Two distinct pools of Src family tyrosine kinases regulate PDGF-induced DNA synthesis and actin dorsal ruffles

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Summary

The mechanism by which the Src family of protein-tyrosine kinases (SFKs) regulate mitogenesis and morphological changes induced by platelet-derived growth factor (PDGF) is not well known. The cholesterol-enriched membrane microdomains, caveolae, regulate PDGF receptor signalling in fibroblasts and we examined their role in SFK functions. Here we show that caveolae disruption by membrane cholesterol depletion or expression of the dominant-negative caveolin-3 DGV mutant impaired Src mitogenic signalling including kinase activation, *Myc* gene induction and DNA synthesis. The impact of caveolae on SFK function was underscored by the capacity of Myc to overcome mitogenic inhibition as a result of caveolae disruption. Using biochemical fractionation we show that caveolae-enriched subcellular membranes regulate the formation of PDGF-receptor-SFK complexes. An additional pool of PDGF-activated SFKs that was insensitive to membrane cholesterol depletion was characterised in non-caveolae fractions. SFK activation outside caveolae was linked to the capacity of PDGF to induce F-actin rearrangements leading to dorsal ruffle formation. Inhibition of phospholipase Cγ (PLCγ), sphingosine kinase and heterotrimeric Gi proteins implicates a PLCγ–sphingosine-1-phosphate–Gi pathway for PDGF-induced SFK activation outside caveolae and actin assembly. In addition, the cytoplasmic tyrosine kinase Abl was identified as an important effector of this signalling cascade. We conclude that PDGF may stimulate two spatially distinct pools of SFKs leading to two different biological outcomes: DNA synthesis and dorsal ruffle formation.

Key words: Src, PDGF, Caveolae, DNA synthesis, F-actin cytoskeleton, Abl

Introduction

Src family protein-tyrosine kinases (SFKs) play important roles in signal transduction induced by growth factors (Bromann et al., 2004). For example the three members Src, Fyn and Yes have been implicated in platelet-derived growth factor (PDGF) signalling leading to DNA synthesis, actin cytoskeleton rearrangement and receptor endocytosis (Bromann et al., 2004). We and others have shown that activation of SFK mitogenic function allows phosphorylation of specific substrates involved in *Myc* expression for cell-cycle progression (Bromann et al., 2004). Several of these substrates have been identified including the adaptor Shc (Blake et al., 2000; Gotoh et al., 1997), the transcription factor Stat3 (Bowman et al., 2001), the guanine-exchange factor Vav2 (Chiariello et al., 2001) and the cytoplasmic tyrosine kinase Abl (Furstoss et al., 2002). The latter operates on Rac-JNK and Rac-Nox pathways for *Myc* induction (Boureux et al., 2005). Abl mitogenic substrates are however unknown.

In addition to mitogenesis, PDGF induces morphological changes leading to lateral ruffles for directed cell migration. This process is driven by cortical actin polymerisation and primarily involves the Rac effector Wave2 for activation of the Arp2/3 complex (Suetsugu et al., 2002b). Although the molecular mechanism by which growth factors induce cell migration has been extensively studied, the role of SFK has not been unravelled. Several reports favour a model where they play an important role in this process: PDGF activates Src at the cell periphery and this requires small GTPases and an intact cytoskeleton (Sandilands et al., 2004). Furthermore, knockout of the SFK-binding site in the PDGF receptor α strongly reduces chemotaxis (Rosenkranz et al., 1999). By contrast, the PDGF migratory response is not affected in cells derived from mice lacking Src, Fyn and Yes. Note that those cells were immortalised by the SV40 large-T antigen (Klinghoffer et al., 1999), therefore one cannot exclude the fact that the large-T antigen alleviates the need for SFKs for cell migration as reported for mitogenesis (Broome and Courtneidge, 2000; Furstoss et al., 2002).

PDGF also induces F-actin circular dorsal ruffle formation. This cellular process has been linked to macropinocytosis (Dharmawardhane et al., 2000) but recent data indicated an important role for cell migration into the extracellular matrix (Suetsugu et al., 2003). This cytoskeletal rearrangement is also regulated by cortical actin polymerisation through the activation of the Arp2/3 complex (Suetsugu et al., 2002b) and involves a Wave1-specific pathway (Suetsugu et al., 2003).

Although Rac plays a central role in lateral lamellipodia extension, Suetsugu and colleagues showed that it also regulates dorsal ruffle formation (Suetsugu et al., 2003). Interestingly, they suggested that both cellular responses are independent and that the latter involves an additional Rac-independent pathway. Most information on intracellular signals upstream or parallel to Rac has been provided by one group who report the existence of Ras, Rac and Rab5 pathways, all required for ruffle formation (Innocenti et al., 2003; Lanzetti et al., 2004; Scita et al., 1999). In addition, Cbl has also been implicated as a negative regulator of this PDGF response and this effect was dependent upon SFK (Scaife et al., 2003). This raises the idea that in addition to mitogenesis, SFK could regulate dorsal actinic rearrangement.

How growth factors use SFKs for transmitting various cell responses is largely unknown. Specificity of signal transduction may involve specific substrates. Alternatively, specificity could be achieved spatially through recruitment of a specific pool of SFKs within the cell. SFKs are localised in distinct subcellular compartments including perinuclear membranes, early endosomes and the plasma membrane. Surprisingly, PDGF-induced SFK activation occurs during recruitment to the plasma membrane with maximal activity at the cell periphery (Sandilands et al., 2004). This suggests that the bulk of SFK signalling is initiated within this region of the cell. Interestingly, several mechanisms have been reported for SFK activation by growth factors: either by a direct interaction with the receptor (Bromann et al., 2004) or by a transactivation mechanism (Rosenfeldt et al., 2001). In the case of PDGF, SFKs associate with the receptor by interaction of their SH2 domain (Twamley et al., 1992) with the phosphotyrosines Y579 and Y581 of the receptor (Mori et al., 1993). Alternatively, it has been suggested that PDGF can also induce SFK activation through the activation of a pertussis-toxin-sensitive G protein (Chen et al., 1994; Conway et al., 1999; Rosenfeldt et al., 2001). In addition, Spiegel and colleagues proposed that sphingosine 1-phosphate (S1P), a lipid second messenger and ligand for the seven transmembrane receptors of the EDG family (Rosenfeldt et al., 2001) is involved in this signalling process. According to their model, PDGF induces a PLCγ/Ca^{2+} pathway that allows recruitment of sphingosine kinase to the membrane for S1P production (Olivera et al., 1999). S1P then binds to the G-protein-coupled EDG1 receptor allowing further activation of SFKs. This signalling crosstalk has been associated with PDGF-induced directed cell movement, although the role of S1P-induced SFK activation was not addressed (Hobson et al., 2001). The contribution of each mechanism for SFK signalling is however not known, but this would favour a model in which a growth factor activates distinct pools of SFK for signalling.

Cholesterol-enriched membrane microdomains (CEMMs) are organelles with specific physical features distinct from the contiguous membrane (Simons and Toomre, 2000). Caveolae define a subclass of these membrane structures in non-lymphoid cells with a diameter of 50-100 nm and represent the major – if not exclusive – CEMMs present in fibroblasts. They are composed of caveolins as main structural proteins, cholesterol and sphingolipids, and a number of signalling molecules including growth factor receptors and SFKs (Pike, 2005). The potential role of non-caveolae CEMMs in signal transduction is the subject of intense debate (Douglass and

Vale, 2005; Munro, 2003). By contrast, a large body of evidence indicates that caveolae function as lipid scaffolds to regulate signal transduction including cell growth (Pike, 2005). For example, most PDGFRs expressed in quiescent fibroblasts reside in caveolae and cholesterol present in these structures regulates part of the tyrosine phosphorylation and signalling induced by PDGF (Liu et al., 2000). Accordingly, caveolin-1 has been identified as an important regulator of cell growth and protein inactivation has been linked to tumorigenesis (Razani and Lisanti, 2001). Here we show that in addition to mitogenesis, SFKs are also required for PDGF-induced dorsal ruffle formation. We also provide evidence for a spatial regulation of SFK signalling induced by PDGF: a first pool initiated from caveolae and regulated by membrane cholesterol for mitogenesis, and a second pool regulated by a S1P-EDG-Gi protein pathway outside caveolae for F-actin assembly. We conclude that a spatial compartmentalisation mechanism induced by PDGF may allow regulation of SFK signalling leading to distinct biological outcomes: cell growth and morphological change.
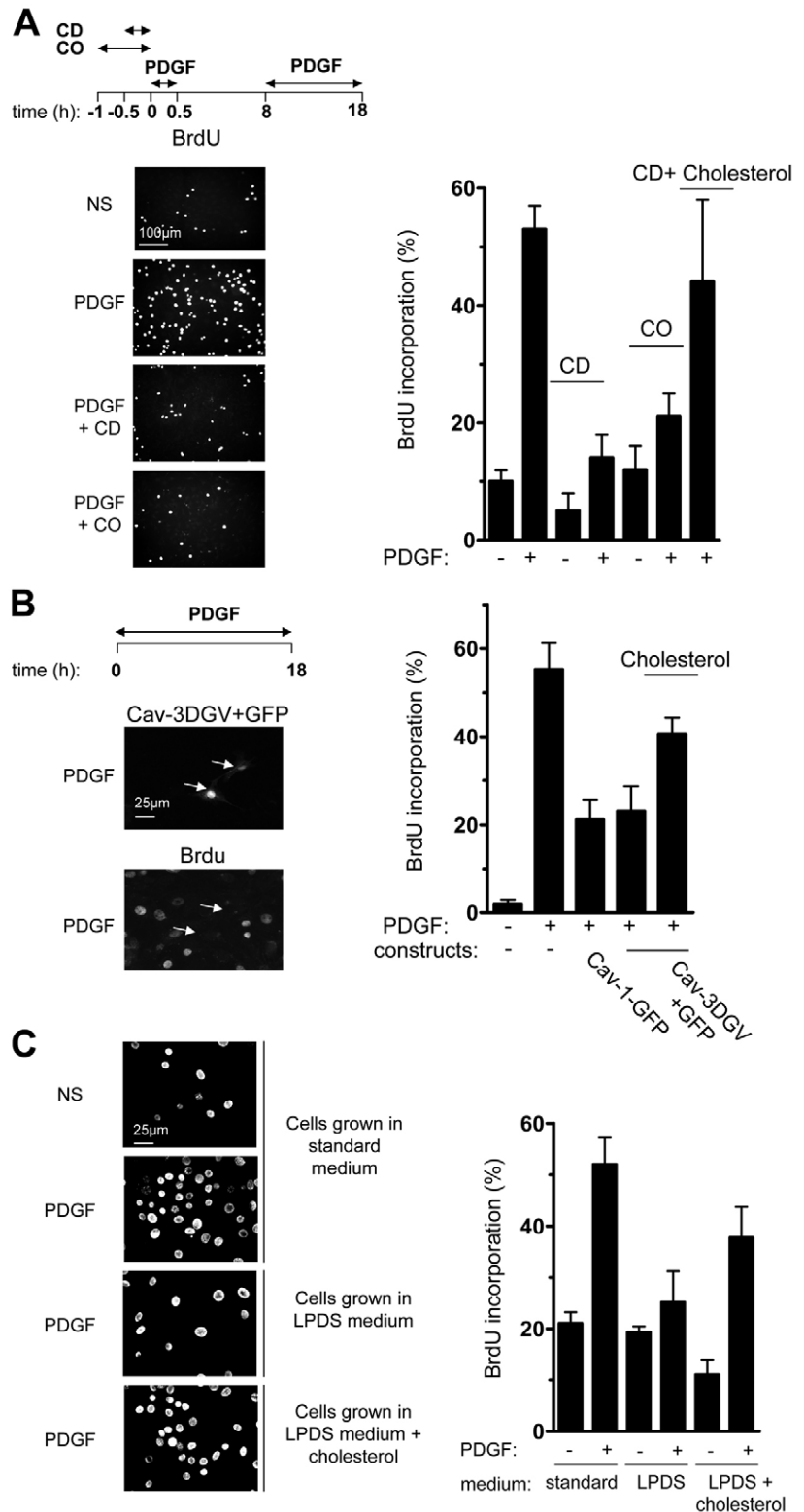
Results
A role for caveolae in mitogenesis
We first sought the role of caveolae in the PDGF mitogenic response. To this end, cholesterol was depleted from the plasma membrane using short treatment and low concentration of cholesterol depleting agents methyl-β-cyclodextine (CD) and cholesterol oxidase (CO) as described by Smart and Anderson (Smart and Anderson, 2002). CD is known to bind cholesterol while cholesterol oxidase converts membrane cholesterol to cholest-4-en3-one (Smart and Anderson, 2002). In these conditions, they specifically deplete cholesterol from membrane microdomains without affecting cytoplasmic content (Smart and Anderson, 2002). Since DNA synthesis occurs after >12 hours of cell stimulation, short drug pretreatment was inappropriate owing to replenishment of membrane cholesterol (not shown); therefore we designed a discontinuous stimulation assay to circumvent this drawback (see Fig. 1A). This was based on a recent report from Jones and Kazlauskas showing that mitogenesis can be mimicked by two pulses of PDGF (Jones and Kazlauskas, 2001). Quiescent cells were treated with indicated drugs for 0.5-1 hour, stimulated with PDGF for 30 minutes and followed by a second pulse of PDGF for 8-18 hours. DNA synthesis was monitored by adding bromo-deoxyuridine in the medium. In these conditions, the mitogenic response was similar to that obtained with continuous growth factor stimulation. An example of such experiments and statistical analyses is shown in Fig. 1A. PDGF induced 50% of the cells to enter S phase and this response was inhibited by CD and CO treatment. Those effects were attributed to cholesterol depletion because soluble cholesterol addition rescued mitogenesis. We concluded that membrane cholesterol regulates mitogenic signals during the first pulse of growth factor stimulation. This data was next confirmed in a continuous stimulation approach. Cellular cholesterol was chronically depleted by growing cells in lipoprotein-deficient serum (LPDS). We observed a marked reduction in DNA synthesis induced by PDGF (80%). Again, this inhibition was due to cholesterol depletion as adding back soluble cholesterol gave a significant rescue (Fig. 1C). Finally, the role of caveolae was further confirmed by overexpressing a dominant-negative

Fig. 1. Membrane cholesterol and Cav-3DGV inhibit PDGF-induced DNA synthesis in fibroblasts. (A) CD and CO inhibit PDGF-induced DNA synthesis. Top panel: discontinuous stimulation for PDGF-induced DNA synthesis. Bottom panel: an example (left) and the statistical analysis (right) of the inhibitory effects of cholesterol depleting agents on PDGF-induced BrdU incorporation. Quiescent NIH 3T3 cells grown on coverslips were treated or not (NS) with CD (10 mM) or CO (0.5 U/ml) as indicated, rinsed and stimulated with PDGF (25 ng/ml) in the presence of BrdU. In some cases, cells have been incubated with CD in the presence of 25 μM soluble cholesterol before stimulation with PDGF (CD+Cholesterol). (B) Caveolin-1-GFP (Cav-1-GFP) and caveolin-3DGV (Cav-3DGV) inhibit mitogenesis induced by continuous PDGF stimulation. Top panel: continuous stimulation for PDGF-induced DNA synthesis. Bottom panel: an example (left) and the statistical analysis (right) of the inhibitory effect of indicated caveolin constructs on PDGF-induced BrdU incorporation. Cells transfected with the indicated constructs were made quiescent and stimulated for 18 hours with PDGF with or without soluble cholesterol (20 μM) as indicated and in the presence of BrdU. GFP was co-transfected with Cav-3DGV to visualise transfected cells. An example of BrdU incorporation obtained from Cav-3DGV transfected cells is indicated by arrows. (C) PDGF-induced DNA synthesis is reduced in cells grown in LPDS. NIH 3T3 cells were grown in 5% fetal calf serum (standard) or 5% LPDS in the presence or absence of soluble cholesterol (25 μM) as indicated for 30 hours and serum-starved for 24 hours before PDGF stimulation. An example (left) and the statistical analysis (right) of the inhibitory effects of chronically depleting cholesterol on PDGF-induced BrdU incorporation. Cells were fixed and BrdU incorporation was analysed by immunofluorescence as described in the Materials and Methods. The means and the s.d. from three to five independent experiments are shown.

N-terminal truncation mutant of caveolin-3 (Cav-3DGV), known to reduce caveolae levels in the cell membrane (Roy et al., 1999). Transfected cells were synchronised in G0 and stimulated for 18 hours with PDGF allowing cell-cycle re-entry. As shown in Fig. 1B, Cav-3DGV reduced the induction of DNA synthesis by 60%. Specificity was shown by the capacity of free cholesterol to blunt Cav-3DGV function (Roy et al., 1999) and substantially overcome mitogenic inhibition. Similar results were obtained when overexpressing caveolin-1, previously described to inhibit mitogenesis (Galbiati et al., 2001). We concluded that caveolae regulate the PDGF mitogenic response.

### A role for caveolae in Src mitogenic signalling

We next examined the intracellular signals affected by membrane cholesterol depletion. CD treatment did not affect receptor activation as assessed by its in vitro kinase activity and tyrosine phosphorylation content (Fig. 2A), confirming that membrane cholesterol does not impact on receptor catalytic activation (Liu et al., 2000). We also did not observe any change of PDGF-induced Ras and ERK activation (Fig. 2B), suggesting that membrane cholesterol does not impinge on the Ras pathway. Nevertheless, CD induced a substantial increase in basal signals including Ras and ERKs. This may be

A

PDGF: - + - +
CD: - - + +

^{32}P-PDGFR-

ip: αPDGFRβ

PDGF: - + - +
CD: - - + +   M$_r$(X10^3)

-180
-115
-82
-64
-50
-37

wb: 4G10

total cell lysate

Fig. 2. Membrane-cholesterol depletion does not affect PDGF-induced receptor, Ras and ERK activation. (A) CD does not affect PDGF receptor activation. NIH 3T3 cells were serum starved, treated with CD, rinsed and stimulated with PDGF for 10 minutes as indicated. PDGFRβ was immunoprecipitated from indicated lysate with αPR4 followed by in vitro kinase assay (left panel). The position of [^{32}P]PDGFRβ is shown. Total cell lysates treated as indicated were directly subjected to western blotting with anti-phosphotyrosine antibody (4G10) (right panel). The location of molecular markers (M$_r$) is also shown. (B) CD does not affect PDGF-induced Ras (left panel) and ERK (right panel) activation. Lysates were either directly subjected to western blotting with anti-Ras (total Ras), anti-ERK (ERK) and anti-phospho ERK (pERKs) antibodies or incubated with Sepharose-bound GST-RalGDS binding domain and western blotting with anti-Ras antibodies (Ras-GTP) as indicated.

B

PDGF: - + - +
CD: - - + +

-RasGTP

-total Ras

PDGF: - + - +
CD: - - + +

-pERKs

-ERKs
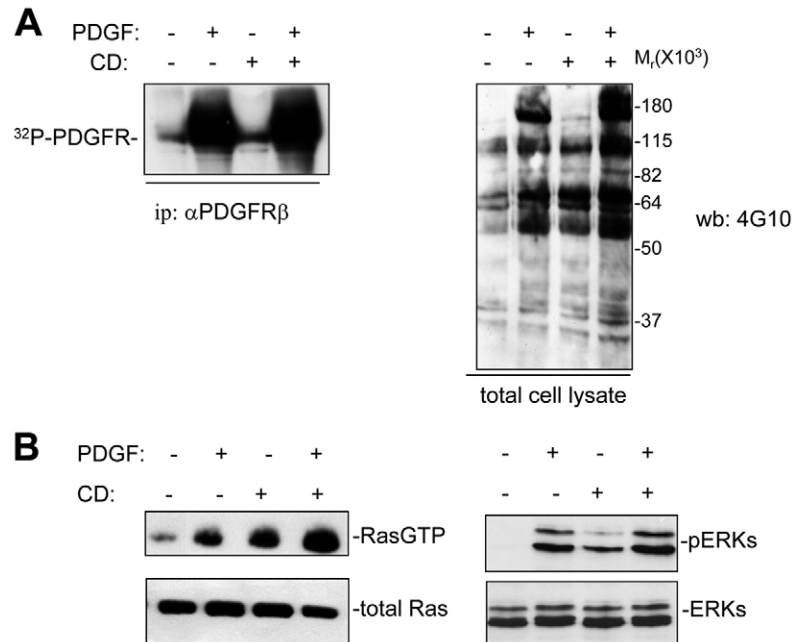
attributed to the capacity of membrane cholesterol to regulate GTPase-activating proteins and specific phosphatases that ensure low basal signalling (Wang et al., 2003). Similarly, CD also induces a significant increase in basal phosphotyrosine content as observed when treating cells with the tyrosine phosphatase inhibitor vanadate. This suggests that CD also inhibits tyrosine phosphatases in vivo. In contrast to the Ras pathway, Src signalling was affected by cholesterol membrane depletion: CD reduced PDGF-induced SFK activation and phosphorylation of the Src-substrate Stat3 on Y705 (Fig. 3). Recovery of cells for >2 hours allows replenishment of membrane cholesterol pools (not shown). In these conditions, a substantial SFK activation was obtained, confirming that inhibition was not due to any toxic effect of the drug (Fig. 3A).

Myc has been described as a important target gene of SFK signalling during growth-factor stimulation (Bromann et al., 2004). Accordingly, membrane cholesterol depletion inhibited the increase of *Myc* mRNA level induced by PDGF. Inhibition was overcome by addition of cholesterol in the medium showing drug specificity (Fig. 3C).

We next evaluated the impact of membrane cholesterol and dominant-negative Cav-3DGV on Src mitogenic function. The group of Kazlauskas suggested that in a discontinuous stimulation assay, the two pulses of PDGF signalling use different intracellular messengers for mitogenesis (Jones and Kazlauskas, 2001). Interestingly, microinjection experiments indicated that SFKs are required for the first 8-10 hours of continuous PDGF stimulation (Roche et al., 1995; Twamley-

Fig. 3. Src mitogenic signalling is affected by membrane cholesterol depletion and Cav-3DGV expression. (A) CD inhibits PDGF-induced SFK activation. SFKs were immunoprecipitated from RIPA cell lysates, which solubilises caveolae, and subjected to an in vitro kinase assay using enolase as a substrate. In addition to conditions used in Fig. 2, kinase activity was also performed on cells treated with CD and left 2 hours for membrane cholesterol replenishment before PDGF stimulation (Recovery) or not. Top panel: an example of SFK activity (^{32}P-Enolase); middle panel: quantification of SFK activity under conditions specified (mean ± s.d. from three independent experiments) and expressed as the ratio of non-stimulated and non-treated cells (control); bottom panel: levels of caveolin-1 and tubulin from RIPA and LB cell lysates. Protein levels were assessed by western blotting of total cell lysates using specific antibodies. (B) CD affects Src-specific tyrosine phosphorylation of Stat3. The level of immunoprecipitated Stat3 and pY$_{705}$Stat3 from indicated cell lysates is shown. (C) Membrane cholesterol depletion inhibits PDGF-induced *Myc* induction. Quiescent cells treated or not with cholesterol-depleting agents in the presence or absence of soluble cholesterol were stimulated for 1 hour with PDGF and total RNA was isolated. Northern blot analysis was performed from indicated RNA using a probe specific for Myc or 26S genes as a control of total RNA level (left panel). *Myc* mRNA level was quantified by real-time quantitative PCR (right panel). The ratio

between the mRNA level and that obtained from quiescent non-treated cells was calculated (Myc response). The mean and the s.d. are shown from three to five independent experiments. (D) SFKs are required for early mitogenic signalling. Quiescent cells were incubated with SU6656 (1 μM) for 1 hour before PDGF stimulation, and subjected to a discontinuous stimulation protocol depicted in top panels. Bottom panels: BrdU incorporation (left) was analysed as described in Fig. 1 and SFK activity (right) was analysed by western blotting of the immunoprecipitated SFK with pY$_{416}$Src antibody specific to the active Src kinases. The ratio between active SFK and SFK levels is shown and is representative of two independent experiments. (E) Expression of Myc overcomes mitogenic inhibition induced by CD. Cells were transfected with a trace amount of GFP construct in the presence of empty vector (mock), and the indicated constructs. Cells were serum starved, treated and stimulated in the presence of BrdU as described in top panel. (F) Expression of Myc overcomes mitogenic inhibition induced by dominant-negative Cav-3DGV. Cells were transfected with trace amounts of GFP construct in the presence of the indicated constructs. Cells were serum starved, and stimulated in the presence of BrdU as described in top panel. BrdU incorporation was analysed and calculated as described in Fig. 1. The mean and the s.d. of three to five independent experiments are shown.

Stein et al., 1993), suggesting that SFK are required for both early and late signalling. Inactivation of SFK by the specific inhibitor SU6656 during the initial phase was sufficient to inhibit DNA synthesis (Fig. 3D, left panel). The short treatment did not affect the capacity of PDGF to activate SFKs during the second pulse of stimulation (Fig. 3D, right panel). Therefore, SFKs may be required for the early events of

mitogenic signalling. We then searched for a functional link between membrane cholesterol and the Src pathway. To this end, cells were transfected with a Myc-expressing construct, synchronised in G0, treated with CD and stimulated with two pulses of PDGF for cell-cycle re-entry. Green fluorescent protein (GFP) was co-expressed as a marker to visualise transfected cells. As shown in Fig. 3E, CD pretreatment did
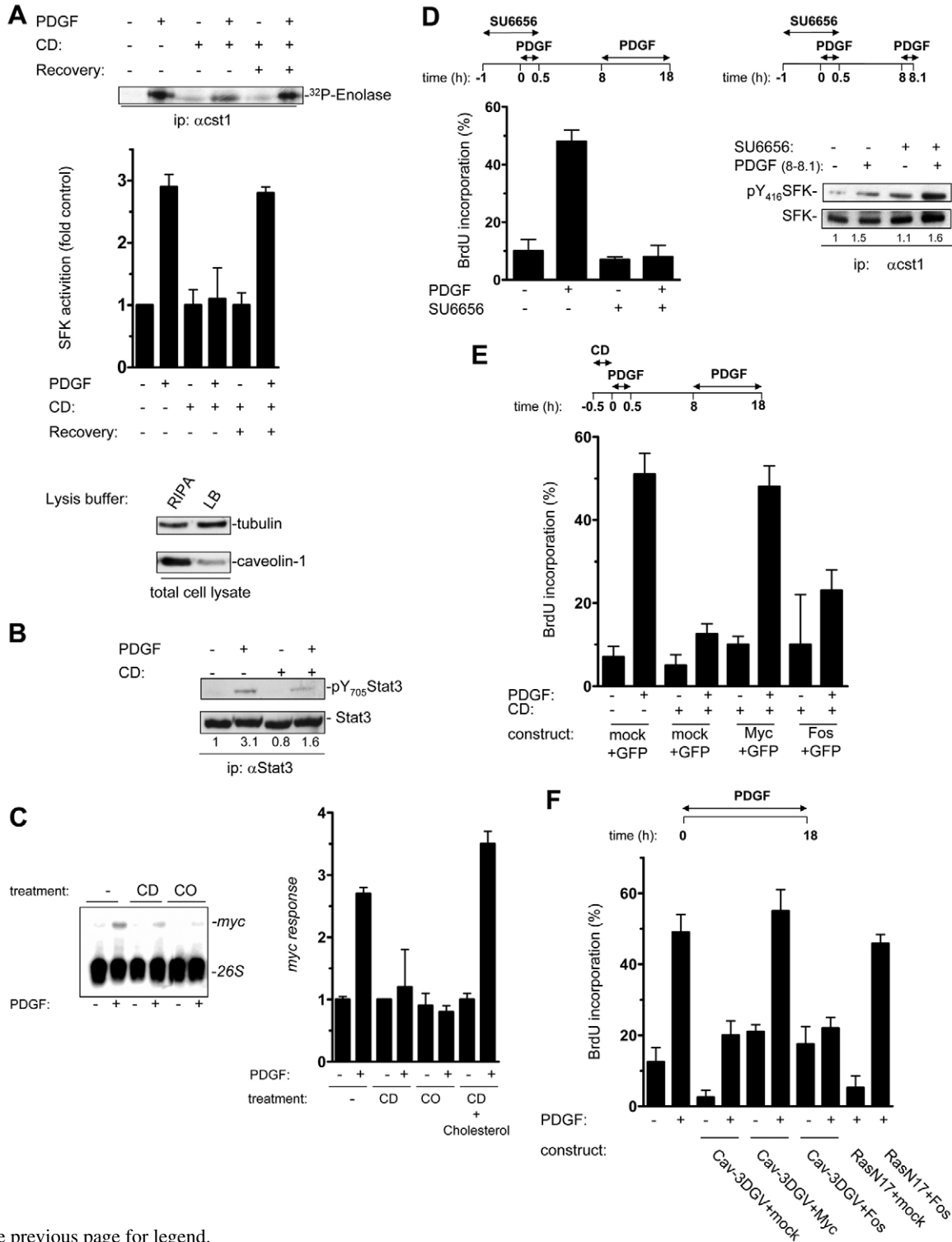
Fig. 3. See previous page for legend.

not inhibit mitogenesis of Myc-expressing cells but did affect control cells. Under our conditions, Myc did not induce DNA synthesis in the absence of growth factor; furthermore, the observed effect was specific to this transcription factor, because forced expression of Fos did not give substantial rescue. By contrast, Fos overcame inhibition induced by the dominant-negative RasN17 mutant showing specificity (Barone and Courtneidge, 1995) (Fig. 3F). The involvement of caveolae in this response was next confirmed by expressing the Cav3-DGV construct. As observed with CD, Myc co-expression bypassed the G1 block induced by this mutant (Fig. 3F). Similar results

were obtained when overexpressing caveolin-1 (not shown). Altogether these data strongly suggest that caveolae regulate Src-induced *Myc* induction for cell-cycle progression.

Two distinct pools of SFKs activated by PDGF

We next characterised SFKs from a purified caveolae-enriched fraction (CEF). Cell lysates were subjected to a Dounce homogenisation for increasing protein solubility, followed by a sucrose gradient fractionation. Under these conditions, CEFs were isolated in the light fractions (2-4) (Fig. 4A). Most of phosphotyrosine content accumulated in heavy fractions
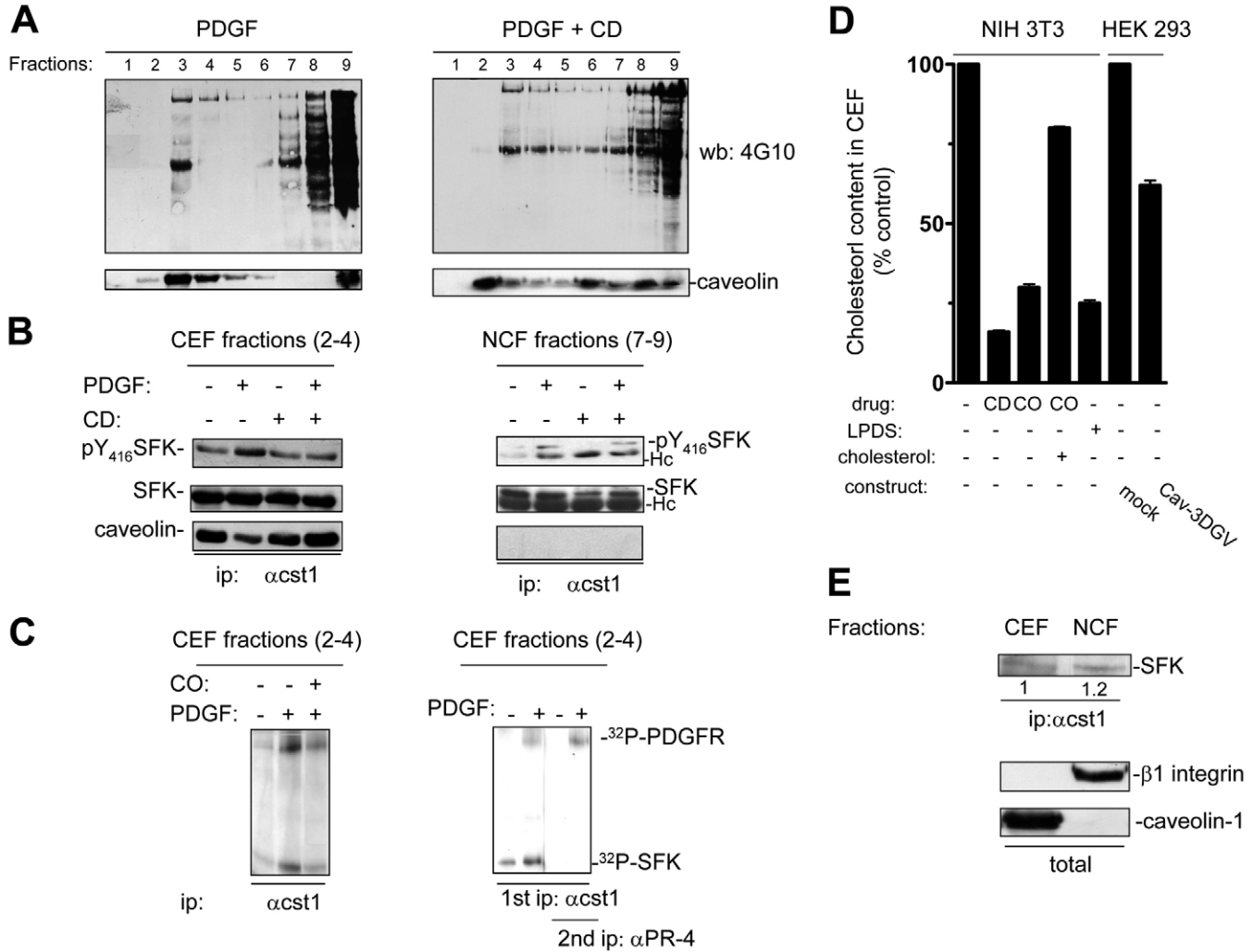
Fig. 4. PDGF stimulates two pools of SFK activities with distinct sensitivity to membrane cholesterol. (A) Purification of CEF. 1% Triton X-100 cell lysates of fibroblasts treated as indicated were subjected to a Dounce homogenisation followed by a sucrose gradient fractionation. Fractions were directly subjected to western blotting with an anti-phosphotyrosine (4G10) and caveolin-specific antibody as indicated. The presence of respective protein as well as the fraction number is shown. (B) PDGF-induced SFK activities in caveolae-enriched (CEF) and non-caveolae (NCF) fractions. CEF (2-4) and NCF (7-9) were pooled and treated as in the Materials and Methods. SFK activities and their association with caveolin were measured by western blotting of immunoprecipitated kinases with pY_{416}Src and caveolin antibody respectively. (C) CO regulates SFK-PDGFR association in CEF. In vitro kinase assay was performed with the immunoprecipitated SFK from CEF. The presence of PDGFR was revealed by re-immunoprecipitation of the labelled proteins using specific antibody (αPR4) as indicated (2nd ip). Antibodies used for immunoprecipitation (ip), cell treatments, SFK, pY_{416}SFK, heavy chains immunoglobulin (Hc), [^{32}P]SFK and [^{32}P]PDGFR are indicated. (D) Cholesterol content in CEF. CEF were isolated as described in Materials and Methods from NIH 3T3 cells treated or grown as indicated and from HEK 293 cells expressing Cav3DGV as indicated. Is shown the cholesterol content in CEF relative to the non treated cells (% control). (E) Levels of SFK in CEF and NCF fractions. SFK were immunoprecipitated from two-thirds of CEF and one-quarter of NCF and subjected to western blotting with cst1 antibody. Caveolin-1 and β1 integrin levels were detected as specific markers of CEF and NFC respectively and assessed by western blotting of total protein fractions. A quantification of SFK levels is indicated.

whereas CEFs mainly contained phosphotyrosine proteins of 60 and 180 kDa (Fig. 4) suggesting the presence of SFKs and the PDGF receptor. Indeed, SFKs could be clearly immunoprecipitated from the light fractions (2-4). Quantification analysis suggested that at least 25% of SFKs are located in caveolae (Fig. 4E). PDGF stimulated SFKs in CEF as shown by phosphorylation of Y416 of the activation loop and the capacity of SFKs to autophosphorylate in vitro (Fig. 4B,C). In addition, PDGF induced SFK-PDGFR complex formation in CEFs as revealed by in vitro kinase assays. The presence of the receptor was further confirmed by reimmunoprecipitation experiments (Fig. 4C). This strongly suggests that activation and association of SFKs with the receptor may occur in caveolae. The effect of CD was next examined on SFK activities. CD treatment decreased the buoyant density of caveolae and tyrosine-phosphorylated proteins present in CEF (Fig. 4A). This was attributed to a flattening of caveolae secondary to cholesterol depletion (Pike, 2005). Indeed CD treatment strongly depleted cholesterol from the CEF (Fig. 4D). Although drug treatment did not affect the level of SFKs in the CEF, it reduced catalytic activation and their association with the receptor (Fig. 4B,C). Furthermore, we observed that caveolin strongly co-precipitated with SFKs and its association was reduced upon PDGF stimulation. However, caveolin dissociation was not observed in CD-treated cells. Because of its negative regulatory effect towards catalytic activity (Li et al., 1996), we believe that SFK regulation also involves caveolin. In addition to the CEF, SFKs were also activated in non-caveolae fractions (fractions 7-9, NCF) (Fig. 4B). However, such stimulation was insensitive to CD and was not associated with caveolin. These results suggest that PDGF additionally induces SFK activation outside caveolae through a distinct mechanism.

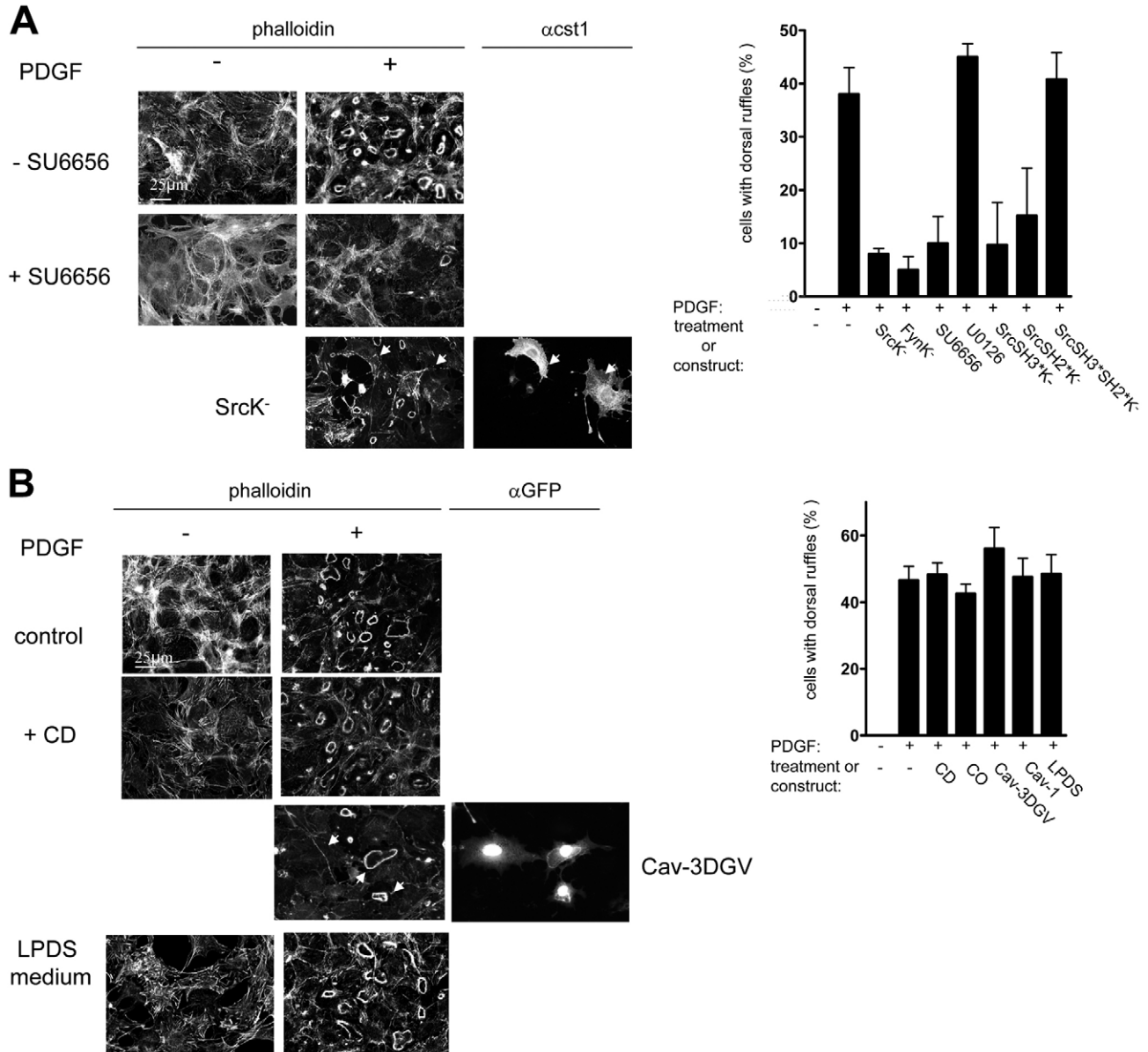## SFKs regulate PDGF-induced actin dorsal ruffles outside caveolae.

We next searched a role for SFKs in PDGF-receptor signalling outside caveolae. In addition to mitogenesis, PDGF induces morphological changes for cell migration and/or invasion. These include formation of lamellipodia and dorsal ruffles (Buccione et al., 2004). We found that the latter required SFKs: 40% of the cells formed dorsal ruffles within 10 minutes of PDGF simulation and this response was inhibited by the SFK inhibitor SU6656 or expression of the kinase-dead mutants SrcK⁻ and FynK⁻ (Fig. 5A). It should be noticed that the inhibitory effect obtained with both dominant-negative constructs indicates that Src and Fyn may have overlapping functions. We next analysed whether the SH2 and SH3 domains of the kinase were necessary for signalling. SrcK⁻ with inactive SH3 (SrcSH3*K⁻) or SH2 domain (SrcSH2*K⁻) were found inhibitory unlike SrcK⁻ allele with both SH2 and SH3 inactivation (SrcSH3*SH2*K⁻). We concluded that both SrcSH2 and SrcSH3 domains are required for signalling leading to dorsal ruffles. In contrast to SFK, the ERK pathway may not be used by PDGF for this morphological response because the MEK inhibitor U0126 had no effect (Fig. 5A). The influence of caveolae was next examined on this cellular response. CD reduced actin stress fibres of quiescent cells in agreement with a role on cytoskeleton rearrangement (Kwik et al., 2003). However membrane cholesterol depletion by treating cells with CD or CO, or growing cells in LPDS

medium had no effect on dorsal ruffle formation (Fig. 5B). Similarly, no effect was observed when overexpressing caveolin-1 and Cav-3DGV constructs (Fig. 5B). We concluded that morphological signals leading to F-actin assembly are not regulated by caveolae.

S1P signalling mediates the PDGF-induced SFK activation for ruffle formation

We next sought the molecular mechanism by which Src regulates this cytoskeleton rearrangement. Spiegel and colleagues suggested that S1P is an important lipid second messenger for PDGF-induced lamellipodia formation and directed cell migration (Hobson et al., 2001). We then surmised that a similar mechanism operates on PDGF-induced dorsal ruffle formation. As shown in Fig. 6A, a short treatment of cells with N,N-dimethylsphingosine (DMS), a specific inhibitor of sphingosine kinase or expression of the kinase inactive form of the kinase 1 (S1K⁻) all prevented this PDGF response. Sphingosine kinase is activated by PDGF through a PLCγ/Ca²⁺-dependent pathway (Olivera et al., 1999). Accordingly, we found that cell treatment with a low dose of the PLC inhibitor U73122 also inhibited this morphological change. Specificity was also shown by the inability of the inactive analogue U73343 to affect cytoskeleton rearrangement. Finally, S1P has been described to bind a seven transmembrane receptor of the EDG family coupled to a heterotrimeric Gi protein for SFK activation (Rosenfeldt et al., 2001). Cell pre-treatment with pertussis toxin (PTx) that ADP-ribosylates heterotrimeric proteins of the Gi family also inhibited dorsal ruffle formation. Altogether, this suggests that dorsal ruffle formation induced by PDGF requires a S1P-Gi-dependent signalling process.

SFK activation by PDGF also implicates the S1P-EDG-Gi cassette (Chen et al., 1994; Rosenfeldt et al., 2001; Waters et al., 2005). Whether this S1P signalling involves the SFK activity identified outside caveolae was next investigated. To this end, SFKs were immunoprecipitated from purified NCF and assayed for an in vitro kinase activity. As shown in Fig. 6C, PDGF induced a threefold increase in kinase activity. Treatment of the cells with DMS, U73122 or PTx, all abrogated PDGF-induced SFK activation. This data fit a model where the non-caveolae pool of activated SFKs originates from an S1P-dependent pathway for dorsal ruffle formation. If our model is true, then PDGF-induced S1P signalling may not regulate the SFK activation in caveolae leading to DNA synthesis. Indeed, none of these inhibitions affected SFK association with the PDGF receptor from CEF (Fig. 6D). Furthermore they poorly affected, if at all, the capacity of PDGF to induce DNA synthesis (Fig. 6B). The existence of two separate pools of SFKs for signalling were next confirmed by an immunofluorescence approach using cells expressing a Src construct and the phosphoY416-specific Src antibody that recognises the activated form of the kinase. As shown in Fig. 7, PDGF-induced Src activation was found associated both with the cell periphery and actin dorsal ruffles. CO membrane cholesterol depletion did not affect dorsal ruffles or Src activity associated with these actinic structures. Accordingly, caveolin-1 was never detected in dorsal ruffles (not shown). However we observed a strong reduction of cells with active Src at the cell periphery. Quantification of the signals indicated that PDGF induced a threefold increase in active SFKs at the cell

Fig. 5. Membrane cholesterol and caveolins do not regulate SFK signalling linked to PDGF-induced dorsal ruffle formation. (A) SFKs are required for PDGF-induced dorsal ruffle formation. Top panel: typical effect obtained with SFK inhibition on PDGF-induced dorsal ruffle formation. Bottom panel: statistical analysis of PDGF response (% of cells with dorsal ruffle) in cells expressing indicated construct or treated with the SFK (SU6656 5 μM) or the MEK (U0126 10 μM) inhibitor as shown. (B) Dorsal ruffle formation is not affected by cholesterol depletion. Top panel: typical effect obtained with membrane cholesterol depletion on PDGF-induced dorsal ruffle formation. Bottom panel: statistical analysis of PDGF response (% of cells with dorsal ruffle) of cells expressing indicated caveolin constructs, treated with indicated cholesterol-depleting agents, or grown in LPDS as shown. NIH 3T3 grown on coverslips were transfected or not with indicated construct, serum-starved, treated with SU6656 (5 μM) for 30 minutes and stimulated with PDGF (15 ng/ml) for 10 minutes. Cells were fixed and processed for actin staining using Rhodamine-phalloidin and ectopic protein expression. Cav-3DGV was co-transfected with trace amounts of GFP construct to visualise transfected cells. Cells expressing SrcK− (A) or Cav-3DGV (B) constructs are indicated by arrows. The mean percentage of cells that formed dorsal ruffles and the s.d. from three to five independent experiments are shown.

periphery (33±12% of total active SFK in PDGF-stimulated cells versus 12±4% in control cells, mean ± s.d., n=5). Such activation was abrogated upon CO treatment (14±1%). By contrast, DMS, U73122 and PTx all impaired Src activation associated with actinic dorsal ruffles while not affecting the pool of active Src at the peripheral membrane. Additionally, DMS induced a strong localisation of active Src in cytoplasmic vesicles that were found colocalised with actin. Frame and colleagues reported that actin polymerisation coordinates Src activation with endosome-mediated delivery to the membrane (Sandilands et al., 2004), suggesting that DMS may also affect the transit of cytoplasmic Src in endosomes to the peripheral structures of the cell. Altogether this data fit with a model in which PDGF uses two distinct pools of SFKs for mitogenesis
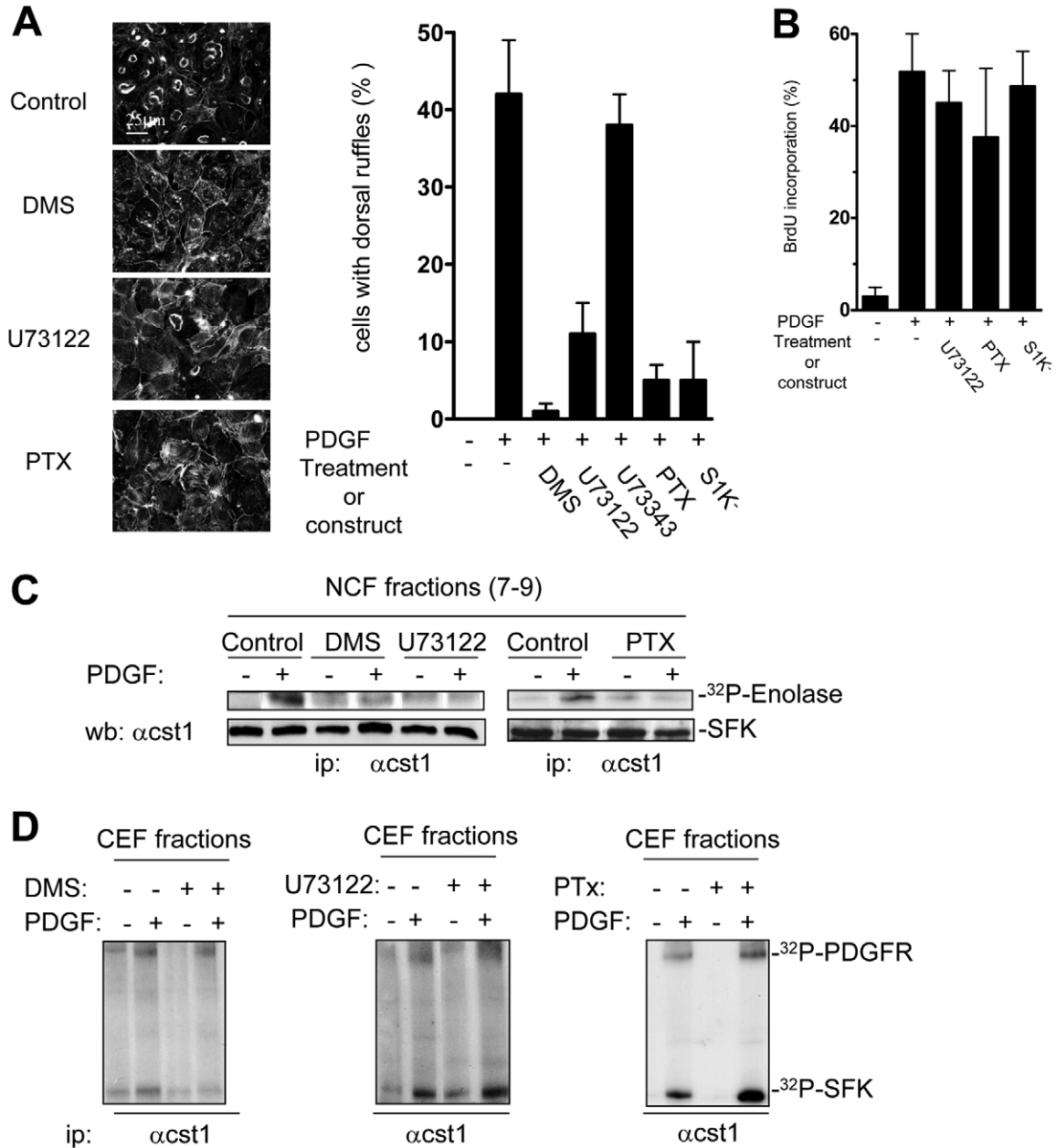
Fig. 6. PDGF-induced dorsal ruffle formation and SFK activation outside caveolae require S1P signalling. (A) S1P signalling regulates PDGF-induced dorsal ruffle formation. Left panel: an illustration of a typical effect obtained with S1P signalling inhibition on PDGF-induced dorsal ruffle formation. Right panel: PDGF response (% of cells with dorsal ruffles) of cells treated with indicated drugs or expressing S1K⁻ as shown. (B) S1P signalling does not affect PDGF-induced BrdU incorporation. Quiescent cells expressing S1K⁻ or treated with indicated drugs were stimulated with PDGF in the presence of BrdU. BrdU incorporation was analysed and calculated as described in Fig. 1. The mean and the s.d. of three to five independent experiments are shown. (C) S1P signalling regulates PDGF-induced SFK activation in NCF. SFKs were immunoprecipitated from NCF as depicted in Fig. 4 with cells treated or not (control) with indicated agents and stimulated or not with PDGF. Levels of SFKs were determined and the in vitro kinase assay performed with denatured enolase as an exogenous substrate (^{32}P-Enolase). (D) S1P signalling does not regulate SFK-PDGFR association in CEF. SFKs were immunoprecipitated from CEF obtained from cells used in panel C and subjected to an in vitro kinase assay. Autophosphorylation of Src kinases (^{32}P-SFK) as well as the presence of the associated PDGFR (^{32}P-PDGFR) are shown. Cell treatment was as follows: DMS (10 μM) for 30 minutes, U73343 and U73122 (1 μM) for 30 minutes and PTx (500 ng/ml) for 4 hours.

and cytoskeletal rearrangement: the first associated with the plasma membrane and the second associated with actinic structures.

Finally, we examined the requirement for Src mitogenic substrates Shc, Stat3 and Abl for ruffle formation. Although non-phosphorylatable alleles of Shc (Shc2Y2F/R394F) and Stat3 (Stat3Y705F) were dominant negative for mitogenesis (Blake et al., 2000; Boureux et al., 2005; Bowman et al., 2001)

they did not influence dorsal ruffle formation (Fig. 8A). Nevertheless, kinase inactive forms of Abl, AblK⁻ and AblPPK⁻, still reduced the PDGF response (Fig. 8A) in agreement with previous reports (Furstoss et al., 2002; Plattner et al., 1999). Therefore SFKs may use both common and specific substrates for dorsal ruffle formation and DNA synthesis. We have previously shown that Abl phosphorylation by SFKs is involved in kinase activation for dorsal ruffle formation (Furstoss et al., 2002). Whether S1P signalling impacts on PDGF-induced Abl activation was also analysed. To this end, Abl outside caveolae was immunoprecipitated from a Triton X-100-extracted cell lysate and assayed for in vitro kinase assay using Crk as a specific substrate. We found that PDGF induced a 2.5-fold increase in Abl catalytic activity as previously reported (Plattner et al., 1999). In addition, treatment of cells with DMS, PTx or a low dose of the PLC inhibitor U73122, all impaired Abl activation. By contrast, they did not affect phosphorylation of the SFK mitogenic substrate

Stat3 on Y705 showing specificity (Fig. 8B). Taken together, these results are consistent with a model where Abl is a downstream element of the S1P signalling pathway for dorsal ruffle formation.

Discussion

SFKs play important roles in PDGF receptor signalling. In this report, we show that PDGF uses two distinct pools of SFKs for mitogenesis and cytoskeleton rearrangement. We first provide evidence for an initiation of Src mitogenic signalling in caveolae in agreement with the strong localisation of these tyrosine kinases in these organelles (Liu et al., 1996). In addition we believe that caveolin defines an additional mechanism for SFK regulation by growth factors in vivo. Indeed caveolin is a major constituent of caveolae that associates with Src and keeps the kinase in an inactive form (Li et al., 1996; and data not shown). The molecular mechanism for negative regulation has not been clearly
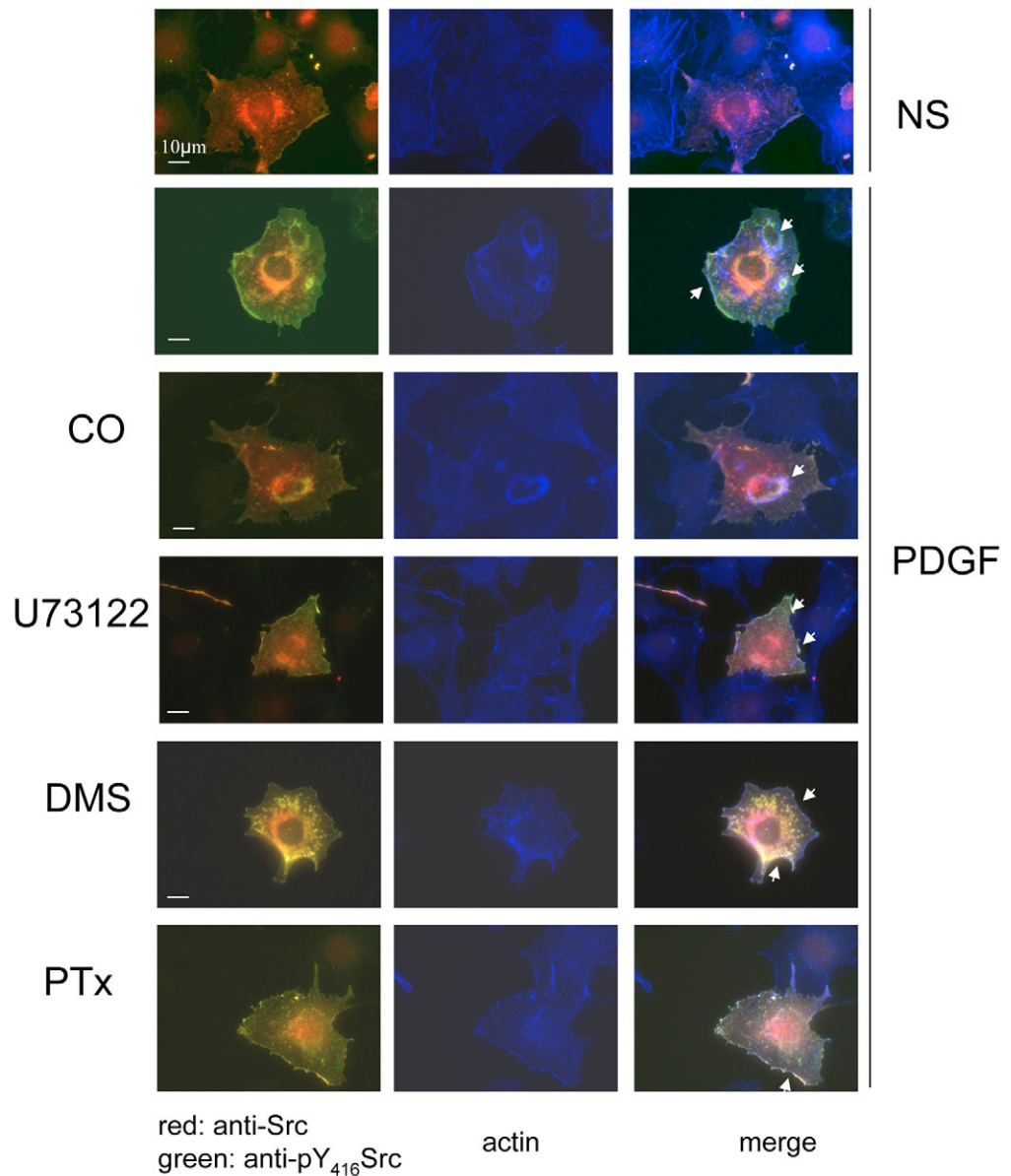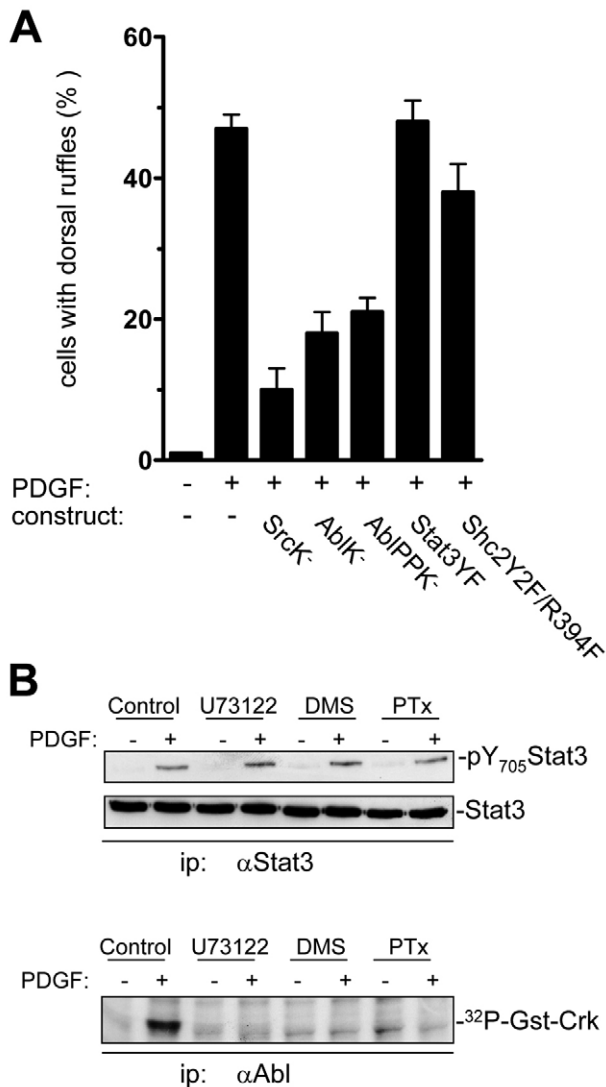

Fig. 7. Spatial regulation of Src activity by membrane cholesterol and S1P signalling in PDGF-stimulated cells. NIH 3T3 were transfected with an avian Src construct, synchronised in G0 by serum starvation, treated or not with indicated agent and stimulated or not (NS) for 10 minutes with PDGF as shown. Src was detected with EC10 antibody (red), active Src with anti-pY$_{416}$ antibody (green) and actin with fluorescent Phalloidin (blue). Left panels: merged imaged of Src and active Src fluorescence. Middle panels: actin. Right panels: merged images of Src, active Src and actin. Pools of active Src at the plasma membrane and associated actinic dorsal ruffles are indicated by arrows.

red: anti-Src
green: anti-pY$_{416}$Src   actin   merge

defined, but in vitro data suggests that it contacts the catalytic core favouring the kinase in an inactive form (Couet et al., 1997). Therefore association of the receptor with SFK may not only open the conformation of the enzyme, but also disrupt caveolin binding for further activation. In addition, caveolae may also modulate substrate phosphorylation by recruiting them close to the kinase (Liu et al., 2000; Wang et al., 2000). Altogether, this molecular mechanism may lead to optimal signalling for gene expression and DNA synthesis. Interestingly, caveolin-1 has been described as a negative regulator of mitogenesis and this function is dependent upon a

Fig. 8. Abl is an effector of the S1P signalling for PDGF-induced dorsal ruffle formation. (A) Abl, but not other Src mitogenic substrates, is required for PDGF-induced dorsal ruffle formation. PDGF response (percentage of cells with dorsal ruffles) of cells expressing indicated constructs. (B) PDGF-induced Abl but not Stat3 activation requires an S1P signalling pathway. Stat3 was immunoprecipitated from cells treated with the indicated drugs and stimulated or not with PDGF as shown. Abl was detected in the same way as Stat3 and then assayed for in vitro kinase activity. The levels of Stat3 and pY$_{705}$Stat3 were assessed by western blotting. [^{32}P]Gst-Crk was also detected.

functional p53 (Galbiati et al., 2001). Similarly we and others identified p53 as a regulator of the Src mitogenic signalling (Broome and Courtneidge, 2000; Furstoss et al., 2002). Therefore a functional link may exist between SFK and caveolin during mitogenesis, probably by operating on the same pathway. In contrast to other extracellular stimuli, SFKs have no major role on PDGF-induced Ras activation (Bromann et al., 2004). The heterogeneous sensitivity of CD reinforces the notion of separate signalling cascades for mitogenesis. Caveolae may be used by the receptor for Src regulation whereas Ras activation largely occurs outside these organelles. Accordingly, recent data suggest that Ki-Ras can stimulate ERK activity in endomembranes including early endosomes and the Golgi complex (Hancock, 2003). Although the Ras isoforms activated by PDGF are not known, the inefficiency of CD to prevent activation may implicate Ki-Ras.

This report also unravels a previously unappreciated function for SFKs in PDGF-induced actin dorsal ruffles. This morphological change has been related to macropinocytosis and protease-dependent cell migration (Suetsugu et al., 2003) suggesting a function for SFKs in those cell responses. In addition our data indicate that PDGF uses a distinct pool of SFKs for this cytoskeleton rearrangement. The latter is localised outside caveolae and is probably associated with actinic structures. Accordingly this pool could be characterised biochemically following fractionation assays indicating that it may be poorly solubilised in standard lysis conditions. This would explain why SFKs were thought to be solely activated by receptor association (Mori et al., 1993). Furthermore, activation of this population may be dependent on the capacity of cells to generate dorsal ruffles. For example Ph cells used by DeMali and Kazlauskas (DeMali and Kazlauskas, 1998) do not form dorsal ruffles (not shown) and most SFK was found activated by receptor association. Our report also proposes a molecular mechanism for activation of this pool of Src kinases, which implicates an S1P-EDG-Gi-SFK signalling cassette as proposed for lamellipodia extension (Rosenfeldt et al., 2001). Therefore, a similar mechanism may exist for dorsal ruffle formation. PDGF-induced S1P formation involves a PLCγ-dependent pathway and indeed a low dose of PLC inhibitor was sufficient to inhibit SFK activation by PDGF outside caveolae. This raises the idea that SFKs are activated through both the association with the receptor and a PLCγ-dependent mechanism (a model is shown in Fig. 9). It should be noted that the molecular mechanism for directed ruffle formation (lateral or dorsal) has not yet been established.

The mechanism by which Src signals for dorsal ruffle formation is largely obscure. Di Fiore et al suggested that PDGF needs a Ras, Rac and a Rab5 pathway for this morphological change (Lanzetti et al., 2004). We found that PDGF-induced Rac activation is regulated by SFK activities suggesting that Rac is a downstream element of this Src morphological signalling (not shown). However rescue experiments with constitutive Rac alleles could not overcome the block induced by SrcK⁻, in agreement with additional Src effectors for ruffle formation. In fact, none of the active Rac, Ras or Rab5 alleles we used – either alone or in combination – could alleviate this inhibition (not shown). Thus an additional intracellular pathway initiated by SFK may be required for dorsal ruffle formation. Alternatively, SFKs may also impact on effectors of some of these regulators for efficient signalling.

Fig. 9. A model illustrating how PDGF uses two distinct pools of SFKs for DNA synthesis and dorsal ruffle formation. PDGF activates SFKs in caveolae (grey) allowing phosphorylation of mitogenic substrates for *Myc* induction and DNA synthesis. PDGF recruits sphingosine kinase (SK1) to the membrane through a PLCγ-dependent pathway for S1P formation. S1P further activates SFK outside caveolae through an EDG receptor coupled to a heterotrimeric Gi protein allowing Abl activation for dorsal ruffle formation. Additional Src substrates (others) may be expected for induction of this morphological change. S, sphingosine.
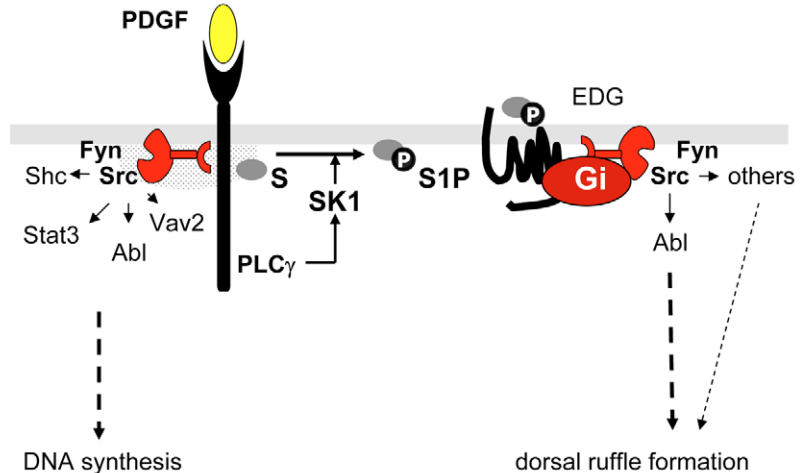
In agreement with that hypothesis, phosphorylation of WASP-like proteins by Src allows high activity in vivo (Suetsugu et al., 2002a). Besides small GTPases, our data indicate an important role of the cytoplasmic tyrosine kinase Abl in this signalling cascade. Abl has been largely implicated in F-actin cytoskeleton rearrangement leading to cell adhesion, directed migration or axon guidance (Woodring et al., 2003). We and others have reported a role for Abl in dorsal ruffle formation (Furstoss et al., 2002; Plattner et al., 1999; Sini et al., 2004), suggesting that it may also have a role in protease-dependent cell migration. Several mechanisms have been proposed for PDGF-induced Abl activation: one implicates Src-induced phosphorylation on Y412 in the activation loop and Y245 in the linker domain for stabilisation of the kinase in an open and active conformation (Furstoss et al., 2002; Plattner et al., 1999). A second mechanism involves an additional PLCγ-dependent pathway for full catalytic activation (Plattner et al., 2003). The mechanism by which PLCγ induces Abl activation is not well understood, but Pendergast et al. surmised the existence of a phosphatidylinositol 4,5-bisphosphate-binding protein for Abl inhibition (Plattner et al., 2003). Our present report provides another mechanism in which Abl activation is regulated by a S1P-SFK dependent pathway. In favour of this hypothesis is the strong inhibition observed with both SFKs and S1P signalling inhibitors. Therefore several mechanisms may exist for PLCγ to regulate Abl catalytic activity in vivo. How Abl transmits the morphological signal for ruffle formation is not known but several substrate candidates have been identified: these include Wave1, adaptor proteins (Nck, CrkII, Abi1/2), PSTPIP and the Rac activator SOS (Leng et al., 2005; Sini et al., 2004; Woodring et al., 2003). Whether any of these substrates are important Abl effectors for dorsal ruffle formation needs to be clarified.

In summary, our data identify a new mechanism for Src mitogenic regulation in caveolae and a Src signalling cascade outside these organelles that mediates PDGF-induced ruffle formation. Except for Abl, none of the Src mitogenic substrates impact on dorsal cytoskeleton rearrangement. Conversely, none of the S1P signalling proteins have an important role in PDGF mitogenic response (Rosenfeldt et al., 2001) (and data not shown). The fact that Abl was used for both cell responses suggests that like Src, two distinct pools of the kinase may be used by the receptor as recently revealed by FRET analysis (Ting et al., 2001). Finally, our results provide strong evidence for a spatial compartmentalisation mechanism for the regulation of SFK signalling leading to distinct biological outcomes: cell growth and morphological change.

Materials and Methods
DNA constructs, antibodies and reagents
Constructs expressing Src, SrcK⁻, Fyn, FynK⁻, AblK⁻, AblPPK⁻ (AblP242E/P249E/K290M), Myc, Fos, Stat3Y705F, Shc2Y2FR394F, RasN17 and GFP have been described (Blake et al., 2000; Boyer et al., 1997; Furstoss et al., 2002; Yamanaka et al., 1996), as have Cav-3DGV (Watson et al., 2001) and Cav-1-GFP (Thomsen et al., 2002). SrcSH3*K⁻ (SrcW118A/K298M), SrcSH2*K⁻ (SrcR175L/K298M) and SrcSH3*SH2*K⁻ (SrcW118A/R175/K298M) were generated by mutagenesis following the manufacturer's instructions (Stratagene). S1K⁻ construct was a generous gift from N. Pyne and S. Pyne (Department of Physiology and Pharmacology, Strathclyde Institute for Biomedical Sciences, Glasgow, UK). Antibodies specific to SFK (cst1) and PDGFRβ (PR4) have been described (Roche et al., 1998). Antibodies specific to pY416Src were from BioSource International, Abl (Ab-3) from Calbiochem, pan-caveolin form Transduction Laboratories, ERKs (SC-94) from Santa Cruz Biotechnology, pY705Stat3 from Cell Signalling, Stat3, Ras and chicken Src (EC10) from Upstate Biotechnology, phospho-ERKs from New England Biolabs and anti-BrdU from Beckton Dickinson. 4G10 was from Prof. Mangeat (CRBM, Montpellier). Rhodamine phalloidin was from Molecular Probes, Bromodeoxyuridine (BrdU) from Boehringer, SU6656 from Calbiochem, CD, soluble cholesterol, Pertussis toxin (PTx), U73122, U73343 and lipoprotein-deficient serum (LPDS) from Sigma, CO from Roche Diagnostic, PDGF-BB and U0126 from Upstate Technology, DMS was from Cayman Chemical Company.

Cell culture, transfection and immunofluorescence
NIH 3T3 cells were cultured and transfected as described (Furstoss et al., 2002). Cells were serum starved for 30 hours according to the protocol described (Jones and Kazlauskas, 2001), then treated as recorded elsewhere (Smart and Anderson, 2002) and stimulated as described in Fig. 1A or as otherwise indicated. Cholesterol-depleting drugs were removed before stimulation. Other drugs such as SU6656, U0126, U73122, U73343, DMS and PTx (500 ng/ml) were left in the medium during stimulation. In chronic cholesterol-depletion experiments, cells were grown in 5% LPDS in the presence or absence of soluble cholesterol (25 μM) for 30 hours and serum starved (0.25% LPDS) for 24 hours before stimulation. BrdU was added to the medium to monitor S-phase entry. Cells were then fixed and analysed for transfected construct expression and BrdU incorporation by immunofluorescence as described (Roche et al., 1995). Circular ruffle formation analysis was performed (Furstoss et al., 2002). The percentage of transfected cells that incorporated BrdU was calculated using the following formula: % of BrdU-positive cells = (number of BrdU-positive transfected cells)/(number of transfected cells)×100. The percentage of transfected cells that have formed dorsal ruffles was calculated using the following formula: % of cells with dorsal ruffle = (number of ruffle-positive transfected cells)/(number of transfected cells) ×100. For each coverslip, about 150-200 cells were analysed. For each cell, the % of active Src present at the cell periphery = relative Src signal × [(pY416Src signal at the cell periphery)/(pY416Src signal of the cell)] ×100. Images were recorded with a motorised DMRA

microscope (Leica Microsystem) using a 63× plasmapochromatic oil-immersion objective (NA 1.32). Acquisition was performed with a coolsmap HQ camera (Photometrics) driven by the Metamorph 6.2r4 acquisition software (Molecular Devices).

Biochemistry

Cells were lysed in lysis buffer (1% NP40) or RIPA buffer (1% Triton X-100, 1% deoxycholate) as described (Furstoss et al., 2002) except for isopicnic gradient fractionation. Immunoprecipitation, re-immunoprecipitation, western blotting and in vitro kinase assays were performed as decribed (Furstoss et al., 2002; Kypta et al., 1990) and quantified using ImageQuant TL software (Molecular Dynamics). RasGTP was precipitated using GST-Ral GDS-Ras binding domain (Busca et al., 2000). Fractionation experiments were performed essentially as described (Anderson, 1998) with minor modifications. Briefly, 10^8 cells were incubated with vehicle or 25 ng/ml PDGF for 10 minutes at 37°C, rinsed with PBS, scraped in ice-cold PBS-containing 1 mM vanadate and pelleted. Pellets were next suspended in ice-cold 2× lysis buffer containing 1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 75 U/ml aprotinin and 1 mM vanadate, for 20 minutes. Cell suspension was homogenised in a tight-fitting Dounce homogeniser with 10 strokes and centrifuged for 5 minutes at 1300 *g* to remove nuclei and large cellular debris. The supernatant was subjected to (5.0-42.5%, w/v) sucrose gradient centrifugation in 4 ml tubes. Nine fractions were collected from the top to the bottom of the gradient. In some experiments, fractions 2-4 were pooled and diluted fivefold in 25 mM MOPS (pH 6.5), 150 mM NaCl containing 1% Triton X-100 and centrifuged at 50,000 *g* for 30 minutes at 4°C. Pellets were then suspended in dissociation buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5 mM EGTA, 1 mM vanadate, 50 mM NaF, 75 U/ml aprotinin, 1% NP 40 and 60 mM octylglucoside and incubated for 1 hour on ice before SFK immunoprecipitation with cst1 antibody. Fractions 7-9 were pooled and diluted fivefold in lysis buffer before SFK immunoprecipitation. Cholesterol content in cholesterol-enriched fractions was assessed using the Amplex® Red cholesterol assay kit from Molecular Probes.

Myc mRNA level was measured both by northern blotting (Furstoss et al., 2002) and using real-time quantitative PCR as described (Puceat et al., 2003). After reverse transcription, 10 ng cDNA was used for real-time quantitative PCR, performed with a light cycler and the SYBR Green fast start kit (Roche). The following primers were used: Myc forward 5′-CGGAGGAAAACGACAAGAGG-3′ and reverse 5′-GTG-CTCGTCTGCTTGAATGG-3′; β-tubulin forward 5′-CGGACAGTGTGGCAAC-CAGATCGG-3′ and reverse 5′-TGG CCAAAAGGACCTGAGCGAACGG-3′. Data were normalised using RT-PCR of the β-tubulin mRNA as an index of cDNA content after reverse transcription.

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