We confirmed that the phospho-Y416-Src-specific antibody used detected only a single 60 kDa species in immunoblotting and that this was specifically competed by the appropriate phosphopeptide (not shown). Also, there was no signal in immunofluorescence of PDGF-stimulated SYF$^{-/-}$ cells (Figure 2D, left), further confirming the specificity of the anti-phospho-Y416-Src antibody used.

Thus, Src-WT-GFP is regulated in a manner that is

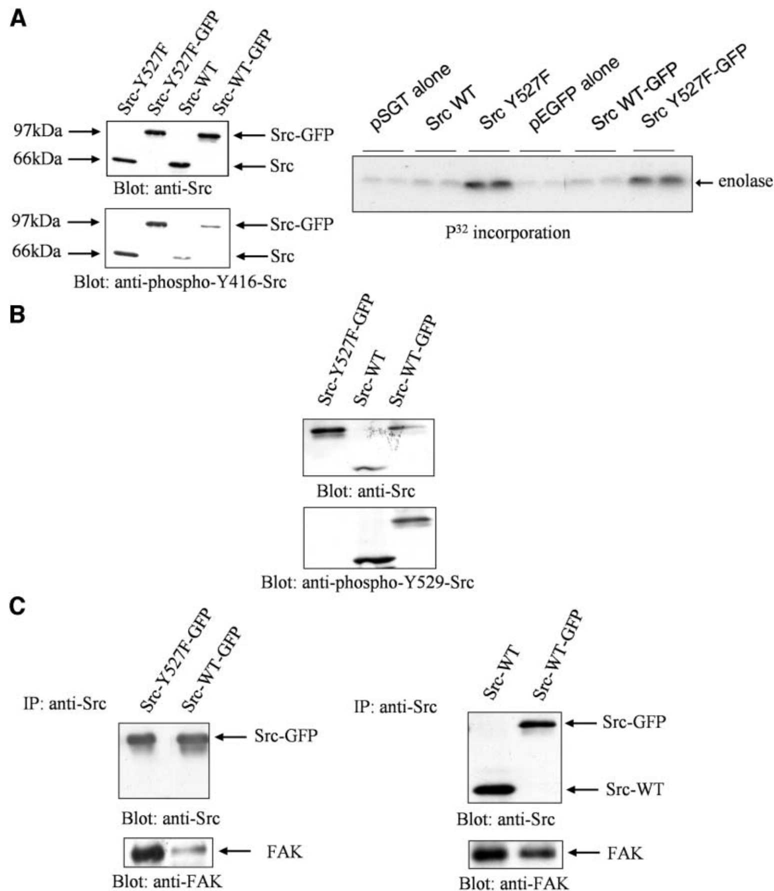

Figure 1. Characterization of a Regulated Wild-Type Src-GFP Fusion Protein

(A) Lysates from SYF$^{-/-}$ mouse embryo fibroblasts (MEFs) expressing Src-Y527F, Src-Y527F-GFP, Src-WT, or Src-WT-GFP were immunoblotted using anti-Src (top) or anti-phospho-Y416-Src (bottom). Lysates were also immunoprecipitated (IP) with anti-Cst-1 antibody and an in vitro kinase assay carried out to measure catalytic activity (right).

(B) Lysates were also immunoblotted using anti-Src (top) or anti-phospho-Y529-Src (bottom).

(C) Src was immunoprecipitated from lysates of SYF$^{-/-}$ cells expressing Src-Y527F-GFP and Src-WT-GFP (left) or Src-WT and Src-WT-GFP (right) using an anti-Src antibody (Src-327) conjugated to agarose. IPs were then immunoblotted using anti-Src (top) and anti-FAK (bottom) antibodies.

indistinguishable from Src-WT, with respect to both activity state, spatial distribution, and availability of its SH domains to bind cellular partners, validating its use as a tool for further study of how Src activity is spatially regulated and the mode of intracellular targeting to peripheral membrane sites.

Src Activation Occurs in Transit to the Membrane and Is Linked to Actin Regulation

Upon close examination of LPA-stimulated Swiss 3T3 cells that overexpress Src-WT-GFP, we found some active Src present in the cytoplasm (Figure 3A, visualized as yellow in merged image), particularly between the perinuclear region (where Src is inactive; green) and the membrane structures (where Src is highly active; red). In many cells, such as that shown in Figure 3A, a gradient of "Src activation" was observed across the cytoplasm, going from inactive (green) through to highly active (orange/red) at the membrane (Figure 3A, right panel). Thus, although only a relatively small proportion of total Src is translocated upon stimulation, it appears that the membrane-associated Src is highly active. Similarly with bradykinin, some active Src is evident in the cytoplasm (Supplemental Figure S2B, yellow in merged image), and Src at the extreme cell periphery is present in discrete structures along filopodia, where it is highly active (Supplemental Figure S2B, orange/red in merged image).

To address the previous link we had made between actin assembly and Src regulation, we examined the effect of cytochalasin D on PDGF-induced activation and translocation of Src-WT expressed in SYF$^{-/-}$ cells. Cytochalasin D inhibited Src membrane translocation; instead, all Src-WT-GFP was retained in the perinuclear region (Figure 3B, green in left panels). Staining with phalloidin confirmed that actin filaments had been disrupted by cytochalasin D treatment (Figure 3B, red in middle panels). Interestingly, costaining with phospho-SrcY416-specific antibody showed that Src was most highly active in PDGF-induced membrane ruffles (Figure 3B, blue in upper right panel) and that activation was impaired in cytochalasin D-treated cells (Figure 3B, lower right panel). Thus, activation of Src also requires an intact actin cytoskeleton. This is consistent with catalytic activation of Src occurring during translocation to the membrane. Immunoblotting also showed that PDGF-induced Src activation was suppressed by cytochalasin D (Figure 3C), by C2 toxin (Supplemental Figure S3A), which specifically interferes with actin polymerization (Barth et al., 2002), and also by latrunculin B, which sequesters G-actin monomers (Supplemental Figure S3B), further suggesting a link between Src activation and actin polymerization. The suppression in Src activation as a consequence of actin-disrupting drugs cytochalasin D and C2 toxin disagrees with a previous report that Src is activated by treatment with cytochalasin D (Lock et al., 1998). Although we do not know the precise

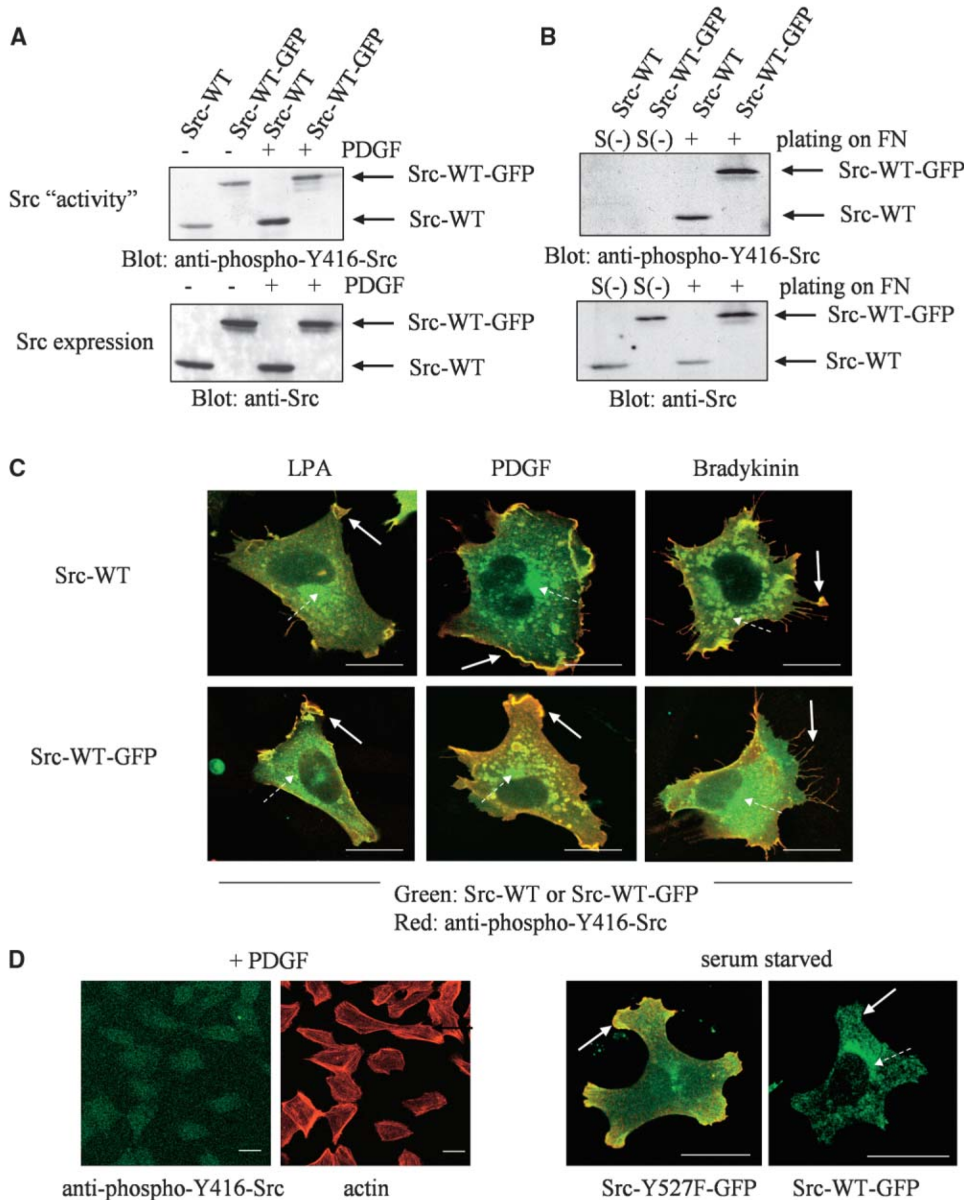

Figure 2. PDGF and Cell Adhesion Activate Src and Src-GFP

(A) $SYF^{-/-}$ cells expressing Src-WT or Src-WT-GFP were serum starved for 16 hr prior to stimulation with PDGF (25 $ngml^{-1}$) for 30 min.

(B) Cells expressing Src-WT or Src-WT-GFP were serum starved for 16 hr before being plated on fibronectin (10 μg/ml) or maintained in suspension for 1 hr.

(C) Cells expressing Src-WT or Src-WT-GFP were serum starved for 16 hr prior to stimulation with LPA (200 $ngml^{-1}$) for 30 min, PDGF (25 $ngml^{-1}$) for 30 min, or bradykinin (100 nM) for 3 min. Src-WT-GFP was visualized by direct fluorescence, Src-WT with an anti-EC10 antibody (FITC secondary), and active Src with anti-phospho-Y416-Src (Texas red secondary).

(D) PDGF-stimulated cells were stained with anti-phospho-Y416-Src (FITC secondary) and with TRITC phalloidin (left), and $SYF^{-/-}$ cells were transfected with Src-Y527F-GFP and Src-WT-GFP, then serum starved for 16 hr (right). Src-WT-GFP and Src-Y527-GFP were visualized by direct fluorescence and active Src with anti-phospho-Y416-Src (Texas red secondary). Arrows indicate active Src at peripheral structures while broken arrows indicate Src retained in the perinuclear region. Scale bars equal 25 μM.

reason for this discrepancy, it may be related to the different cells or to the much higher concentration of cytochalasin D used in the previous study.

An Interfering Mutant of Scar1 Impairs Src Activation and Peripheral Targeting

To address the role of actin polymerization more directly, we made use of an interfering mutant of Scar1 (also known as WAVE1), a member of the WASP/Scar family of adaptor proteins that links upstream signals to actin polymerization. Specifically, we used *myc*-tagged Scar1 WA (three constructs depicted in Supplemental Figure S4A) as a tool that sequesters the Arp2/3 actin nucleation complex that normally binds to the WA region of WASP/Scar proteins (Machesky and Insall, 1998). We confirmed that the overall effect of Scar1 WA was to

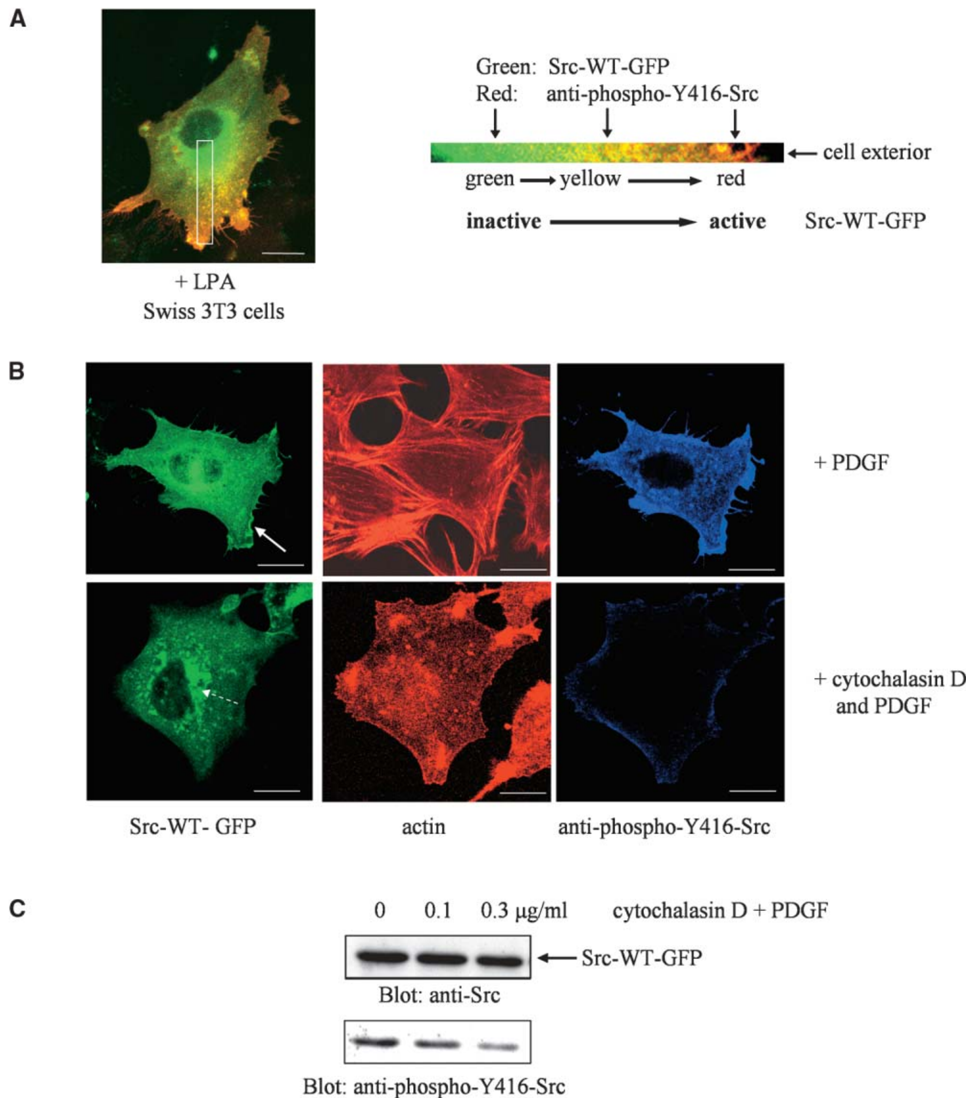

Figure 3. Src Membrane Localization and Activation Are Dependent on the Organized Actin Cytoskeleton

(A) Cells expressing Src-WT-GFP were serum starved and stimulated with LPA. Src-WT-GFP was visualized by direct fluorescence and active Src with anti-phospho-Y416-Src (Texas red secondary).

(B) SYF$^{-/-}$ cells expressing Src-WT-GFP were serum starved for 16 hr, then stimulated with PDGF (top) or treated with cytochalasin D (0.3 μg/ml) for 1 hr before PDGF stimulation (bottom). Src-WT-GFP and TRITC phalloidin were visualized by direct fluorescence and active Src with an anti-phospho-Y416-Src (Cy5 secondary). Arrows indicate Src at the membrane while broken arrows indicate Src held in perinuclear region. Scale bars equal 25 μM.

(C) SYF$^{-/-}$ cells expressing Src-WT-GFP were serum starved for 16 hr prior to treatment with cytochalasin D for 1 hr (0.1 μg/ml or 0.3 μg/ml) and PDGF stimulation. Lysates were then immunoblotted with anti-Src (top) and anti-phospho-Y416-Src (bottom). Src-WT-GFP is shown.

cause disruption of filamentous actin (Supplemental Figure S4A, bottom left) when compared to untransfected cells or cells expressing Scar1 S (Supplemental Figure S4A, top and middle, respectively). In comparison to untransfected cells (Figure 4A, top) or cells expressing a noninterfering Scar1 S mutant (Figure 4A, middle; Machesky and Insall, 1998), Src-WT-GFP was unable to translocate to the membrane in response to PDGF in cells expressing Scar1 WA (Figure 4A, bottom; quantified in Figure 4B). In cells expressing this Scar1 mutant (Figure 4C, right, visualized as red) we found that Src-WT-GFP did not translocate to the membrane (an example is shown in Figure 4C, right), and anti-phospho-Y416-Src staining (blue) was not evident either at the membrane or in the perinuclear region where Src-WT-GFP was retained in an inactive state (Figure 4C, right, broken arrow). For clarity, single images of only anti-phospho-Y416-Src staining are shown (blue in Figure 4C, bottom). This was in contrast to untransfected cells, or cells expressing a Scar1 S mutant, in which PDGF treatment induced some active Src-WT-GFP to translocate to the membrane (Figure 4C, visualized as blue staining, unbroken arrows, left and middle).

Thus, we have identified a clear link between actin filament assembly and both Src membrane targeting and catalytic activation, ensuring that Src never becomes activated in inappropriate subcellular locations. This further suggests that the "outside-in" signaling from

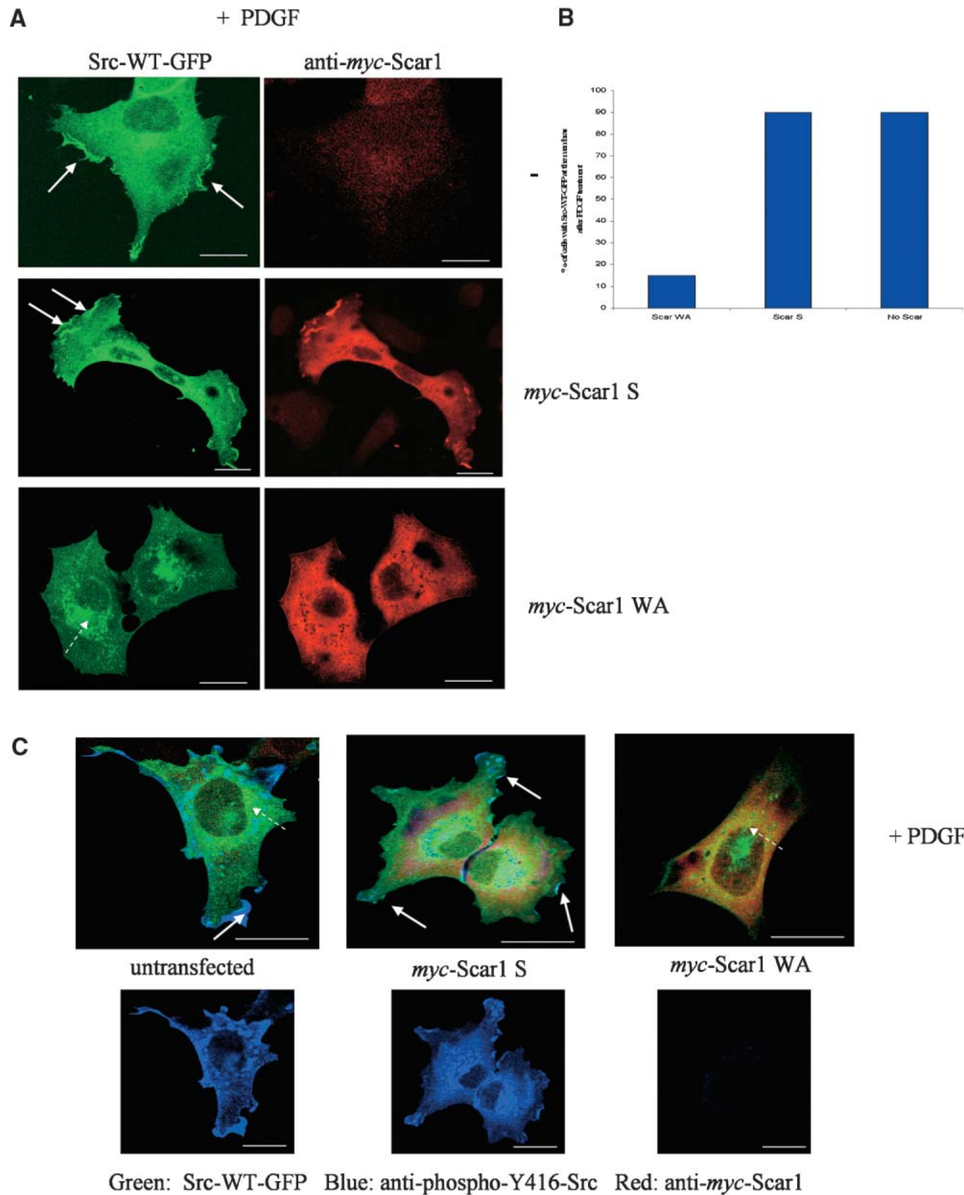

Figure 4. Actin Filament Assembly Is Required for Peripheral Targeting

(A) SYF$^{-/-}$ cells were transfected with Src-WT-GFP and either untransfected or transfected with *myc*-Scar1 S or *myc*-Scar1 WA. After serum starvation for 16 hr, cells were stimulated with PDGF. Src-WT-GFP (left) was visualized by direct fluorescence and Scar1 (right) detected with an anti-*myc* antibody (Texas red secondary). Arrows indicate the presence of Src at the cell periphery or in the perinuclear region.

(B) The graph represents quantification of 100 cells expressing each myc-Scar1 protein that has Src-WT-GFP at periphery after PDGF stimulation and is expressed as a percentage of total cells.

(C) Top panels show cells expressing Src-WT-GFP and either *myc*-Scar1 S or *myc*-Scar1 WA stimulated with PDGF and stained with anti-*myc* antibody (Texas red secondary) and anti-phospho-Y416-Src antibody (Cy5 secondary). Arrows indicate active Src while broken arrows indicate inactive Src. Bottom panels show only active Src (blue). Scale bars equal 25 μM.

ligand bound PDGF receptor that mediates recruitment of active Src to the activated receptor involves signaling to the actin polymerization machinery. However, such "outside-in" signaling may also involve regulation of actin-associated cytoplasmic structures, such as endosomes.

RhoB Colocalizes with Active Src in the Cytoplasm

In the course of the experiments above, we often saw discrete intracellular structures containing Src-WT around the perinuclear region, but particularly out toward the cell periphery. As Src had previously been colocalized with cytoplasmic endosomes (Gasman et al., 2003; Kaplan et al., 1992), we investigated whether Src-WT-GFP expressed in SYF$^{-/-}$ cells colocalized with the endosome-associated Rho GTPase family members RhoB or RhoD. In the case of exogenous *myc*-tagged RhoB, we saw strong colocalization both in the perinuclear region (Figure 5A, visualized as yellow in merged image, left) and in discrete structures throughout the cytoplasm (Figure

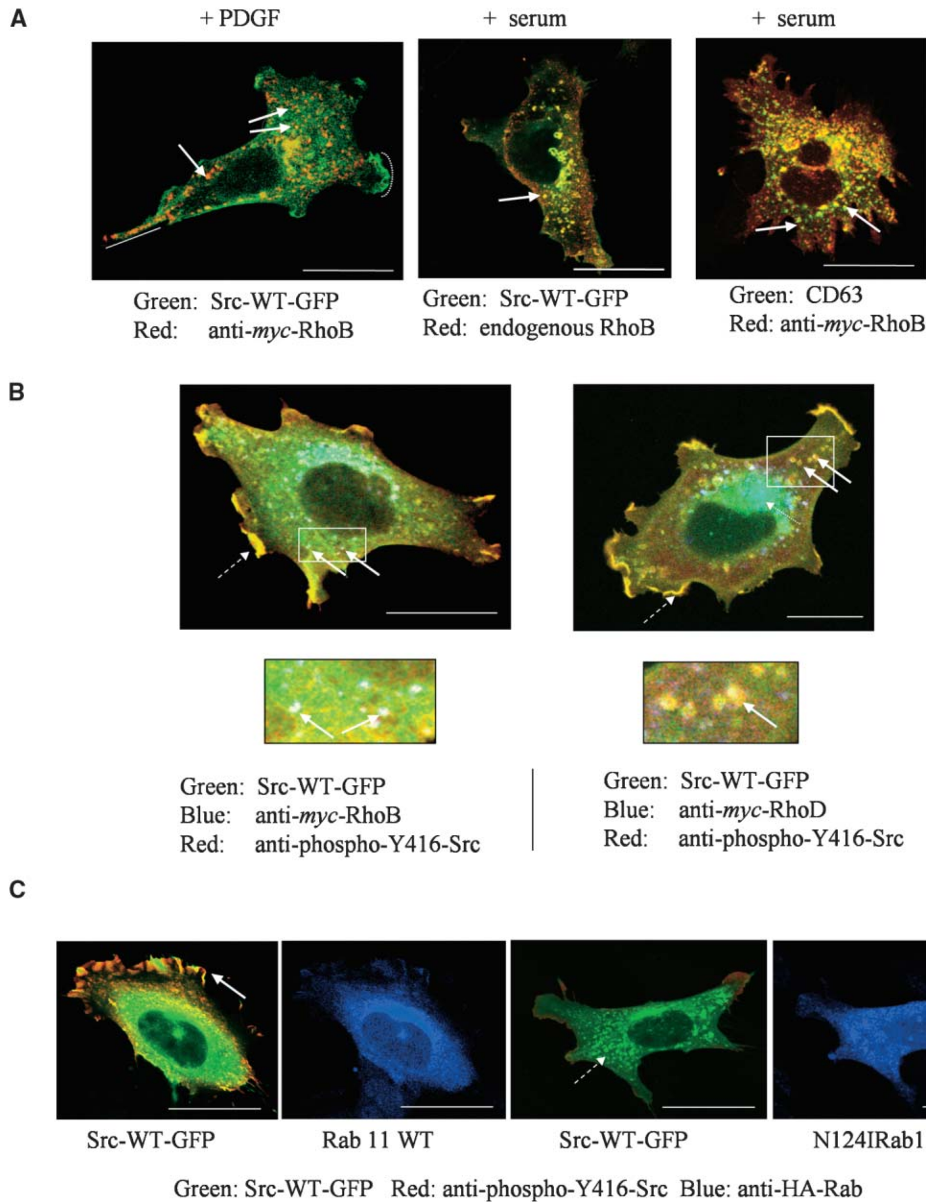

Figure 5. Active Src-WT-GFP Is Associated with RhoB Endosomes in Transit to the Cell Periphery

(A) RhoB$^{+/+}$ MEFs (middle) expressing low levels of Src-WT-GFP were stained for endogenous RhoB using an anti-RhoB antibody (Texas red secondary). SYF$^{-/-}$ cells were transfected with Src-WT-GFP and *myc*-RhoB prior to PDGF stimulation (left) or were transfected with *myc*-RhoB and CD63-GFP (right). Src-WT-GFP and CD63-GFP were visualized by direct fluorescence and *myc*-RhoB was detected with an anti-*myc* antibody (Texas red secondary). Arrows indicate colocalization in discrete structures. Unbroken straight line indicates the pseudopod at the rear of the cell. The broken lines show membrane ruffles at the front of the cell.

(B) Cells expressing Src-WT-GFP and *myc*-RhoB (left) or *myc*-RhoD (right) were stimulated with PDGF. Src-WT-GFP was visualized by direct fluorescence, RhoB and RhoD with an anti-*myc* antibody (Cy5 secondary), and active Src with anti-phospho-Y416-Src (Texas red secondary). Solid arrows indicate active Src in structures outside the perinuclear region of the cell, broken arrows show active Src at the periphery, and dotted arrow shows RhoD.

(C) Cells expressing Src-WT-GFP and either Rab11 WT (left) or dominant-negative N124IRab11 (right) were stimulated with PDGF. Src-WT-GFP was visualized by direct fluorescence, Rab11 with an anti-HA tag antibody (Cy5 secondary), and active Src with anti-phospho-Y416-Src (Texas red secondary). Arrows indicate active Src at the cell membrane and broken arrows indicate Src retained in the cytoplasm. Scale bars equal 25 μM.

5A, arrows, left). For example, we saw a number of Src/RhoB structures aligned along the pseudopod at the rear of the cell (Figure 5A, unbroken straight line, left), whereas Src, but very little RhoB, was visible in membrane ruffles along the front edges of the cell (Figure 5A, broken lines, left). We also saw colocalization of endogenous RhoB and low-level Src-WT-GFP, indicating that Src and RhoB are visible in the same cytoplasmic structures even when not overexpressed (Figure 5A, middle). Also, RhoB colocalized, at least partially,

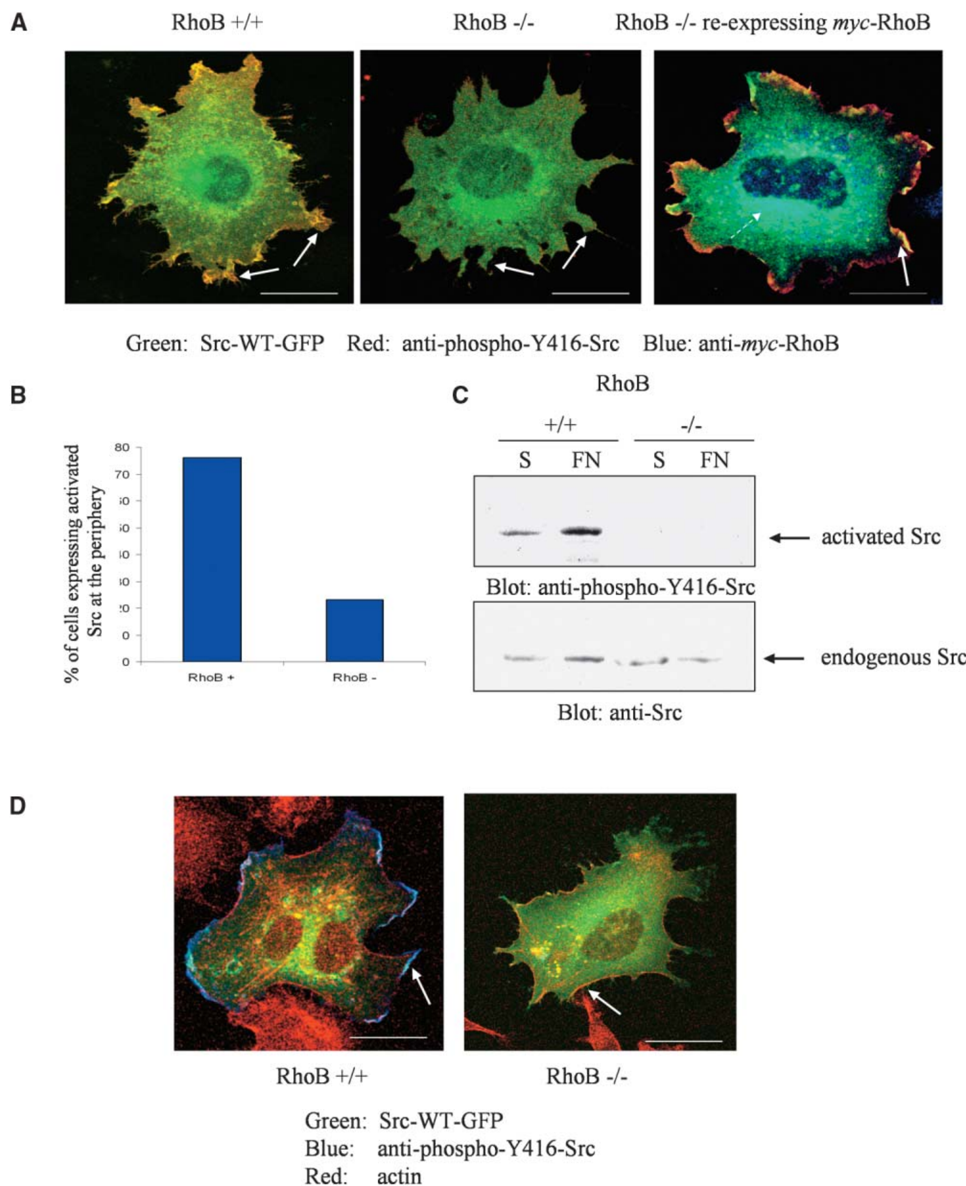

Figure 6. Targeting and Activation of Src Is RhoB Dependent

(A) RhoB$^{+/+}$ MEFs (left) and RhoB$^{-/-}$ MEFs (middle) expressing Src-WT-GFP were serum starved for 16 hr, then plated on fibronectin-coated dishes (10 μg/ml) for 1 hr. *myc*-RhoB was also reintroduced back into RhoB$^{-/-}$ cells prior to plating on fibronectin (right). Cells were stained with anti-phospho-Y416-Src (Texas red secondary), anti-*myc* antibody (Cy5 secondary), and the Src visualized directly. Solid arrows indicate the presence or absence of active Src at the cell periphery while dashed arrows indicate the presence of *myc*-RhoB.

(B) Quantification of cells expressing active Src at the membrane shown as a percentage of 100 cells expressing Src.

(C) Cells were either maintained in suspension or plated on fibronectin for 1 hr. Endogenous Src was detected by immunoblotting using anti-Src (bottom) and anti-phospho-Y416-Src (top).

(D) RhoB$^{+/+}$ (left) and RhoB$^{-/-}$ cells (right) expressing Src-WT-GFP were plated on fibronectin for 1 hr, then stained with anti-phospho-Y416 Src antibody (Cy5 secondary) and with TRITC phalloidin. Arrows indicate membrane ruffles. Scale bars equal 25 μM.

with CD63, a marker of late endosomes that has already been reported to colocalize with endogenous RhoB (Figure 5A, right; Wherlock et al., 2004). Using triple color imaging, we found, as before, that Src-WT-GFP was targeted to the membrane in an active form (Figure 5B, dashed arrows) and that intracellular structures that had moved out from the perinuclear region contained both RhoB and active Src (Figure 5B, left, white in merged image, solid arrows). In contrast to RhoB, we did not find strong colocalization between active Src-WT-GFP and RhoD in the area of cytoplasm between the perinuclear region and the membrane (Figure 5B, right, active Src-WT-GFP visualized as yellow, RhoD in blue in merged image; arrows show active Src in structures in the cytoplasm that lack RhoD), although there was some overlap between inactive Src and RhoD adjacent to the nucleus (Figure 5B, right, dotted arrow). Higher magnification of cytoplasmic regions of different images confirm a lack of RhoD (blue) staining when compared to RhoB in cytoplasmic Src-containing structures (these

were visualized as white in Figure 5B, left). We also found that translocation of Src and concomitant activation was inhibited by expression of dominant-negative mutant of Rab11, but not wild-type Rab11 that controls the perinuclear recycling compartment and recycling of some integrins to the plasma membrane (Figure 5C; Roberts et al., 2004; Ullrich et al., 1996).

RhoB Is Required for Both Catalytic Activation and Peripheral Targeting of Src after Plating on Fibronectin

The colocalization of Src-WT-GFP with RhoB prompted us to ask whether Src was activated, or translocated to the membrane, in RhoB$^{-/-}$ cells. Since the fold activation of Src was greatest when suspended cells were adhered to a substratum (Figure 2), we compared the localization of active Src-WT-GFP in RhoB$^{+/+}$ and RhoB$^{-/-}$ cells after plating. In the majority of RhoB$^{-/-}$ cells, there was no accumulation of Src at the membrane and little visible evidence of Src activation (Figure 6A, middle). Quantification indicated that active Src was present in only about 20% of RhoB$^{-/-}$ cells, as opposed to about 80% of their RhoB$^{+/+}$ counterparts (Figure 6B). Furthermore, although some active Src was detected in 20% of RhoB$^{-/-}$ cells, this was usually a much smaller amount of activated Src than was normally seen in RhoB$^{+/+}$ cells (not shown). Re-expression of exogenous RhoB restored peripheral targeting of activated Src (Figure 6A, right). Adhesion-induced activation of endogenous Src, as judged by immunoblotting with anti-phospho-Y416-Src, was inhibited in RhoB$^{-/-}$ cells, and residual Src activity found in suspended RhoB$^{+/+}$ mouse embryo fibroblasts (MEFs) was inhibited (Figure 6C). RhoB$^{-/-}$ cells were generally less well spread when plated on to fibronectin (compare wild-type MEFs and RhoB$^{-/-}$ counterparts in Figure 6D); this is consistent with a previous report of impaired cell spreading (Liu et al., 2001). However, peripheral actin structures were still evident (Figure 6D, right, arrow), yet no active Src was visible at these peripheral actin structures in RhoB$^{-/-}$ cells (Figure 6D, right) when compared to the targeting of active Src to large membrane ruffles in wild-type MEFs (Figure 6D, left, visualized as blue). Thus, RhoB is a major contributor to control of Src's peripheral targeting and activation upon adhesion.

Src Influences the Actin Dependence of RhoB-Associated Endosomes

Since we have separately implicated RhoB and polymerized actin in the catalytic activation and membrane targeting of Src, and as this is impaired by expression of an interfering mutant of Scar1 (Figure 4), we costained SYF$^{-/-}$ cells expressing Src-WT-GFP, RhoB-CFP, and *myc*-tagged Scar1. Triple color merging indicated that all three could readily be detected in discrete structures in the cytoplasm, showing that Scar1 is also present in Src-containing, RhoB-associated endosomes (Figure 7A). This implies that these endosomes may be directly linked to the actin polymerization machinery in some way. We therefore tested whether the subcellular distribution of RhoB endosomes was affected by disruption of the actin cytoskeleton. Treatment with cytochalasin D did not alter RhoB endosomes, which were distributed throughout the cytoplasm of PDGF-treated SYF$^{-/-}$ cells (Figure 7B, compare blue staining in top right with middle right). What we did find was that RhoB endosomes were tightly restrained in the perinuclear region of cytochalasin D-treated SYF$^{-/-}$ cells in which Src had been reintroduced (Figure 7B, bottom right). Quantification of the number of cells in which RhoB endosomes were visibly held in the perinuclear region by cytochalasin D treatment is shown in Supplemental Figure S5. These findings suggest that the presence of Src in RhoB endosomes renders these endosomes dependent on the actin cytoskeleton for their movement within the cytoplasm. In addition to Scar1, the actin filament regulatory protein mDia2 is also present in the Src-containing RhoB-associated endosomes (not shown), further supporting the idea that these structures are directly linked in some way to actin polymerization events.

Src- and RhoB-Containing Structures in Transit Are Associated with New Polymerized Actin

We simultaneously stimulated Src membrane targeting and actin polymerization. For this we used cells expressing a *ts* v-Src that was rendered kinase defective (KD) to avoid confusion caused by simultaneous activation of a strong v-Src kinase activity. This enabled exquisitely controlled induction of Src membrane targeting by a simple temperature shift. This *ts* v-Src-KD protein is tightly retained in the perinuclear region and synchronously targeted to the cell periphery in an actin-dependent manner upon switch to the permissive temperature (Fincham et al., 2000; Fincham and Frame, 1998). We treated cells expressing this *ts* v-Src-KD protein (which we had also fused to GFP) at the restrictive temperature with cytochalasin D to disrupt the actin cytoskeleton (Figure 8A). Then, we washed out the cytochalasin D to allow simultaneous reformation of polymerized actin filaments and induced membrane targeting of *ts* v-Src-KD-GFP by switch to the permissive temperature. Under these conditions, we could visualize Src-containing structures (by GFP in green) associated with small newly forming bundled actin filaments, termed actin "clouds," in the cytoplasm between the perinuclear region and the cell membrane (examples shown in Figure 8B). This is reminiscent of images that described endocytic vesicles moving at the tips of actin tails in cultured mast cells (described in Merrifield et al., 1999) and may be analogous to the actin-based structures associated with some pathogens that is also controlled by tyrosine phosphorylation (Frischknecht et al., 1999; Newsome et al., 2004). In addition, we visualized RhoB-containing structures associated with short actin clouds after simultaneous serum addition and cytochalasin D removal (Figure 8C) and demonstrated that association of these RhoB-containing structures with actin clouds does not occur in SYF$^{-/-}$ cells (Figure 8C, right). Although stimulation of Src-WT-GFP translocation in response to serum is less synchronous than the tight *ts* v-Src regulation, we have also visualized similar Src-WT-GFP-containing structures at the tips of associated actin clouds (Figure 8D). Moreover, actin clouds associated with the Src-containing structures were not evident upon expression of the Scar1 WA mutant that impairs actin assembly (Figure 4; Supplemental Figure S4). Thus, it appears that under some circumstances at least, Src- and RhoB-containing cytoplasmic structures are directly linked to

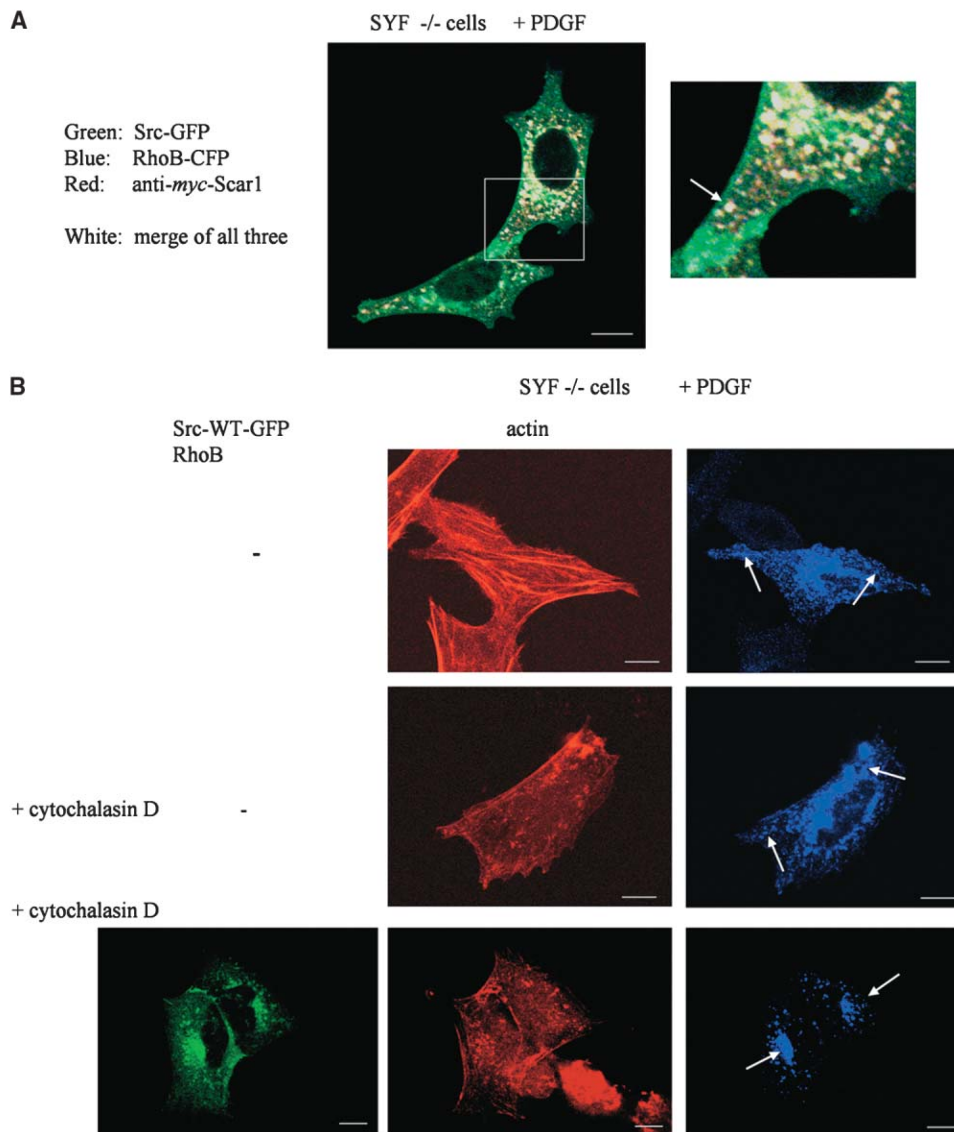

Figure 7. RhoB-Associated Endosome Distribution Is Actin Dependent when Src Is Present

(A) SYF$^{-/-}$ cells expressing Src-WT-GFP, RhoB-CFP, and *myc*-Scar1 were stimulated with PDGF. Src-WT-GFP and RhoB-CFP were visualized directly and Scar1 with an anti-myc antibody (Texas red secondary). Arrows indicate colocalization of all three proteins.

(B) Cells expressing *myc*-RhoB (top and middle) or both Src-WT-GFP and *myc*-RhoB (bottom) were serum starved for 16 hr. Cells were then stimulated with PDGF and left untreated (−) or treated (+) with cytochalasin D (0.3 μg/ml) for 1 hr before PDGF stimulation. Src-WT-GFP and TRITC phalloidin were visualized directly and RhoB with an anti-*myc* antibody (Cy5 secondary). Arrows indicate position of RhoB-associated endosomes. Scale bars equal 25 μM.

short actin clouds after synchronous induction of translocation and new actin polymerization. Time-lapse imaging shows that the endosome structures associated with the actin clouds are motile within the cytoplasm (Supplemental Movie S1). At longer times after cytochalasin D removal, endosome-associated actin clouds appear to coalesce to form stress fiber-like structures and endosomes can be visualized moving while associated with these (Supplemental Movie S2). Thus, RhoB/Src and Scar1-containing endosome structures associate with actin, and it is possible that these proteins functionally cooperate to permit endosome-associated actin assembly and actin-dependent movement away from the perinuclear region. This is visibly linked to stimulus-induced delivery of the required amount of active Src to the plasma membrane in a RhoB-dependent manner. In a different context, F-actin has previously been visualized in association with endosomes and time-lapse imaging, suggesting that the F-actin spots may be responsible for endosome propulsion (Kaksonen et al., 2000).

Discussion

Stimulus-Induced Src Activity and Translocation

Src family kinases are required for mitogenic signaling downstream of receptors for PDGF, epidermal growth factor (EGF), and colony stimulating factor-1 (CSF-1) (Roche et al., 1995; Twamley-Stein et al., 1993), as well as downstream of integrin ECM receptors. All three of the ubiquitous Src kinases are activated, albeit rather

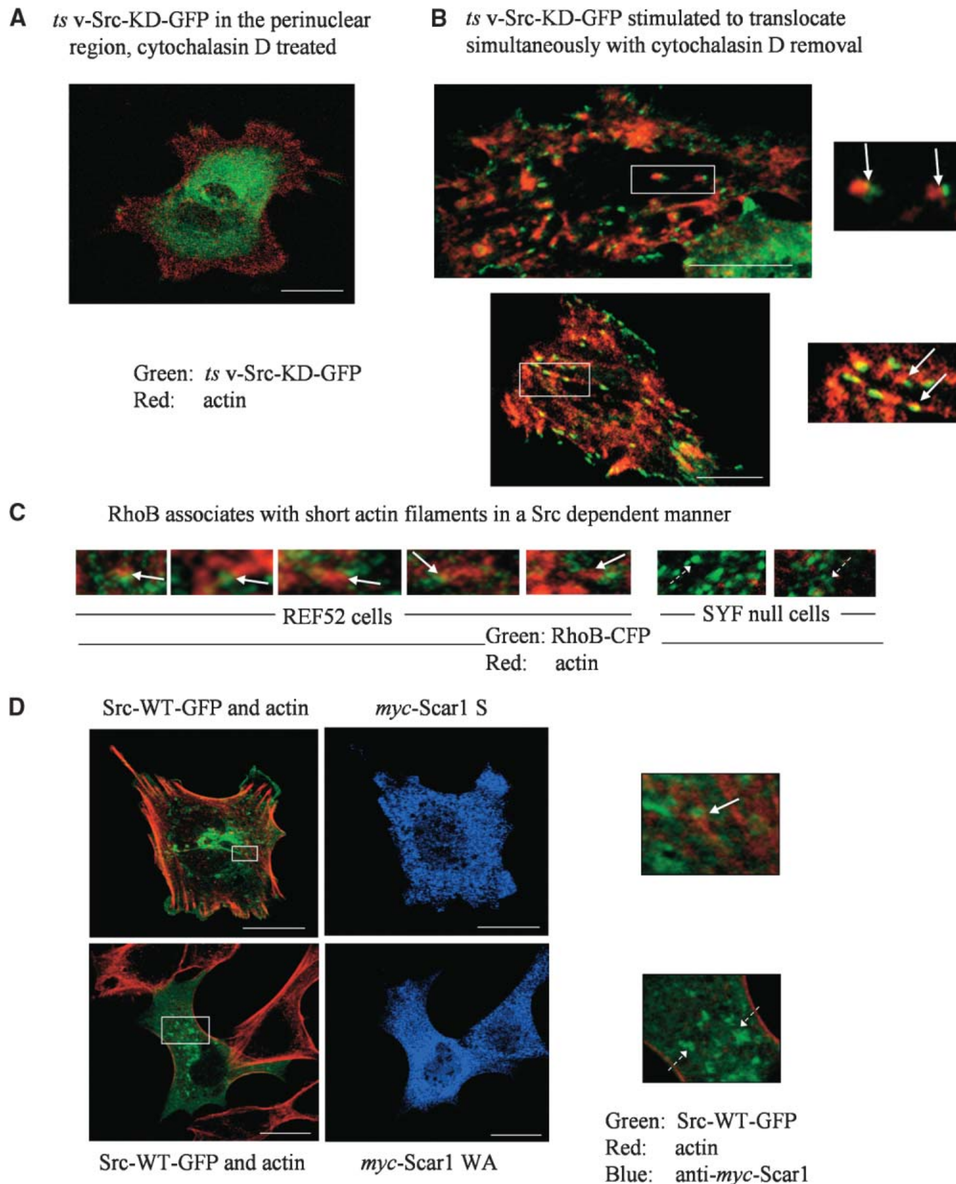

Figure 8. New Actin Polymerization Is Associated with Src in Transit to the Cell Periphery

(A) REF52 cells expressing *ts* v-Src-KD-GFP and Ds-Red actin were treated with cytochalasin D (0.3 μg/ml) for 1 hr at the restrictive temperature of 39°C.

(B) Cells expressing *ts* v-Src-KD-GFP and Ds-Red actin were treated with cytochalasin D for 1 hr at 39°C, then washed twice in DMEM plus 10% FBS and switched to the permissive temperature of 35°C for 30 min to synchronize initiation of Src translocation and actin repolymerization. Both proteins were visualized by direct fluorescence.

(C) REF52 cells (left) and $SYF^{-/-}$ (right) cells expressing CFP-RhoB and Ds-Red-actin were treated with cytochalasin D for 1 hr, then the cytochalasin D was washed out for 20 min. Both proteins were visualized by direct fluorescence.

(D) $SYF^{-/-}$ cells expressing Src-WT-GFP and *myc*-Scar1 S (top) or *myc*-Scar1 WA (bottom) were treated with cytochalasin D (0.3 μg/ml) for 1 hr, then maintained in DMEM plus 10% FBS for 20 min. Src and actin were visualized directly and Scar1 with anti-*myc* antibody (Cy5 secondary). Solid arrows indicate association with small newly polymerizing actin filaments, while broken arrows indicate the absence of association. Scale bars equal 25 μM.

modestly, after growth factor treatment, and about 5%–10% of Src molecules become transiently associated with activated PDGF-receptors (Abram and Courtneidge, 2000; Kypta et al., 1990). In the case of PDGF-receptor signaling, Src is thought to induce mitogenesis via Ras-independent signaling to Myc (Barone and Courtneidge, 1995), whereas Src may phosphorylate STAT transcription factors needed for mitogenic signaling by the EGF receptor (Olayioye et al., 1999). Although there is considerable information on downstream effectors, the nature of the signals that cause ligand bound PDGF-receptor, or occupied integrin, to trigger Src activation, and translocation from perinuclear membranes to the plasma membrane are much less well understood.

One common event downstream of both PDGF- and EGF-receptor activation appears to be translocation of Src to the actin cytoskeleton (Weernink and Rijksen, 1995). Interestingly, proper mitogenic signaling downstream of the PDGF receptor requires the Src SH3 domain (Broome and Hunter, 1996; Erpel et al., 1996), which we have shown, in the context of v-Src, to be essential for actin association and membrane translocation (Fincham et al., 2000). Src is also translocated to the cytoskeleton during thrombin-induced platelet activation (Oda et al., 1992; Wong et al., 1992), implying that Src activation is commonly linked to association with the actin cytoskeleton. Here, we show that some Src activation in response to PDGF occurs in the cytoplasm, probably during transit to the plasma membrane, and the actin cytoskeleton is necessary for both membrane translocation and optimal activation of Src. This implies that catalytic activation and actin-dependent peripheral targeting are coregulated after growth factor or integrin stimulation.

Integrin stimulation is also reported to induce transient activation of Src during spreading of fibroblasts (Kaplan et al., 1995). In our experiments, adhesion of cells resulted in robust activation and membrane translocation of Src that was re-expressed in SYF$^{-/-}$ MEFs. The low level of Src activity in suspended cells (in which actin is disorganized) supports a key role for the actin cytoskeleton in stimulus-induced Src activation.

Involvement of RhoB-Associated Endosomes

Numerous studies have observed Src present at intracellular membranes as well as at the plasma membrane. For example, v-Src or c-Src have variously been colocalized with the nuclear envelope and juxtanuclear reticular membrane structures (Krueger et al., 1980), specifically in perinuclear vesicles in this region of the cell (Redmond et al., 1992), with Golgi membranes in CHO cells (Bard et al., 2002), with intracellular membranes of osteoclasts (Horne et al., 1992), and with endosomes in the trans-Golgi region of fibroblasts (Kaplan et al., 1992). Thus, v-Src, and a substantial proportion of c-Src, colocalize around the nucleus with endosomal membranes (Fincham et al., 1996, 2000; Kaplan et al., 1992), while a smaller proportion also locates at the plasma membrane. However, it has not been established whether, and if so how, Src activity differs at these distinct subcellular locations. Here we show that perinuclear Src is generally inactive. After stimulation with PDGF, or plating cells on fibronectin, some Src is present in an active form in cytoplasmic structures and at peripheral membranes. Moreover, a gradient of Src activity is often observed between the perinuclear region and peripheral membrane structures after stimulation. In contrast to normal Src, the constitutively active mutant SrcY527F protein is largely recruited into peripheral structures, presumably because the SH3/2 domains of active Src constitutively bind cellular partners that control membrane targeting (Fincham et al., 2000; Timpson et al., 2001).

The endosomal structures containing active Src colocalized with RhoB, a Rho GTPase family member known to reside at the cytosolic face of endocytic vesicles and to be involved in EGF-receptor trafficking (Gampel et al., 1999; Robertson et al., 1995). By contrast, we did not find active Src associated with endosomes that colocalized with RhoD, although it was recently reported that RhoD causes activation and endosomal recruitment of Src by a mechanism involving hDia2C (Gasman et al., 2003). Although the reason for these differences is unknown, individual cell types may use different Rho GTPases to move Src around in the cell, reflecting a level of redundancy between endosome-regulating members of the Rho GTPase family. Such redundancy might explain why Src can translocate to the cell periphery in about 20% of RhoB-deficient cells, and also why RhoB is dispensable for mouse development (Liu et al., 2001). In our study, Src activity was stimulated after recruitment to cytoplasmic endosomes that also contained RhoB and overlapped with the late endosomal marker CD63 (Escola et al., 1998). Furthermore, Src targeting and activation were blocked by a dominant-negative mutant of Rab11, suggesting that Src is recruited into the perinuclear recycling compartment that is controlled by Rab11. A model is presented in Supplemental Figure S7. This is consistent with inactive Src residing in the perinuclear region and being “picked up” from that location for transport to peripheral membranes by Rab11-dependent endosomes. Active Src was not generally visualized in the perinuclear region, implying that Src was activated after recruitment to endosomes had been initiated.

One question raised by these findings is whether or not the active Src we visualized in association with endosome structures was in transit to the cell periphery after stimulation, or being internalized for recycling or degradation. This was addressed by use of RhoB$^{-/-}$ cells, in which we found that Src was neither translocated to the membrane nor activated normally in about 80% of cells after plating on fibronectin. This indicates that Src-containing RhoB-associated cytoplasmic endosomes are required for the targeting of active Src outwards from the perinuclear region to the cell periphery.

Actin Dependence of Src Activation and Membrane Translocation

Our data show that Src activation and peripheral targeting requires the cellular actin network to be intact. In fibroblasts, RhoA-induced actin filaments are needed for Src to move through the cytoplasm from the juxtanuclear region to the plasma membrane (Fincham et al., 2000; Timpson et al., 2001). Our finding that another Rho family member, RhoB, is needed demonstrates that coordinated activities of different Rho proteins regulate Src intracellular trafficking. Here, we show that actin polymerization is also required. We found that Scar1, a key regulator of actin polymerization, is present in Src-associated, RhoB-associated endosomes. Moreover, an interfering mutant of Scar1 that sequesters Arp2/3 and blocks actin nucleation inhibited Src activation and peripheral targeting. However, a number of lines of evidence demonstrate the existence of actin tracks for the movement of subsets of vesicles from the trans-Golgi area to the apical membrane in epithelial cells (Jacob et al., 2003). There are also indications that actin filament nucleation by endomembranes may be an early event in the transportation of vesicular cargo along actin filaments, and that this may involve unconventional low

molecular weight myosins (Raposo et al., 1999; Taunton, 2001). Interestingly, pinosomes provide an example of vesicles that carry the machinery needed for actin assembly, and these move through the cytoplasm of mast cells at the tips of short-lived actin tails, some of which originate in the middle of cells (Merrifield et al., 1999). Although we have not determined whether Src-containing structures use actin tracks or short actin tails to move through the cytoplasm, we did find that Src could localize at the tips of short actin clouds in the cytoplasm of cells in which Src targeting was tightly synchronized with recovery of actin polymerization. Similar newly polymerized actin clouds are associated with RhoB endosomes, implying that Src recruitment to these endosomes may induce actin nucleation events (perhaps via Scar1 or mDia2 that we also found to be present [not shown]) that lead to the formation of associated actin clouds and actin-dependent movement. In keeping with this, we found that the movement of RhoB-associated endosomes out of the perinuclear region became actin-dependent when Src was re-expressed in $SYF^{-/-}$ cells. Thus, Src may "hijack" a subset of cytoplasmic endosomes to regulate movement through the cytoplasm in an actin-dependent manner.

Conclusions and Perspectives

One essential process in directional cell responses is the movement of required signal transduction proteins to the cell surface. Our findings imply a new role for RhoB in controlling the stimulus-induced peripheral targeting of Src. The role for RhoB-associated endosomes in outward movement of Src to the plasma membrane and inward movement of EGF-receptor (Gampel et al., 1999) indicates that these endosomes are bidirectional with regard to moving signaling proteins through the cytoplasm. Furthermore, the RhoB dependence of Src activation and translocation to the plasma membrane could explain why RhoB-deficient cells exhibit adhesion and migration defects (Liu et al., 2001). Interestingly, RhoB is induced by v-Src and is an immediate-early gene induced by EGF or PDGF (Jahner and Hunter, 1991), suggesting that stimuli requiring Src activity induce expression of this Rho protein that critically coordinates Src activation and targeting. This further suggests the possibility that RhoB is an important component of "outside-in" signaling pathway from ligand bound growth factor receptors that stimulates Src activation and translocation to the activated receptors. In addition, RhoB and Src are needed for overlapping biological responses, for example, serum-response factor-induced transcription from the serum response element (Hakak and Martin, 1999; Lebowitz et al., 1997; Tominaga et al., 2000). This raises the possibility that the requirement for RhoB is to permit translocation of active Src to the plasma membrane. Together with the reported RhoB dependence of intracellular Akt trafficking (Adini et al., 2003), our findings imply that there may be a general role for RhoB in the delivery of signaling proteins to specific subcellular locations.

Experimental Procedures

Plasmids

CA10-SrcWT, SrcY527F, and RhoD were kind gifts from P. Schwartzberg (NIH, Bethesda, MD), K. Kaplan (MIT, Cambridge, MA), and C. Murphy, Medical School, University of Ioannina, Greece), respectively. Scar-1 constructs were kindly provided by L. Machesky (Biological Sciences, University of Birmingham, UK).

The c-Src-linker proteins were generated by the ligation of two fragments: one fragment corresponding to the first 1618 nucleotides of pRSP-Src-WT digested with Xho1 and Bsu361 and the second fragment corresponding to the remaining nucleotides with the addition of a tandem Gly-Ser-Gly-Ser after the Leu533 and a BamHI site by PCR (oligos 5′-CACCCTCTATGGCCGGTTCACCA-3′ and 5′-TTGGATCCGAGCCGGAGCCTAGGTTCTCTCCAGGCTGG-3′). The c-Src-linker proteins were then ligated into pEGFP-N1 vector (BD Biosciences, Oxford, UK) digested with Xho1 and BamH1. Src-Y527F-GFP was made the same way using a pRSP-Src-Y527F construct as a template.

Cell Culture

Swiss 3T3 cells, Src/Yes/Fyn deficient ($SYF^{-/-}$) cells, REF52s, RhoB-deficient mouse embryo fibroblasts ($RhoB^{-/-}$ MEFs), and the relevant $RhoB^{+/+}$ control MEFs were routinely grown in DMEM supplemented with 10% FCS. Cells were plated onto glass chamber slides or 90 mm tissue culture dishes 24 hr prior to transfection. $SYF^{-/-}$ cells were transiently transfected using FuGene6 liposomal reagent (Roche Diagnostics Ltd, Sussex, UK) under serum-free conditions. All other cells were transfected using Polyfect Transfection Reagent (Qiagen, Crawley, UK) then serum starved for 16 hr. Cells were either treated with LPA (200 ng/ml^{-1}) for 30 min (Sigma, Poole, UK), PDGF (25 ng/ml^{-1}) for 30 min (TCS Biologicals, Botolph Claydon, UK), or bradykinin (100 nM) for 3 min (Sigma) or were plated on fibronectin (BD Biosciences) (10 μg/ml) for 1 hr.

Protein Immunoblotting

Cells were lysed in IP buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 5 mM EGTA, 5 mM EDTA, 1 mM vanadate, 25 mM NaF, 1 mM PMSF, and 10 μg/ml aprotinin) and centrifuged at 13,000 rpm and 4°C for 15 min. Immunoblotting was carried out using 10 μg of lysate per sample, the amount of protein being determined using a Micro BCA Protein Assay Kit (Perbio, Cheshire, UK). Proteins were separated by SDS-10% polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, blocked with 5% low-fat milk in TBS-0.2% Tween 20 (Sigma), and probed with anti-Src at 1:1000 (CN Bioscience, Nottingham, UK) in 3% BSA/TBS-0.2%Tween 20, anti-phospho-Y416-Src antibody at 1:1000 (NEB, Hertfordshire, UK) or with anti-phospho-Y529-Src antibody at 1:1000 (Calbiochem, San Diego, CA). Detection was by incubation with HRP-conjugated secondary antibodies, and visualization by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Immunoprecipitation

The amount of Src in each sample was judged by immunoblotting, and equal amounts of Src (approx. 1 mg of lysate) were incubated with 15 μl of anti-Src antibody conjugated to agarose overnight at 4°C (CN Bioscience). IPs were then washed three times in IP buffer and resuspended in 2× electrophoresis sample buffer. Immunoblotting was carried out as before using anti-Src antibody or anti-FAK antibody (BD Biosciences) at 1:1000 in 5% low-fat milk in TBS-0.2%Tween 20.

Kinase Assay

IPs were carried out using 200 μg of lysate and 3 μg of anti-Cst1 antibody (Kypta et al., 1990) overnight at 4°C. IPs were then washed three times in IP buffer and twice in kinase buffer (1 M PIPES, 0.5 M $MnCl_2$, 1 M DTT, and 1 mM vanadate). Samples were incubated with kinase buffer, enolase, and ATP for 5 min at 30°C until the reaction was stopped by the addition of sample buffer. Samples were then run on an SDS-PAGE gel and autoradiography carried out.

Disruption of Actin Cytoskeleton

$SYF^{-/-}$ cells were treated with cytochalasin D (0.3 μg/ml) for 1 hr (Sigma) prior to treatment with PDGF or to being washed out with DMEM plus 10% FBS for 20 min. REF52 cells were transiently co-transfected with v-Src-KD-GFP and Ds-Red actin and incubated overnight at 39°C. Cells were then treated with cytochalasin D (0.1 μg/ml) for 1 hr at 39°C, washed twice in DMEM plus 10% FCS, and

incubated for 30 min at 35°C to initiate actin repolymerization and v-Src translocation. This experiment was also carried out with CFP-RhoB and DsRed actin at 37°C for 20 min.

Immunofluorescence

Cells were washed twice in cold phosphate-buffered saline (PBS) and fixed in 3% paraformaldehyde. They were then washed in PBS/100 nM glycine and permeabilized with PBS/0.1% saponin/20 mM glycine. After blocking with PBS/0.1% saponin/10% FCS, cells were incubated with primary antibodies for 1 hr. Antibodies were anti-Src mAb EC10 to detect exogenously expressed chicken Src proteins, anti-9E10 mAb to detect *myc*-tagged proteins (both Upstate Biotechnology, Lake Placid, NY), anti-phospho-Y416-Src to detect active Src (New England Biolabs, Hertfordshire, UK), anti-HA mAb to detect Rab11 constructs, and TRITC phalloidin to detect actin (Sigma). Nonconjugated antibody detection was by reaction with species-specific FITC, Cy5, or Texas red-conjugated secondary antibodies for 45 min (Jackson ImmunoResearch, Luton, UK). pECFP-Endo (RhoB-CFP) and pEYFP-actin, which was recloned into a DsRed vector (all BD Biosciences), were detected by direct fluorescence. Cells were visualized using a confocal microscope (Leica UK Ltd, Milton Keynes, UK). When using phospho-specific antibodies, PBS was replaced by tris-buffered saline (TBS).

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References

Abram, C.L., and Courtneidge, S.A. (2000). Src family tyrosine kinases and growth factor signaling. Exp. Cell Res. *254*, 1–13.

Adamson, P., Paterson, H.F., and Hall, A. (1992). Intracellular localization of the P21rho proteins. J. Cell Biol. *119*, 617–627.

Adini, I., Rabinovitz, I., Sun, J.F., Prendergast, G.C., and Benjamin, L.E. (2003). RhoB controls Akt trafficking and stage-specific survival of endothelial cells during vascular development. Genes Dev. *17*, 2721–2732.

Bard, F., Patel, U., Levy, J.B., Jurdic, P., Horne, W.C., and Baron, R. (2002). Molecular complexes that contain both c-Cbl and c-Src associate with Golgi membranes. Eur. J. Cell Biol. *81*, 26–35.

Barone, M.V., and Courtneidge, S.A. (1995). Myc but not Fos rescue of PDGF signalling block caused by kinase-inactive Src. Nature *378*, 509–512.

Barth, H., Roebling, R., Fritz, M., and Aktories, K. (2002). The binary *Clostridium botulinum* C2 toxin as a protein delivery system: identification of the minimal protein region necessary for interaction of toxin components. J. Biol. Chem. *277*, 5074–5081.

Broome, M.A., and Hunter, T. (1996). Requirement for c-Src catalytic activity and the SH3 domain in platelet-derived growth factor BB and epidermal growth factor mitogenic signaling. J. Biol. Chem. *271*, 16798–16806.

Erpel, T., Alonso, G., Roche, S., and Courtneidge, S.A. (1996). The Src SH3 domain is required for DNA synthesis induced by platelet-derived growth factor and epidermal growth factor. J. Biol. Chem. *271*, 16807–16812.

Escola, J.M., Kleijmeer, M.J., Stoorvogel, W., Griffith, J.M., Yoshie, O., and Geuze, H.J. (1998). Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J. Biol. Chem. *273*, 20121–20127.

Fincham, V.J., and Frame, M.C. (1998). The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. EMBO J. *17*, 81–92.

Fincham, V.J., Unlu, M., Brunton, V.G., Pitts, J.D., Wyke, J.A., and Frame, M.C. (1996). 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. *135*, 1551–1564.

Fincham, V.J., Brunton, V.G., and Frame, M.C. (2000). The SH3 domain directs acto-myosin-dependent targeting of v-Src to focal adhesions via phosphatidylinositol 3-kinase. Mol. Cell. Biol. *20*, 6518–6536.

Flynn, P., Mellor, H., Casamassima, A., and Parker, P.J. (2000). Rho GTPase control of protein kinase C-related protein kinase activation by 3-phosphoinositide-dependent protein kinase. J. Biol. Chem. *275*, 11064–11070.

Frischknecht, F., Cudmore, S., Moreau, V., Reckmann, I., Rottger, S., and Way, M. (1999). Tyrosine phosphorylation is required for actin-based motility of vaccinia but not *Listeria* or *Shigella*. Curr. Biol. *9*, 89–92.

Gampel, A., Parker, P.J., and Mellor, H. (1999). Regulation of epidermal growth factor receptor traffic by the small GTPase rhoB. Curr. Biol. *9*, 955–958.

Gasman, S., Kalaidzidis, Y., and Zerial, M. (2003). RhoD regulates endosome dynamics through Diaphanous-related Formin and Src tyrosine kinase. Nat. Cell Biol. *5*, 195–204.

Hakak, Y., and Martin, G.S. (1999). Cas mediates transcriptional activation of the serum response element by Src. Mol. Cell. Biol. *19*, 6953–6962.

Hauck, C.R., Hunter, T., and Schlaepfer, D.D. (2001). The v-Src SH3 domain facilitates a cell adhesion-independent association with focal adhesion kinase. J. Biol. Chem. *276*, 17653–17662.

Horne, W.C., Neff, L., Chatterjee, D., Lomri, A., Levy, J.B., and Baron, R. (1992). Osteoclasts express high levels of pp60c-src in association with intracellular membranes. J. Cell Biol. *119*, 1003–1013.

Jacob, R., Heine, M., Alfalah, M., and Naim, H.Y. (2003). Distinct cytoskeletal tracks direct individual vesicle populations to the apical membrane of epithelial cells. Curr. Biol. *13*, 607–612.

Jahner, D., and Hunter, T. (1991). The ras-related gene rhoB is an immediate-early gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts. Mol. Cell. Biol. *11*, 3682–3690.

Kaksonen, M., Peng, H.B., and Rauvala, H. (2000). Association of cortactin with dynamic actin in lamellipodia and on endosomal vesicles. J. Cell Sci. *113*, 4421–4426.

Kaplan, K.B., Swedlow, J.R., Varmus, H.E., and Morgan, D.O. (1992). Association of p60c-src with endosomal membranes in mammalian fibroblasts. J. Cell Biol. *118*, 321–333.

Kaplan, K.B., Bibbins, K.B., Swedlow, J.R., Arnaud, M., Morgan, D.O., and Varmus, H.E. (1994). 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. *13*, 4745–4756.

Kaplan, K.B., Swedlow, J.R., Morgan, D.O., and Varmus, H.E. (1995). c-Src enhances the spreading of src−/− fibroblasts on fibronectin by a kinase-independent mechanism. Genes Dev. *9*, 1505–1517.

Krueger, J.G., Wang, E., Garber, E.A., and Goldberg, A.R. (1980). Differences in intracellular location of pp60src in rat and chicken cells transformed by Rous sarcoma virus. Proc. Natl. Acad. Sci. USA *77*, 4142–4146.

Kypta, R.M., Goldberg, Y., Ulug, E.T., and Courtneidge, S.A. (1990). Association between the PDGF receptor and members of the src family of tyrosine kinases. Cell *62*, 481–492.

Lebowitz, P.F., Du, W., and Prendergast, G.C. (1997). Prenylation of RhoB is required for its cell transforming function but not its ability to activate serum response element-dependent transcription. J. Biol. Chem. *272*, 16093–16095.

Liu, A.X., Rane, N., Liu, J.P., and Prendergast, G.C. (2001). RhoB is dispensable for mouse development, but it modifies susceptibility

to tumor formation as well as cell adhesion and growth factor signaling in transformed cells. Mol. Cell. Biol. *21*, 6906–6912.

Lock, P., Abram, C.L., Gibson, T., and Courtneidge, S.A. (1998). A new method for isolating tyrosine kinase substrates used to identify fish, an SH3 and PX domain-containing protein, and Src substrate. EMBO J. *17*, 4346–4357.

Machesky, L.M., and Insall, R.H. (1998). Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. Curr. Biol. *8*, 1347–1356.

Mellor, H., Flynn, P., Nobes, C.D., Hall, A., and Parker, P.J. (1998). PRK1 is targeted to endosomes by the small GTPase, RhoB. J. Biol. Chem. *273*, 4811–4814.

Merrifield, C.J., Moss, S.E., Ballestrem, C., Imhof, B.A., Giese, G., Wunderlich, I., and Almers, W. (1999). Endocytic vesicles move at the tips of actin tails in cultured mast cells. Nat. Cell Biol. *1*, 72–74.

Newsome, T.P., Scaplehorn, N., and Way, M. (2004). Src mediates a switch from microtubule- to actin-based motility of vaccinia virus. Science *306*, 124–129.

Oda, A., Druker, B.J., Smith, M., and Salzman, E.W. (1992). Association of pp60src with Triton X-100-insoluble residue in human blood platelets requires platelet aggregation and actin polymerization. J. Biol. Chem. *267*, 20075–20081.

Olayioye, M.A., Beuvink, I., Horsch, K., Daly, J.M., and Hynes, N.E. (1999). ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. J. Biol. Chem. *274*, 17209–17218.

Raposo, G., Cordonnier, M.N., Tenza, D., Menichi, B., Durrbach, A., Louvard, D., and Coudrier, E. (1999). Association of myosin I alpha with endosomes and lysosomes in mammalian cells. Mol. Biol. Cell *10*, 1477–1494.

Redmond, T., Brott, B.K., Jove, R., and Welsh, M.J. (1992). Localization of the viral and cellular Src kinases to perinuclear vesicles in fibroblasts. Cell Growth Differ. *3*, 567–576.

Roberts, M.S., Woods, A.J., Dale, T.C., Van Der Sluijs, P., and Norman, J.C. (2004). Protein kinase B/Akt acts via glycogen synthase kinase 3 to regulate recycling of alpha v beta 3 and alpha 5 beta 1 integrins. Mol. Cell. Biol. *24*, 1505–1515.

Robertson, D., Paterson, H.F., Adamson, P., Hall, A., and Monaghan, P. (1995). Ultrastructural localization of ras-related proteins using epitope-tagged plasmids. J. Histochem. Cytochem. *43*, 471–480.

Roche, S., Koegl, M., Barone, M.V., Roussel, M.F., and Courtneidge, S.A. (1995). DNA synthesis induced by some but not all growth factors requires Src family protein tyrosine kinases. Mol. Cell. Biol. *15*, 1102–1109.

Taunton, J. (2001). Actin filament nucleation by endosomes, lysosomes and secretory vesicles. Curr. Opin. Cell Biol. *13*, 85–91.

Timpson, P., Jones, G.E., Frame, M.C., and Brunton, V.G. (2001). Coordination of cell polarization and migration by the Rho family GTPases requires Src tyrosine kinase activity. Curr. Biol. *11*, 1836–1846.

Tominaga, T., Sahai, E., Chardin, P., McCormick, F., Courtneidge, S.A., and Alberts, A.S. (2000). Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. Mol. Cell *5*, 13–25.

Twamley-Stein, G.M., Pepperkok, R., Ansorge, W., and Courtneidge, S.A. (1993). The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. Proc. Natl. Acad. Sci. USA *90*, 7696–7700.

Ullrich, O., Reinsch, S., Urbe, S., Zerial, M., and Parton, R.G. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. J. Cell Biol. *135*, 913–924.

Weernink, P.A., and Rijksen, G. (1995). Activation and translocation of c-Src to the cytoskeleton by both platelet-derived growth factor and epidermal growth factor. J. Biol. Chem. *270*, 2264–2267.

Wherlock, M., Gampel, A., Futter, C., and Mellor, H. (2004). Farnesyltransferase inhibitors disrupt EGF receptor traffic through modulation of the RhoB GTPase. J. Cell Sci. *117*, 3221–3231.

Wong, S., Reynolds, A.B., and Papkoff, J. (1992). Platelet activation leads to increased c-src kinase activity and association of c-src with an 85-kDa tyrosine phosphoprotein. Oncogene *7*, 2407–2415.