The membrane targeting and spatial activation of Src, Yes and Fyn is influenced by palmitoylation and distinct RhoB/RhoD endosome requirements

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Summary

Src activation is a tightly regulated process which requires RhoB endosome-associated actin assembly and transit to the cell periphery. We show here that although two other ubiquitous Src family kinases (SFKs) Yes and Fyn also require intact actin filaments for peripheral membrane targeting, they display distinct spatial activation and endosomal requirements. Unlike Src, both Yes and Fyn are constitutively membrane-localized to some extent, and Fyn is present in RhoD-positive endosomes whereas Yes does not visibly colocalize with either of the endosomal markers RhoB or RhoD. By modulating amino acid acceptor sites for palmitoylation in Src, Yes and Fyn, we show that Src S3C/S6C, which is palmitoylated (unlike wild-type Src) behaves in a manner more similar to Fyn, by predominantly colocalizing with RhoD endosomes, and the targeting of both Fyn and Src S3C/S6C is inhibited by siRNA-mediated knockdown of RhoD. Moreover, Fyn C3S/C6S, which is no longer palmitoylated, behaves much more like Src by colocalizing with RhoB endosomes and by requiring RhoB for activation and membrane translocation. These data imply that distinct modes of spatial activation and membrane delivery, at least partly under the control of specific acylation attachment sequences and endosome sub-type requirements, define distinct properties of the three ubiquitously expressed SFKs.

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Introduction

The Src family of non-receptor tyrosine kinases (SFK) is composed of eight members, three of which are ubiquitously expressed: Src, Yes and Fyn. Structurally, these proteins are highly homologous and all consist of an amino-terminal site of myristylation, a unique domain, SH3 and SH2 domains, a kinase domain (SH1) and a carboxyl-terminal regulatory region (Thomas and Brugge, 1997). Owing to the similarities in structure and their ubiquitous expression Src, Yes and Fyn are able to perform overlapping functions and in some cases can compensate for the loss of the other kinases (Fukui et al., 1991; Roche et al., 1995; Thomas et al., 1995). However, the phenotypes of single knockout mice, and of cells derived from these, are quite different, suggesting a degree of biological specificity and unique function (Soriano et al., 1991; Stein et al., 1994). For example, signalling in thymocytes is impaired in $Fyn^{-/-}$ mice (Gauen et al., 1994), whereas $Yes^{-/-}$ (also known as $Yes1^{-/-}$) mice have a reduced ability to transcytose the polyimmunoglobulin receptor (pIG) (Luton et al., 1999). Distinct from both these, $Src^{-/-}$ mice develop osteopetrosis due to defective osteoclast function (Soriano et al., 1991). These findings, together with the viable phenotypes of individual Src-, Fyn- or Yes-deficient mice, and the observed embryonic lethality of mice that are deficient in all three kinases (Klinghoffer et al., 1999), indicate that these SFKs have distinct properties, but display a degree of functional redundancy. However, the molecular basis for uniqueness of function of Src, Yes and Fyn has never been elucidated.

We have investigated the possibility that the distinctiveness of individual SFKs may be explained by differences in the way in which activation and sub-cellular localization is regulated. In contrast to Src, both Yes and Fyn contain cysteine residues downstream of the myristylated glycine residue (at position 2), and these cysteines are sites of palmitoylation (Resh, 1994). Incorporation of one or more palmitate moieties is reported to facilitate localization of Yes and Fyn to detergent-resistant membrane fractions rich in glycolipids, sphingolipids, cholesterol and glycosylphosphatidylinositol (GPI)-linked proteins known as lipid rafts (Shenoy-Scaria et al., 1994). Src is not palmitoylated and localizes to the plasma membrane, focal adhesions and endosomes (Garber et al., 1983; Courtneidge et al., 1980; Kaplan et al., 1992). These earlier findings suggested that palmitoylation may be a determinant of the delivery of individual SFKs to particular membrane micro-domains, although how palmitoylation contributes to the peripheral translocation of Src, Fyn and Yes, or regulation of their enzymatic activity, is not known.

Recently, we used a novel Src-GFP fusion protein to show that Src is most likely activated in RhoB-positive endosomes and that associated actin filaments are required for intracellular

targeting to occur efficiently (Sandilands et al., 2004). Here, we examine the control of Yes and Fyn activation and peripheral membrane targeting and conclude that the spatial regulation and endosomal requirement of the SFKs are distinct. In particular, we show that palmitoylation state is at least one determinant of the targeting of Src and Fyn to specific endosomal sub-compartments defined by the presence of RhoB or RhoD, and is involved in the precise mode of intracellular targeting and spatial activation. We propose that the distinctive spatial activation of the ubiquitous SFKs, and subsequent delivery to particular membrane sites, specifies their different properties.

Results

Src, Fyn and Yes exhibit distinct spatial regulation

The targeting of Src to the plasma membrane is a tightly regulated process that requires the SH2 and SH3 domains but is kinase independent (Kaplan et al., 1994; Fincham et al., 2000). However, the activation of Src does requires membrane translocation and stimulation of actin re-arrangements induced by agents such as LPA, via RhoA-mediated stress fibre formation, platelet-derived growth factor (PDGF), via Rac1-mediated lamellipodia, or bradykinin, via Cdc42-induced filopodia (Timpson et al., 2001). We set out to address whether the spatial activation of Yes or Fyn was regulated in a similar manner to that of Src. To do this we utilized cells derived from Src-Yes-Fyn-deficient mice ($SYF^{-/-}$) (Klinghoffer et al., 1999), expressed the three ubiquitous SFKs individually and compared the intracellular targeting and spatial activation of each protein. Activity was detected using an anti-phospho-Y416-Src antibody which does not detect any auto-phosphorylated SFK family members in untransfected $SYF^{-/-}$ cells maintained in serum, as shown by western blot (Fig. S1, left panels in supplementary material) and immunofluorescence (Fig. S1, right panels in supplementary material) thus confirming specificity. This antibody is therefore a useful tool that allows us to examine the precise localization of the active forms of SFK proteins.

As previously shown for Src-WT-GFP (Sandilands et al., 2004), Src was retained in the perinuclear region of serum-free $SYF^{-/-}$ cells in an inactive state in most cells (around 70%), as shown by lack of staining with the anti-phospho-Y416-Src antibody (representative images shown in Fig. 1A). Upon stimulation with PDGF, a proportion of active Src was targeted to membrane ruffles via endosomal vesicles in which active Src is present [in around 90% of cells; Fig. 1A, and in Sandilands et al. (Sandilands et al., 2004)]. Active Fyn was present at the cell periphery in both serum-free (in 97% of cells) and PDGF-stimulated cells (in 95% of cells; Fig. 1A), and in endosomal structures (Fig. 1A, right panels, broken arrow). Yes was detected at the plasma membrane of serum-deprived cells (95%) and PDGF-treated cells (98%), in both an active and inactive state (Fig. 1A, middle panels, solid arrows). In particular, discrete membrane regions, or patches, containing active Yes were apparent (Fig. 1A). This is in contrast to active Src and Fyn, which localize in a much more uniform way along ruffles at the plasma membrane although it is possible that inactive SFK is also trafficked to the plasma membrane and is present at these sites. Similar results to those for the targeting of the individual SFKs to PDGF-induced membrane ruffles were obtained when $SYF^{-/-}$ cells were stimulated instead with LPA or with bradykinin, except that the peripheral structures at which the SFKs localized were focal adhesions or filopodia, respectively (data not shown). It should be noted that we

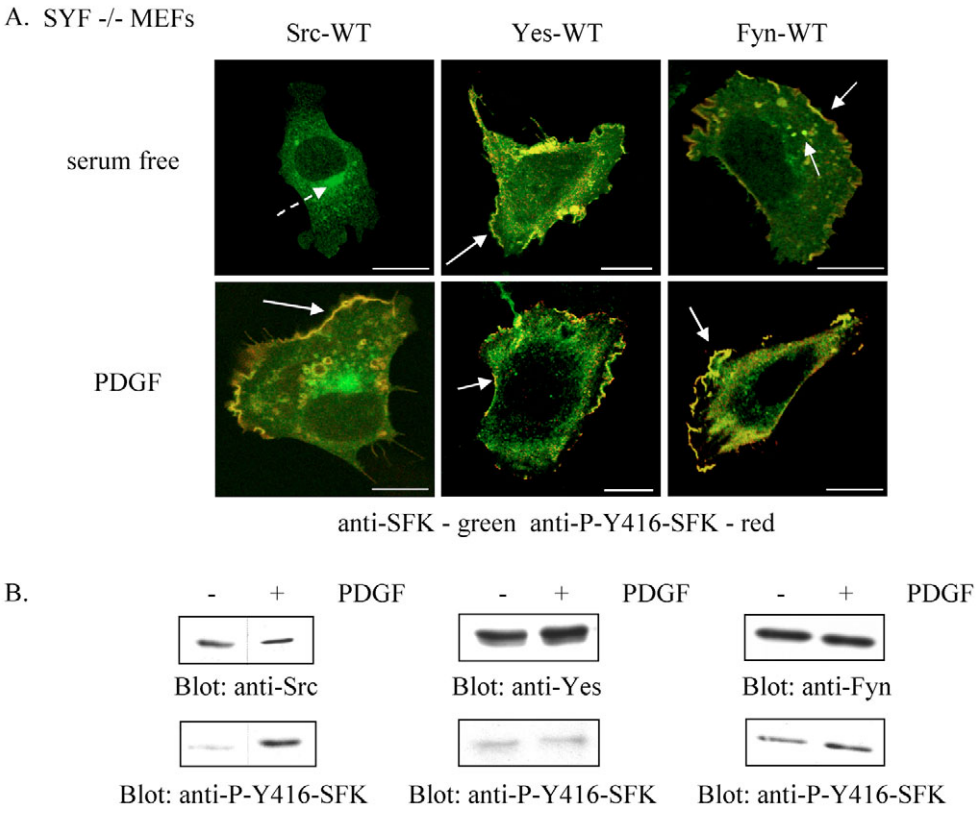

Fig. 1. Src, Fyn and Yes are distinctly spatially regulated. (A) $SYF^{-/-}$ cells expressing Src-WT (wild-type Src), Yes-WT or Fyn-WT were maintained in serum-free medium (upper panels) or stimulated with PDGF (lower panels). Localization of active SFK was detected using an anti-P-Y416-SFK antibody (Texas Red secondary) and total protein using an anti-Src, anti-Yes or anti-Fyn antibody (FITC secondary). Solid arrows indicate SFK at the cell periphery whereas broken arrows indicate SFKs at the perinuclear region of the cell. Bars, 25 μm. (B) Western blot analysis of $SYF^{-/-}$ cells expressing Src-WT (left panels), Yes-WT (middle panels) or Fyn-WT (right panels) in serum-free medium (–) or stimulated with PDGF. Activation was detected using an anti-P-Y416-SFK antibody (lower panels). Quantification shown in Table S1 in supplementary material.

sometimes observe a general diffuse staining of each SFK that may represent a pool of cytoplasmic protein, possibly resulting from overexpression and saturation of the perinuclear endosomes.

SYF$^{-/-}$ cells re-expressing Src, Yes or Fyn were maintained in serum-free medium (Fig. 1B, –) or stimulated with PDGF (+), and activity was measured by immunoblotting using anti-phospho-Y416 SFK as a marker of activation. We found that exogenous Src and Fyn were activated upon addition of PDGF, whereas no increase in Yes activity was evident, although it is possible that Yes is activated much earlier than either Src or Fyn (Fig. 1B). Similar results were found when LPA was used as the stimulus (data not shown).

Since we intended to use expressed proteins for the remainder of this study, it was important to establish that the higher levels of Src, Yes and Fyn were localized and activated in a similar manner to the endogenous proteins. We therefore used antibodies that specifically detect the unique domains of Src (via amino acids 2-17), Yes (amino acids 10-193) and Fyn (amino acids 1-132) and found that the localization of endogenous SFKs in mouse embryo fibroblasts (MEFs) mirrored that of exogenously expressed proteins (Fig. S2A in supplementary material).

Lysates of these cells were immunoprecipitated with either specific anti-Src, anti-Yes or anti-Fyn antibodies, and immunoblotted with anti-phospho-Y416-Src that recognizes all three of the SFKs in their active forms (Fig. S2B in supplementary material). This showed that both Src and Fyn were modestly activated in response to PDGF, although endogenous Yes was not visibly activated (Fig. S2B in supplementary material). Taken together, these data show that exogenous Src, Fyn and Yes are activated in a similar manner to their endogenous counterparts.

Activation of all three SFKs requires an intact actin cytoskeleton

We showed that Src-WT-GFP was unable to translocate to the periphery of SYF$^{-/-}$ cells in which the actin had been destroyed by treatment with cytochalasin D (Sandilands et al., 2004). Moreover, the restoration of actin filament assembly following washout of cytochalasin D was sufficient to target Src to the plasma membrane (Sandilands et al., 2004). To address whether actin was also required for targeting and activation of the other SFKs, given the differences between them (Fig. 1), we examined the effect of cytochalasin D treatment on the targeting of active Src, Yes and Fyn in PDGF-treated cells. We found that actin disruption inhibited the activation and membrane targeting of all three SFKs, and under these conditions they were retained in the perinuclear region in an inactive state (Fig. 2A). In addition, we found that disruption of cytoplasmic actin filaments by placing cells in suspension was associated with substantial loss of SFK auto-phosphorylation in all cases, and this was restored on adhesion to fibronectin (Fig. 2B). Since we had shown that viral Src and

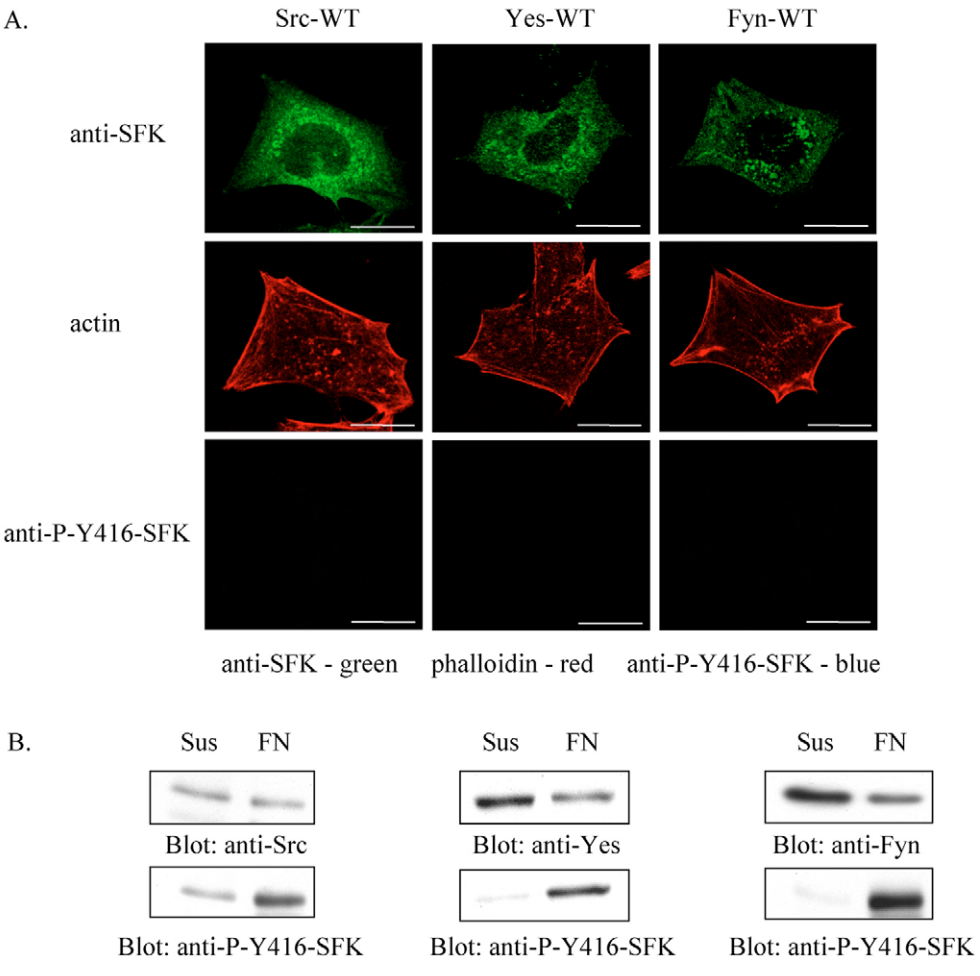

Fig. 2. Activation of all three SFKs requires an intact actin cytoskeleton. (A) SYF$^{-/-}$ cells expressing Src-WT, Yes-WT or Fyn-WT were treated with cytochalasin D (0.3 μg/ml) for 1 hour prior to PDGF stimulation. Localization of active SFK was detected with an anti-P-Y416-SFK antibody (Cy5 secondary), total protein with an anti-Src, anti-Yes or anti-Fyn antibody (FITC secondary) and actin with TRITC-phalloidin. Bars, 25 μm. (B) Lysates from SYF$^{-/-}$ cells expressing Src-WT, Yes-WT or Fyn-WT maintained in suspension (Sus) or plated on fibronectin (FN) for 1 hour (10 μg/ml) were immunoblotted using anti-Src, anti-Yes, anti-Fyn (upper panels) and anti-P-Y416-SFK antibodies (lower panels). Quantification shown in Table S1 in supplementary material.

cellular Src appear to be propelled through the cytoplasm at the tips of newly polymerized actin filaments (Sandilands et al., 2004), reminiscent of endocytic vesicles moving at the tips of actin tails in cultured mast cells (Merrifield et al., 1999), we simultaneously enabled membrane targeting and actin polymerization by treating cells with cytochalasin D for 1 hour then washing it out for 15 minutes at the same time as adding serum. Under these conditions, we could visualize Src-, Yes- and Fyn-containing structures associated with small newly forming bundled actin filaments, termed actin 'clouds', in the cytoplasm between the perinuclear region and the cell membrane (Fig. S3 in supplementary material). These experiments imply that although the activation and targeting of Src, Yes and Fyn are distinctly controlled, they have a common requirement for actin filament assembly. Inhibition of membrane translocation by disruption of actin is accompanied by impaired catalytic activation, suggesting that although the three kinases are differently responsive to stimuli, their targeting and activation are always linked.

RhoB is involved in the intracellular targeting of Src but not that of Yes and Fyn

We previously showed that Src-WT-GFP was present in vesicular structures that contain the endosome-associated RhoB and that the peripheral targeting and concomitant activation of this protein was suppressed in RhoB-deficient ($^{-/-}$) cells (Sandilands et al., 2004). Here we show that in PDGF-stimulated SYF$^{-/-}$ cells, Src strongly colocalized with RhoB-positive late endosomes and more weakly with RhoD-containing early endosomes (Fig. 3A, left panels). However, cytoplasmic Fyn appeared to be more strongly localized to RhoD-positive endosomes with lesser visible colocalization

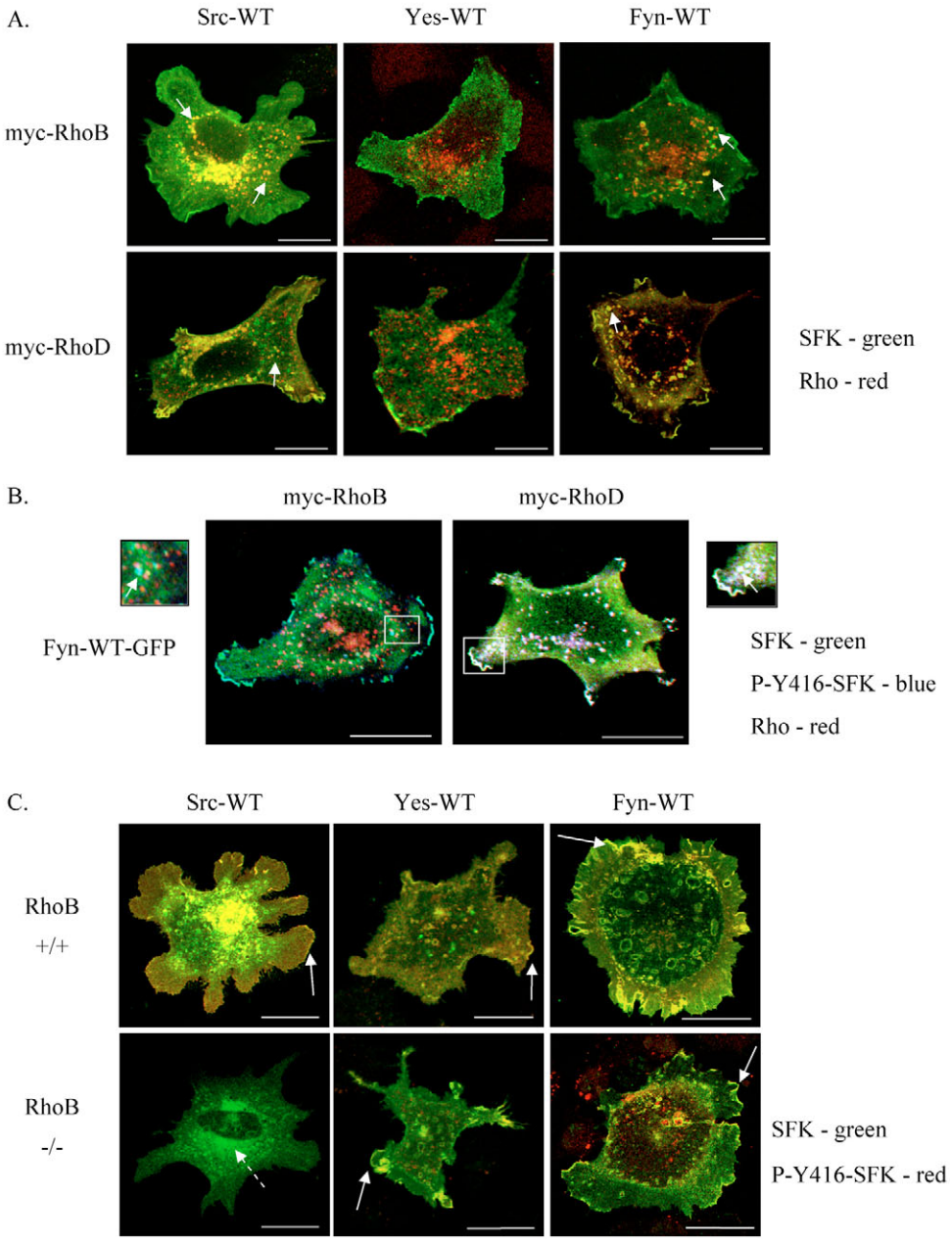

Fig. 3. RhoB is involved in the intracellular targeting of Src but not of Yes and Fyn. (A) The colocalization of Myc-RhoB (upper panels) and Myc-RhoD (lower panels) with Src-WT, Yes-WT or Fyn-WT was studied in SYF$^{-/-}$ cells stimulated with PDGF. Solid arrows indicate colocalization. (B) SYF$^{-/-}$ cells expressing Fyn-WT-GFP and Myc-RhoB or Myc-RhoD were stimulated with PDGF and stained for activated SFK. (C) RhoB$^{+/+}$ (upper panels) or RhoB$^{-/-}$ (lower panels) cells expressing Src-WT, Yes-WT or Fyn-WT were plated on fibronectin for 1 hour and stained for activated SFK. Solid arrows indicate localization of active protein at the cell periphery whereas broken arrows indicate inactive SFK retained in the perinuclear region. Localization of active SFK was detected using an anti-P-Y416-SFK antibody (Texas Red or Cy5 secondary), total protein using an anti-Src, anti-Yes or anti-Fyn antibody (FITC secondary) and Myc-Rho with an anti-Myc antibody (Texas Red secondary). Bars, 25 μm. Quantification shown in the Tables S1 and S2 in supplementary material.

with RhoB-containing structures (Fig. 3A, right panels). Distinct from both Src and Fyn there was no obvious colocalization of Yes in endosomal structures that contained either RhoB or RhoD (Fig. 3A, middle panels).

We next used a Fyn-WT-GFP protein that has an amino acid linker peptide between the Fyn carboxyl terminus and the GFP moiety, using a similar strategy to the one we described previously to generate a normally regulated Src-WT-GFP (Sandilands et al., 2004). Active Fyn-WT-GFP colocalized with RhoD (shown by white 'endosome' staining in Fig. 3B) but not with RhoB (shown by lack of white 'endosome' staining in Fig. 3B), suggesting that Fyn is activated and translocated in RhoD-containing endosomes, rather than the RhoB-containing endosomes used by Src.

The more predominant colocalization of Fyn with RhoD prompted us to ask whether activation and peripheral translocation of Fyn was, like Src, RhoB dependent (Sandilands et al., 2004). When $RhoB^{+/+}$ cells were plated onto fibronectin, Src, Yes and Fyn were all targeted to the cell periphery (in 75%, 92% and 95% of cells, respectively; Fig. 3C, upper panels). However, in $RhoB^{-/-}$ cells, Src was retained in an inactive state in the perinuclear region (in 85% of cells; Fig. 3C, lower left panel) while Yes and Fyn were still able to translocate (in 95% and 94% of cells, respectively; Fig. 3C, lower right panels). We observed similar results when adherent cells were stimulated with PDGF (data not shown). This indicates that the peripheral targeting and activation of Fyn and Yes were not, like Src, dependent upon RhoB.

These findings imply that whereas RhoB controls Src's peripheral targeting and concomitant activation upon adhesion or mitogen stimulation, it is not so important for the targeting of activated Fyn or Yes. However, in the case of Fyn, targeting and activation is more likely to be mediated by RhoD endosomes.

Palmitoylation status contributes to RhoB-dependence of intracellular targeting

Palmitoylation is the covalent attachment of fatty acids to cysteine residues of membrane proteins and all members of the SFK family have this modification except for Src and Blk (Koegl et al., 1994). Fatty acylation of SFKs has been shown to influence their interactions with cellular components. Fyn, for example is palmitoylated at cysteines 3 and 6 (Shenoy-Scaria et al., 1993; Alland et al., 1994), a modification which is thought to influence its ability to bind to cellular membranes (Shenoy-Scaria et al., 1994). Mutation of these sites is reported to cause redistribution of Fyn from the plasma membrane to intracellular membranes (Wolven et al., 1997). To determine whether palmitoylation modulation could contribute to the localization of SFKs to different endosomal sub-types, we generated Fyn and Yes mutants that could not be palmitoylated (Fyn-GFP C3S/C6S and Yes C3S) (see Wolven et al., 1997) and a mutant of Src that would be palmitoylated (Src-GFP S3C/S6C) (see Alland et al., 1994). In previous studies, it was reported that the mutations introduced had the predicted effect on palmitoylation.

When these proteins were expressed in serum-starved, or PDGF-stimulated, $SYF^{-/-}$ cells, we detected Src-GFP S3C/S6C at the cell periphery and in endosomal structures in its active form (Fig. 4A, left panels), properties that were more reminiscent of wild-type Fyn (Fig. 1A). Conversely, Fyn-GFP C3S/C6S was detected only in intracellular endosomes in serum-free medium and only at the plasma membrane after PDGF treatment (Fig. 4A, right panels), regulated behaviour that was more reminiscent of wild-type Src (Fig. 1A). Whereas Yes-WT is rarely detected in endosomes, inactive Yes C3S, like wild-type Src, was now obviously present in endosomes in the perinuclear region of unstimulated cells and active Yes C3S was visible in endosomes and at the plasma membrane upon stimulation with PDGF (Fig. 4A, middle panels). Thus, enabling palmitoylation of Src causes spatial activation to be regulated in a manner more reminiscent of Fyn and Yes, whereas removing palmitoylation potential from Fyn and Yes causes them to be regulated in a Src-like manner in regard of PDGF stimulation, suggesting an important role for palmitoylation in membrane targeting and catalytic activation of the individual SFKs.

To address whether modulating palmitoylation status of SFKs affected their sub-cellular localization, we cotransfected palmitoylation mutant proteins into $SYF^{-/-}$ cells along with either Myc-RhoB or Myc-RhoD. Src-GFP S3C/S6C, in the active state, now colocalized with RhoD endosomes (Fig. 4B, second panel), instead of RhoB endosomes (Fig. 4B, left panel), with which wild-type Src would normally colocalize (see Fig. 3A). Whereas active Fyn-GFP C3S/C6S could still colocalize with RhoD endosomes to some extent, (Fig. 4B, right panel), this mutant was now much more robustly colocalized with RhoB endosomes (Fig. 4B, right panels). This was in contrast to active wild-type Fyn, which more predominantly colocalized with RhoD endosomes (see Fig. 3B).

To confirm that RhoB and RhoD are not likely to be involved in the targeting or activation of Yes we looked for colocalization between these Rho GTPases and endosomal Yes that was present upon washout of cytochalasin D, conditions under which the endosomal localization of Yes is most obvious (as described in Fig. S3 in supplementary material). Neither wild-type Yes, nor the palmitoylation Yes C3S mutant, colocalized with either RhoB or RhoD (data not shown). Thus, Yes differs from Fyn and Src by not obviously colocalizing with RhoB or RhoD, and this is not changed upon modulation of the palmitoylation attachment residue.

In $RhoB^{+/+}$ cells, the Src-GFP S3C/S6C pro-palmitoylation mutant was constitutively active at the plasma membrane (in 88% of cells; Fig. 4C, upper panels); however, in contrast to Src-WT, active Src-GFP S3C/S6C was now efficiently translocated to peripheral membranes in $RhoB^{-/-}$ cells (in 83%; Fig. 4C, lower left panel). This implies that palmitoylation was sufficient to drive Src's delivery to the membrane, in a RhoB-independent manner. Conversely, whereas active Fyn and the non-palmitoylation mutant Fyn-GFP C3S/C6S protein were both present at the cell periphery in $RhoB^{+/+}$ cells (in 92% and 80% of cells, respectively; Fig. 3C and Fig. 4C, upper right panels), there was no obvious accumulation of the Fyn-GFP C3S/C6S mutant at the membrane and no visible evidence of activation (Fig. 4C, lower right panel) in $RhoB^{-/-}$ cells. These data show that a mutation that impairs palmitoylation causes activation and peripheral translocation of Fyn to become RhoB dependent. Thus, palmitoylation status contributes to the predominant cytoplasmic endosome sub-type with which Src and Fyn associate, and RhoB dependence of activation and targeting. In the case of Yes, the Yes C3S palmitoylation

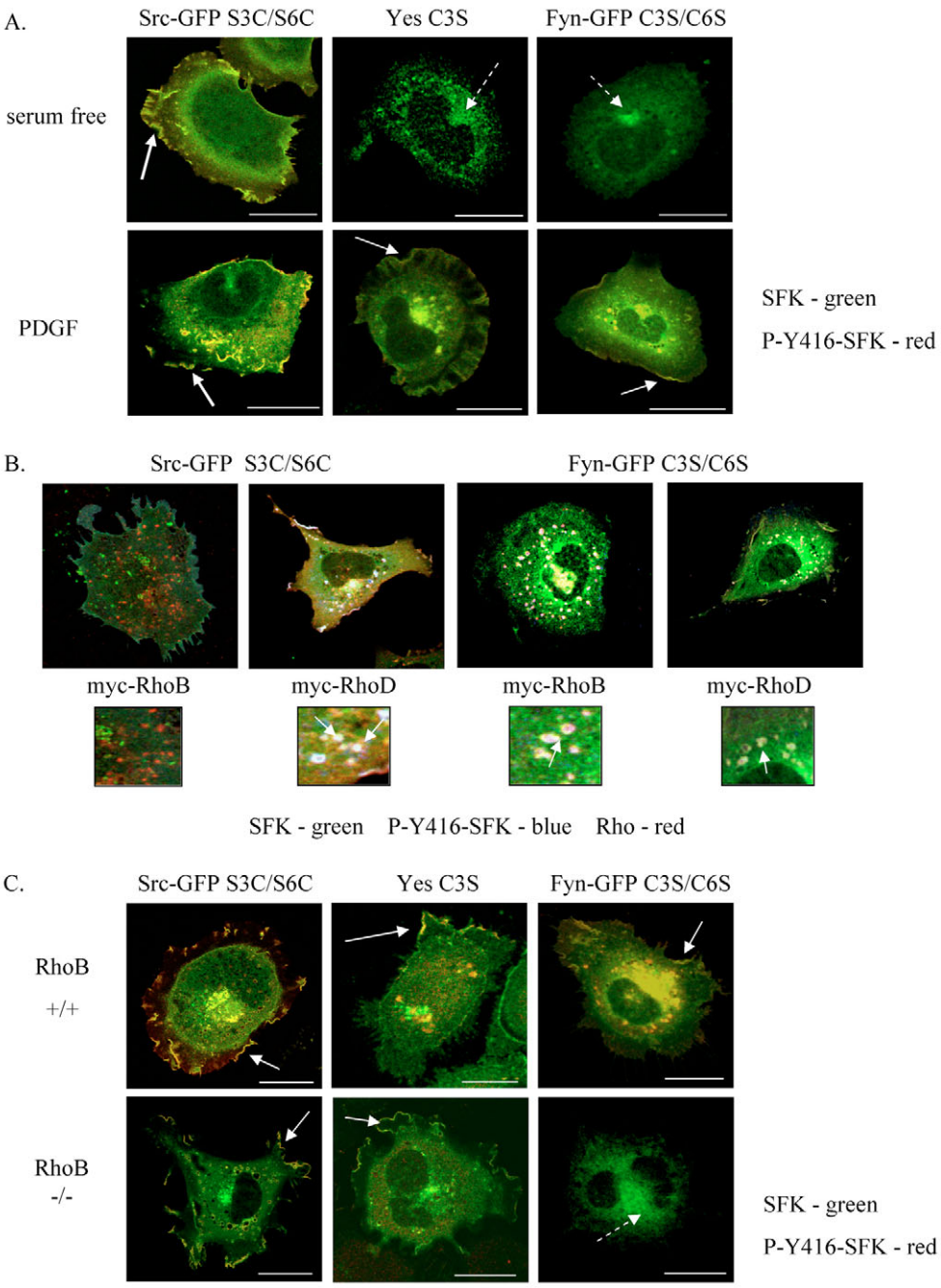

Fig. 4. Palmitoylation contributes to RhoB-dependence of intracellular targeting. (A) SYF$^{-/-}$ cells expressing Src-GFP S3C/S6C, Yes C3S or Fyn-GFP C3S/C6S were maintained in serum-free medium (upper panels) or stimulated with PDGF (lower panels). Solid arrows indicate localization of active SFK at cell periphery whereas broken arrows indicate inactive protein at the perinuclear region. (B) SYF$^{-/-}$ cells expressing Src-GFP S3C/S6C or Fyn-GFP C3S/C6S with Myc-RhoB or Myc-RhoD were stimulated with PDGF. Solid arrows in higher magnification images indicate colocalization between active SFK and Myc-Rho. (C) RhoB$^{+/+}$ (upper panels) or RhoB$^{-/-}$ cells (lower panels) expressing Src-GFP S3C/S6C, Yes C3S or Fyn-GFP C3S/C6S were plated on fibronectin for 1 hour. Solid arrows indicate active SFK at the cell periphery and broken arrows inactive SFK at the perinuclear region. Localization of active SFK was detected using an anti-P-Y416-SFK antibody (Texas Red or Cy5 secondary), total protein using an anti-Yes antibody (FITC secondary) and Myc-Rho was visualized using an anti-Myc antibody (Texas Red secondary). Bars, 25 μm. Quantification shown in the Tables S1 and S2 in supplementary material.

mutant was present at the cell periphery in both RhoB$^{+/+}$ and RhoB$^{-/-}$ cells (in 100% of cases for each cell type; Fig. 4C, middle panels) confirming that regardless of palmitoylation status, and in contrast to Src and non-palmitoylated Fyn, RhoB does not play a major role in the targeting and of Yes. Moreover, this confirms that trafficking and spatial activation are distinctly regulated for Src, Fyn and Yes.

Translocation/activation of Fyn-GFP and Src-GFP S3C/S6C is impaired by RhoD knockdown

We next suppressed expression of RhoD RNA in SYF$^{-/-}$ cells by using specific RhoD oligonucleotides, and compared the results with those of a scrambled siRNA control. Knockdown was judged by RT-PCR, since there are no suitable antibodies to monitor endogenous RhoD levels (Fig. 5A,B,C). These cells were cotransfected with either Fyn-WT-GFP (Fig. 5A), Src-GFP S3C/S6C (Fig. 5B), the palmitoylated forms of Fyn and Src that we had found to efficiently colocalize with RhoD and Src-WT-GFP (Fig. 5C). In SYF$^{-/-}$ cells expressing a non-targeting siRNA control (control siRNA), active Fyn-WT-GFP was visualized at the cell periphery (in 100% of cells; Fig. 5A). The number of cells in which active Fyn-GFP was targeted to the cell periphery was reduced (to around 40%) when cotransfected with RhoD-specific siRNA. Similarly, in the case of Src-GFP S3C/S6C, decreased expression of RhoD was linked to a reduction in the number of cells in which active kinase was translocated to the cell periphery (to 56% of cells; Fig. 5B). These cell numbers are likely to represent an

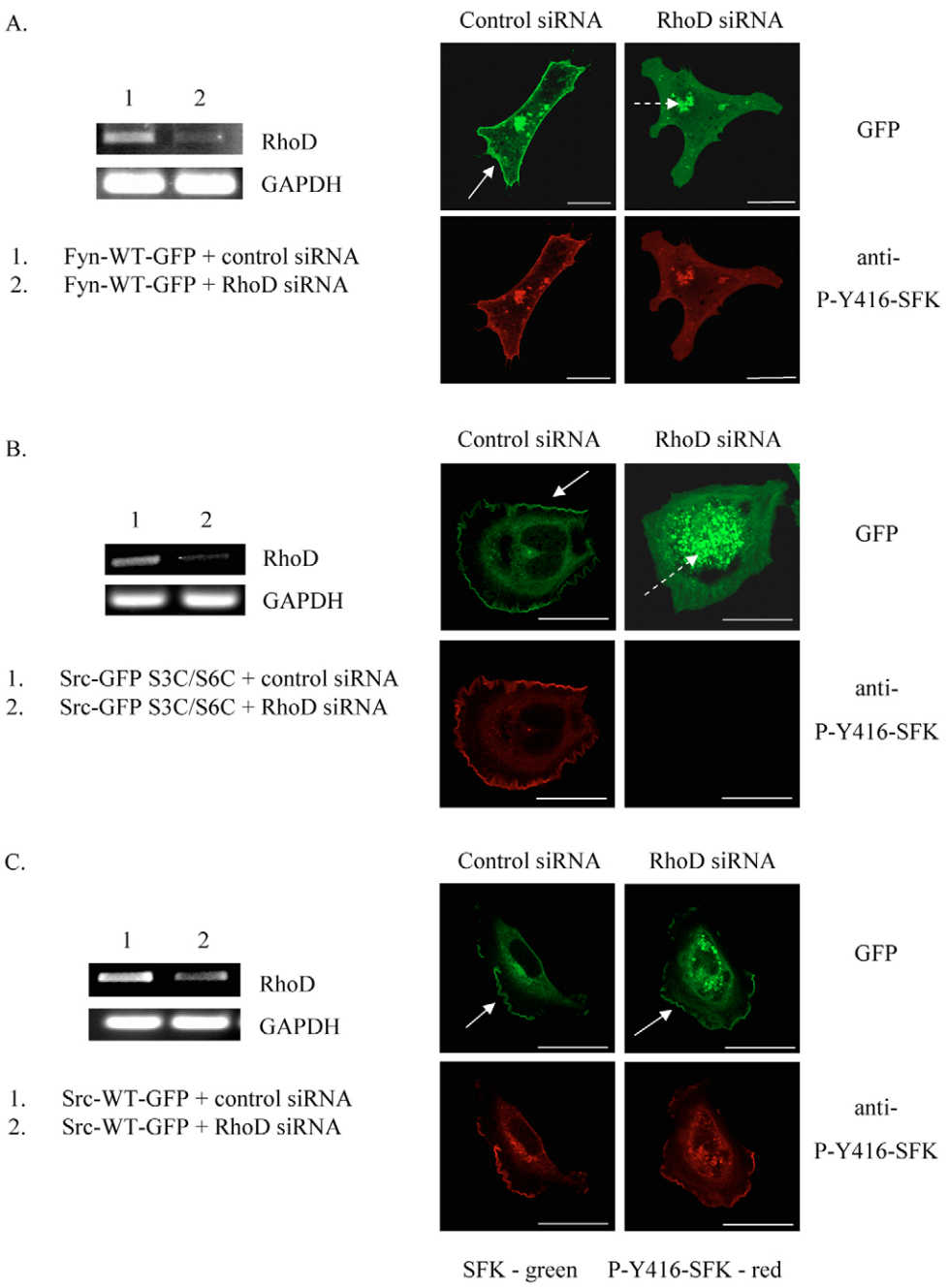

Fig. 5. Knockdown of RhoD impairs translocation of Fyn-GFP and Src-GFP palmitoylation mutant. cDNA from SYF$^{-/-}$ cells expressing (A) Fyn-WT-GFP and an siRNA control or Fyn-WT-GFP and RhoD siRNA or (B) Src-GFP S3C/S6C and an siRNA control or Src-GFP S3C/S6C and RhoD siRNA or (C) Src-WT-GFP and an siRNA control or Src-WT-GFP and RhoD siRNA was reverse transcribed (left panels). Cells from the same experiment were plated for 6 hours in serum-containing medium then stained for anti-P-Y416-SFK (Texas Red secondary). Solid arrows indicate SFK at the cell periphery whereas broken arrows show SFK retained in the perinuclear region. Bars, 25 μm.

underestimate of the reduced ability of RhoD knockdown to interfere with efficient targeting of active Fyn and Src S3C/S6C, since RhoD is not efficiently suppressed in all cells in the population. In SYF$^{-/-}$ cells expressing Src-WT-GFP, knockdown of RhoD siRNA did not affect the translocation of active kinase to the cell periphery (still translocated in 95% of cells), similar to cells expressing the non-targeting siRNA control (93% of cells; Fig. 5C).

Use of a farnesyl transferase inhibitor (FTI) disrupts RhoB-, but not RhoD-dependent trafficking of SFKs

Farnesyl transferase inhibitors (FTIs) are a class of biologically active anticancer drugs that inhibit farnesylation of many proteins such as Ras and RhoB via inhibition of the enzyme farnesyl transferase (Gibbs et al., 1994; Kohl et al., 1995). Since RhoB, but not RhoD is farnesylated, the administration of an FTI such as L744832 has been shown to affect the function of this Rho protein in particular (Du and Prendergast, 1999; Liu et al., 2000), and therefore provides a test of the RhoB-dependence of membrane targeting. In order to ascertain whether FTIs would influence the intracellular trafficking and activation of SFK, we treated SYF$^{-/-}$ cells expressing wild-type SFKs, or their respective palmitoylation mutants, with L744832 prior to stimulation with PDGF. We found that Src-WT-GFP and Fyn-GFP C3S/C6S were maintained in the perinuclear region of the cells in an inactive state (Fig. 6, upper left panel and lower right panel, broken arrows) in the majority of cells (86% and 90%, respectively in comparison to 6% and

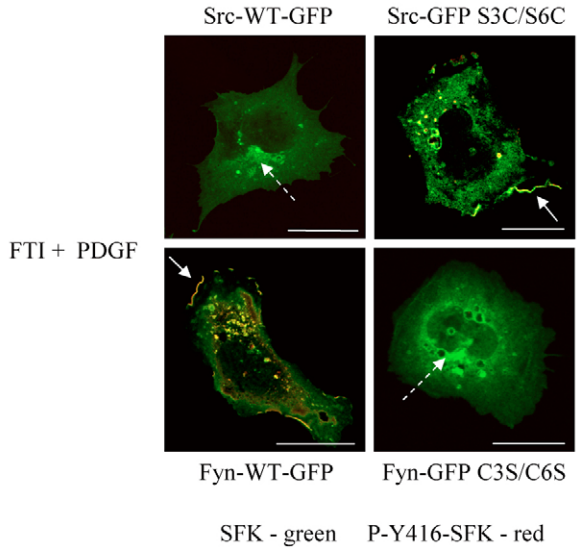

Fig. 6. FTI inhibitor disrupts RhoB-, but not RhoD-, dependent trafficking of SFKs. $SYF^{-/-}$ cells expressing Src-WT-GFP, Fyn-WT-GFP, Src-GFP S3C/S6C or Fyn-GFP C3S/C6S were treated with 10 μM L-744832 for 2 hours prior to stimulation with PDGF. Active SFK was detected using anti-P-Y416-SFK (Texas Red secondary). Solid arrows indicate SFK at cell periphery whereas broken arrows indicate SFK retained in perinuclear region. Bars, 25 μm. Quantification shown in Table S1 in supplementary material.

10% of PDGF-stimulated cells). The intracellular targeting of Fyn-WT-GFP and Src-GFP S3C/S6C, however, were unaffected by treatment with the FTI (Fig. 6, lower left and upper right panels, solid arrows) since active SFK was detected at the cell periphery in most cells (98% and 96% compared to 100% and 92% of PDGF-stimulated cells). We subsequently tried to confirm the results of this experiment by biochemical fractionation but found that the proportion of Src-WT-GFP present in cytosolic or membrane fractions was not affected by treatment with FTI inhibitor prior to PDGF stimulation (Fig. S4 in supplementary material). This is because inactive Src in FTI-treated cells is not held 'diffusely' in the cytoplasm but in endosomal membrane structures which fractionate with membrane components (M) rather that the cytoplasmic soluble protein fraction (C). Thus, the inability of the biochemical fractionation protocol to distinguish between plasma membrane and intracellular membranes means that this experiment was not revealing.

Overall this data is entirely consistent with our observations using molecular intervention, and shows that the trafficking of non-palmitoylated SFKs is dependent upon RhoB.

Discussion

Together with our colocalization data and studies in RhoB-deficient cells, our findings with RhoD knockdown indicate that palmitoylation specifies the largely RhoD-dependent translocation of Fyn and the pro-palmitoylation Src S3C/S6C mutant protein, whereas lack of palmitoylation specifies the largely RhoB-dependent translocation of Src. Both processes are actin dependent and linked to spatially regulated kinase activation. Although Yes translocation and activation are apparently independent of RhoB and RhoD, this is likely to involve another endosome sub-type that is, as yet, undefined. Palmitoylation is therefore one determinant of specific endosomal targeting of Src and Fyn, since modulating palmitoylation changes the predominant endosome sub-type to which the individual kinases localize, and the dependence of activation and peripheral membrane translocation on RhoB and RhoD. However, palmitoylation is not the sole determinant, since Yes, like Fyn, is also palmitoylated but is not stably associated with either RhoB- or RhoD-containing endosomes, even upon alteration of palmitoylation potential. This implies that other properties of the SFKs, perhaps the SH3 domains, which are also needed for proper intracellular targeting (Fincham et al., 2000) and which bind different cellular proteins, may also contribute. Previous work has shown that both myristylation and palmitoylation are required for methylation of Fyn, a process that is functionally significant since methylation-deficient Fyn does not promote cell adhesion and spreading (Liang et al., 2004). However, we were not able to establish a role for methylation, since treatment with a general methylation inhibitor 5-Aza-2′deoxycytidine (Liang et al., 2002) did not affect localization of Fyn-GFP even at high concentrations (data not shown).

The functions of SFKs are partially overlapping, and all three require to be knocked out to cause severe developmental defects and embryonic lethality (Klinghoffer et al., 1999). In keeping with redundant functions, we found that re-expression of the palmitoylation-modulated mutants of Src, Fyn and Yes could all support restoration of signalling to tyrosine phosphorylation of focal adhesion kinase (FAK; specifically on FAK-Y-861; not shown) as well as their wild-type counterparts (not shown), implying that for some signalling functions, membrane targeting of any one of the SFKs, by whatever means, is sufficient. Despite this, each of the three kinases is distinctly regulated regarding where they are activated, their precise endosomal requirements and whether they require cell stimulation. Since Src, Fyn and Yes are differentially required for particular biological processes our results suggest that their distinctiveness may come, at least in part, from their different modes of spatial regulation and targeting. Interestingly, there are a number of reports that palmitoylated SFKs reside in lipid rafts (e.g. Kasai et al., 2005) and signal from specific membrane microdomains. Fyn, in particular, is thought to signal from lipid rafts in thymocytes (Maksumova et al., 2005; Filipp and Julius, 2004), whereas it is also proposed that Fyn associates with cholesterol-rich membrane domains via the membrane adaptor caveolin-1 and that it is from such membranous regions that Fyn contributes to integrin signalling (Wary et al., 1998). It could be that the presence of the kinases in different sub-cellular cytoplasmic compartments provides a means of tightly and spatially controlling Src, Fyn and Yes, thus ensuring that activation of the particular kinases occurs only at the correct sub-cellular location and membrane compartment, conferring functional specificity.

One interesting question that our work raises is how activation of Src kinases, which we believe to occur in intracellular pools during transit to peripheral membranes, is spatially regulated – particularly since the activating signal is initially received at the plasma membrane. We have some preliminary evidence that this may involve an amplification loop whereby there is activation of a basal pool of Src which is constitutively localized at the plasma membrane, and this

triggers the 'hijacking' of a subset of recycling endosomes from the perinuclear recycling compartment to cause concomitant activation of more Src- and actin-dependent delivery, via these recycling endosomes, to the plasma membrane. Together with the systems that downregulate Src activity after the peak of ligand-induced signalling, this would implicate precise amplification of Src activity and membrane translocation, involving the endosomal and actin networks, in the delivery of proper signal amplitude and duration. This is a hypothesis that we intend to test in future work.

Materials and Methods

Plasmids

CA10-SrcWT, pSG5-Fyn-WT and Myc-RhoD were kind gifts from P. Schwartzberg (NIH, Bethesda, MD), G. Superti Furga (EMBL, Heidelberg, Germany) and C. Murphy (Medical School, University of Ioannina, Greece), respectively.

The Fyn-WT-linker protein was generated by PCR (oligos 5′-GAA TTC ATG GGC TGT GTG CAA TGT AAG G-3′ and 5′-GGA TCC GAG CCG GAG CCC AGG TTT TCA CCA GGT TGG TAC TGG GG-3′) and cloned into EGFPN-1 (BD Clontech, Oxford, UK). Generation of the Src-GFP and Fyn-GFP (also in EGFP-N1 vectors) palmitoylation mutants has been described by Alland et al. (Alland et al., 1994) and Wolven et al. (Wolven et al., 1997), respectively. Yes C3S mutant was generated from Yes-WT pBabe by PCR using the following primers: 5′-ATG GGC TCC ATT AAA AGT AAA GAA AAC AAA AGT CCA GCC-3′ and 5′-TTA TAA ATT TTC TCC TGG CTG GTA CTG-3′ then cloned into pBabe puro and sequenced.

Cell culture

Src-Yes-Fyn-deficient ($SYF^{-/-}$) cells, 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 coverslips or 60 mm tissue culture dishes 24 hours 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 hours. Cells were treated with PDGF (25 ng/ml) for 30 minutes (TCS Biologicals, Botolph Claydon, UK) or were plated on fibronectin (BD Biosciences, Oxford, UK; 10 μg/ml) for 1 hour. $SYF^{-/-}$ cells were treated with cytochalasin D (0.3 μg/ml) for 1 hour (Sigma, Poole UK) prior to washout with DMEM plus 10% FBS for 15 minutes to initiate actin re-polymerization and SFK translocation. L744832 was added at 10 μM for 2 or 24 hours (Biomol Int, Exeter, UK) prior to stimulation with PDGF.

Immunoprecipitation (IP)

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 16,000 ***g*** and 4°C for 15 minutes. Samples (500 μg) were incubated with 3 μl of anti-Src 2-17 (CR UK, London, UK), anti-Yes or anti-Fyn (BD Transduction, Oxford, UK) overnight at 4°C. IPs were then washed three times in IP buffer and re-suspended in 2× electrophoresis sample buffer. Immunoblotting was carried out as described below.

Protein immunoblotting

Each sample for immunoblotting contained 20 μg of lysate, 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, Poole UK) and probed with anti-Src (CN Bioscience, Nottingham, UK), anti-Yes or anti-Fyn (BD Transduction, Oxford, UK) in 3% BSA-TBS-0.2% Tween 20 or with anti-phospho-Y416-Src antibody at 1:1000 (NEB, Hertfordshire, UK). Protein was detected by incubation with HRP-conjugated secondary antibodies, and visualization by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Immunofluorescence

Cells were washed twice in cold Tris-buffered saline (TBS) and fixed in 3% paraformaldehyde. They were then washed in TBS-100 nM glycine and permeabilized with TBS-0.1% saponin-20 mM glycine. After blocking with TBS-0.1% saponin-10% FCS, cells were incubated with primary antibodies for 1 hour. Antibodies were anti-Src mAb EC10 to detect exogenously expressed chicken Src proteins (Upstate Biotechnology, Lake Placid, NY), anti-Yes and anti-Fyn antibodies to detect Yes and Fyn (BD Transduction, Oxford, UK), anti-Src 2-17 antibody (CR UK) to detect endogenous Src, anti-9E10 mAb to detect Myc-tagged proteins (Upstate Biotechnology), anti-phospho-Y416-Src to detect active Src (NEB) and TRITC-phalloidin to detect actin (Sigma). Non-conjugated antibody was detected by reaction with species-specific FITC-, Cy5- or Texas Red-conjugated secondary antibodies for 45 minutes (Jackson ImmunoResearch, Luton, UK). Cells were examined using a confocal microscope (Leica UK Ltd, Milton Keynes, UK). Each experiment was repeated a minimum of three times, 100 cells were counted for each condition for quantification purposes and an image selected that represented the phenotype of the majority of cells. Quantification using a Student's two-tailed *t*-test to determine statistical significance is shown in Tables S1 and S2 in supplementary material.

RhoD siRNA

200 nM RhoD siGENOME SMART pool or non-targeted siRNA control (Dharmacon, Lafayette, CO) were electroporated into $SYF^{-/-}$ cells using Nucleofect V (Amaxa GmbH, Cologne, Germany) and incubated for 48 hours. Fyn-WT-GFP, Src-WT-GFP or Src-GFP S3C S6C were then transfected using FuGene6 (Roche Diagnostics Ltd) and cells incubated for a further 24 hours.

Total RNA was prepared from $SYF^{-/-}$ cells using an RNA extraction kit (Qiagen, Sussex, UK). An equal amount of total RNA (1 μg) isolated from untransfected, control siRNA or RhoD siRNA cells was reverse transcribed using SuperScript First Strand System for RT-PCR (Invitrogen, Paisley, UK). PCR was then performed using the following primers to amplify an 800 bp region of RhoD: 5′-AGC GCT ATA ATG CCA CTC TGC AG-3′ and 5′-GGA TAA ATA GGA TGA TCA CGG ACG GGG C-3′. PCR conditions were as follows: 95°C for 10 minutes, 30 cycles of 95°C for 45 seconds, 56°C for 30 seconds, 72°C for 2 minutes then 1 cycle of 72°C for 7 minutes. GAPDH primers were used as a control and were a kind gift from Joanne Thurlow (Beatson Institute, Glasgow, UK).

Cell fractionation

$SYF^{-/-}$ cells expressing Src-WT-GFP were treated with L744832 for 2 hours prior to stimulation with PDGF. Cells were re-suspended (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM NaCl, 10 mM KCl, 100 μg/ml DNAse I and protease inhibitors) then lysed by homogenization. Lysates were centrifuged at 700 ***g*** for 10 minutes to remove unbroken cells then the supernatant centrifuged for 1 hour at 100,000 ***g*** to obtain a cytosolic fraction (C) and a membrane fraction (M).

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