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Src family kinases are required for integrin but not PDGFR signal transduction

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Src family kinases (SFKs) have been implicated as important regulators of ligand-induced cellular responses including proliferation, survival, adhesion and migration. Analysis of SFK function has been impeded by extensive redundancy between family members. We have generated mouse embryos harboring functional null mutations of the ubiquitously expressed SFKs Src, Yes and Fyn. This triple mutation leads to severe developmental defects and lethality by E9.5. To elucidate the molecular mechanisms underlying this phenotype, SYF cells (deficient for Src, Yes and Fyn) were derived and tested for their ability to respond to growth factors or plating on extracellular matrix. Our studies reveal that while Src, Yes and Fyn are largely dispensable for platelet-derived growth factor (PDGF)-induced signaling, they are absolutely required to mediate specific functions regulated by extracellular matrix proteins. Fibronectin-induced tyrosine phosphorylation of focal adhesion proteins, including the focal adhesion kinase FAK, was nearly eliminated in the absence of Src, Yes and Fyn. Furthermore, consistent with previous reports demonstrating the importance of FAK for cell migration, SYF cells displayed reduced motility *in vitro*. These results demonstrate that SFK activity is essential during embryogenesis and suggest that defects observed in SYF triple mutant embryos may be linked to deficiencies in signaling by extracellular matrix-coupled receptors.

Keywords: cell migration/FAK/integrins/PDGF/Src family kinase

Introduction

The past several years have witnessed an intense effort to identify the biochemical, cellular and physiological role(s) of Src family protein tyrosine kinases (SFKs) (Brown and Cooper, 1996; Lowell and Soriano, 1996; Thomas and Brugge, 1997). Eight murine SFKs have been identified to date. While Hck, Fgr, Blk and Lck, are restricted to hematopoetic cell lineages, Lyn is expressed in these and in neuronal tissues, and Src, Yes and Fyn are expressed ubiquitously (Brown and Cooper, 1996; Lowell and Soriano, 1996; Thomas and Brugge, 1997). Targeted gene deletions of Src or Fyn, individually, pairwise with each other or with Yes, lead to perinatal death or postnatal defects in specific cell types (Soriano *et al*., 1991; Appleby *et al*., 1992; Stein *et al*., 1992, 1994). The frequency of perinatal lethality is increased in mice harboring compound mutations, suggesting redundancy of biological functions of the SFKs in development (Stein *et al*., 1994). A requirement for SFKs in mouse embryonic development, however, has not been demonstrated directly.

Src, Yes and Fyn may promote normal development by executing various cellular functions initiated by activation of cell surface receptors (Brown and Cooper, 1996; Thomas and Brugge, 1997). SFKs have been implicated as essential signaling components in a diverse array of cellular functions, including growth factor-induced proliferation and gene expression, extracellular matrix-promoted adhesion and spreading, migration and protection from apoptosis (Brown and Cooper, 1996; Lowell and Soriano, 1996; Parsons and Parsons, 1997; Thomas and Brugge, 1997). Two particularly well-characterized ligand–receptor systems which appear to require SFKs for optimal function include the platelet-derived growth factor (PDGF)–PDGF receptor (PDGFR) system and the fibronectin (FN)–integrin system. Importantly, targeted deletion of either PDGF ligand (A or B) or PDGFR (α or β) results in embryonic lethality, underscoring the importance of all components of this system for normal mammalian embryogenesis (Leveen *et al*., 1994; Soriano, 1994, 1997; Bostrom *et al*., 1996). Similarly, targeted deletion of FN, and specific subtypes of its receptor components, the α and β integrins, leads to lethality at distinct stages of gestation (Hynes, 1996). If Src, Yes and Fyn are important for signaling from PDGFRs or integrins, they should also be important for embryonic development. However, their importance might be masked in single and double mutant embryos by functional redundancy.

Biochemical evidence linking SFKs to PDGFR function was established initially by demonstrating that stimulation of fibroblasts with PDGF BB promotes PDGFβR association with Src, Fyn and Yes, serine and tyrosine phosphorylation of these kinases, and a 2- to 5-fold increase in SFK catalytic activity (Ralston and Bishop, 1985; Gould and Hunter, 1988; Kypta *et al*., 1990). The interaction is mediated by binding of the SFK SH2 domains to phosphorylated tyrosine residues 579 and 581 found within the juxtamembrane domain of the human PDGFβR (Twamley *et al*., 1992; Mori *et al*., 1993) and the analogous tyrosine residues on the PDGFαR (Gelderloos *et al*., 1998; Hooshmand-Rad *et al*., 1998).

One of the primary functions of SFKs in response to PDGFR activation is thought to be promotion of cell cycle progression. PDGF-induced entry into S-phase is severely diminished by microinjection of fibroblasts with a specific

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interfering antibody, catalytically inactive forms of Src or Fyn, or SH3 domain mutants of Src (Twamley-Stein *et al*., 1993; Roche *et al*., 1995b; Erpel *et al*., 1996). Similar results are achieved by overexpressing catalytically inactive Src or Src SH3 domain mutants in cells lacking endogenous Src (Broome and Hunter, 1996). The microinjection approach has also identified a requirement for Src in PDGF-induced expression of *myc*, an event which occurs prior to, and is a prerequisite for DNA synthesis in response to PDGF (Barone and Courtneidge, 1995). Furthermore, G_2–M transition can be blocked by microinjection of anti-SFK reagents (Roche *et al*., 1995a). These results suggest that SFKs play crucial roles in driving cells through multiple check points of the cell cycle. However, several recent studies have challenged the idea that SFK function is required for PDGF-driven mitogenesis. In contrast to the results described above, PDGFR mutants that selectively fail to bind to and activate SFKs are fully capable of mediating PDGF-triggered DNA synthesis (DeMali and Kazlauskas, 1998; Gelderloos *et al*., 1998; Hooshmand-Rad *et al*., 1998; Sachsenmaier *et al*., 1999) and induction of *myc* expression (DeMali and Kazlauskas, 1998; Gelderloos *et al*., 1998; Sachsenmaier *et al*., 1999). Two of these studies suggest that while SFKs may not be required for entry into S-phase, they are essential for mediating optimal tyrosine phosphorylation of SH2-containing proteins bound to activated PDGFRs (DeMali and Kazlauskas, 1998; Gelderloos *et al*., 1998).

Strong biochemical evidence has implicated SFKs in integrin-mediated signaling at specialized points of cell–matrix contact known as focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996; Parsons, 1996; Hanks and Polte, 1997). SFKs localize to focal adhesions upon activation of integrins by extracellular matrix proteins such as FN, and this event is concomitant with rapid tyrosine phosphorylation of focal adhesion proteins (Burridge and Chrzanowska-Wodnicka, 1996; Parsons, 1996; Hanks and Polte, 1997). Recruitment of SFKs to integrin-based signaling complexes can occur through association with various components of the complex, but is thought to occur primarily via interaction with another tyrosine kinase, focal adhesion kinase (FAK) (Cobb *et al*., 1994; Schlaepfer *et al*., 1994; Schlaepfer and Hunter, 1996). FAK is heavily phosphorylated in response to integrin activation, predominantly on Tyr397 which conforms to the consensus binding sequence for Src SH2 domains (Schaller *et al*., 1994). This residue is the primary site of FAK autophosphorylation *in vitro*, and may be autophosphorylated *in vivo*, although phosphorylation by an exogenous kinase has not been ruled out (Schaller *et al*., 1994; Schlaepfer *et al*., 1994; Calalb *et al*., 1995). Tyrosine to phenylalanine mutation of Y397 not only disrupts association with Src, but dramatically reduces phosphorylation of FAK at multiple tyrosine residues, reducing the ability of FAK to associate with other signaling proteins such as Grb2 (Calalb *et al*., 1995; Schlaepfer and Hunter, 1996). This suggests that SFKs may be required to phosphorylate FAK at secondary tyrosine residues in response to integrin activation. Indeed, FN-induced phosphorylation of the focal adhesion protein $p130^{cas}$ is greatly reduced in cells lacking either Src or Fyn, while phosphorylation of other proteins including FAK is only modestly attenuated (Bockholt and Burridge, 1995; Vuori *et al*., 1996; Schlaepfer *et al*., 1997). Thus it appears that SFKs contribute to the phosphorylation of focal adhesion proteins, but the extent of this contribution has been difficult to determine due to possible redundancy between SFKs and FAK.

To address the requirement for SFKs in PDGF- and FN-induced cellular functions, we derived cell lines from mice which harbor targeted mutations for all these kinases. In this study, we show that embryos deficient for Src, Yes and Fyn die at mid-gestation. Furthermore, cells lacking functional Src, Yes and Fyn (SYF cells) were found to be fully competent to respond to PDGF in assays for substrate phosphorylation, *myc* induction, entry into S-phase, cell proliferation and chemotaxis. In contrast, FN-induced tyrosine phosphorylation of focal adhesion proteins including FAK was reduced dramatically. This deficiency in integrin-mediated signaling may be responsible for the reduced motility displayed by the SYF cells in wound healing and haptotaxis assays, and may provide a molecular mechanism underlying the SYF mutant phenotype.

Results

src$^{-/-}$ *yes*$^{-/-}$ *fyn*$^{-/-}$ (SYF) mutant embryos

Embryos were derived by breeding mice homozygous mutant for *yes* and heterozygous mutant for *src* and *fyn*. In this cross, triple mutant progeny are expected to occur at a frequency of 1 in 16. Since double homozygous mutation of *src* and *fyn* or *src* and *yes* leads to perinatal death, pregnant females initially were sacrificed between E10.5 and E12.5 for embryo harvest. No homozygous SYF embryos were recovered after a brief initial screen of 48 embryos. In contrast, triple homozygous mutant SYF embryos were recovered at the expected frequency (7/118) at E9.5. All triple mutant embryos exhibited severe developmental defects and were dead or dying. The two most severely affected embryos were approximately one-fifth the size of normal embryos, and were undergoing resorption. The other five triple mutant embryos were also severely retarded, and were approximately one-third to one-half the size of normal embryos (Figure 1). These embryos had not ‘turned’, a process which occurs between E8.5 and E9.5 and results in an inversion of the orientation of the germ layers. Other abnormalities were also detected, including a wavy neural tube, blood-filled blebs beneath the epidermis and a tuberous allantois which was not attached to the chorion. Despite these defects, all of the seven embryos recovered had yolk sacs that appeared normal and were well vascularized, and the five larger embryos showed normal beating of the heart. The severity of the SYF triple mutant phenotype at E9.5 is consistent with our inability to recover these embryos at later stages and suggests that lethality occurs between E9 and E10. As these kinases are co-expressed broadly in the embryo, these results indicate that SFKs play a crucial role in early mammalian embryonic development and suggest a high degree of functional redundancy between Src, Yes and Fyn.

Derivation and characterization of SYF triple mutant cells

Since SFKs have been demonstrated as downstream effectors of many cell surface receptor signal transduction pathways (Brown and Cooper, 1996), it is possible that

Fig. 1. Phenotypic abnormalities in SYF triple mutant E9.5 embryos. (**A**) A dorsal view of an SYF E9.5 embryo next to a wild-type littermate. Note the reduced size and the wavy neural tube denoted by the asterisk. (**B**) A lateral view of an E9.5 SYF triple mutant embryo. The arrow marks the presence of a blood-filled bleb.

Fig. 2. SYF cells express the PDGFR but do not display Src, Yes and Fyn kinase activity. Confluent, quiescent SYF cells (SYF), cells expressing endogenous Src (Src^{++}) or SYF cells re-expressing c-Src (c-Src) were left unstimulated or were stimulated with the indicated concentration of PDGF BB. Cell lysates were either subjected immediately to SDS–PAGE or were immunoprecipitated with the cst.1 antibody which recognizes Src, Yes and Fyn (Kypta *et al*., 1990). Lysates were transferred to Immobilon and blotted for (**A**) PDGFβR (30 A, 1:1000), (**B**) RasGAP (70.3, 1:5000) or (**C**) c-Src (327, 1:100). The decrease in levels of PDGFR observed in the ninth lane was due to a decrease in the amount of total protein loaded determined by comparison with the RasGAP blot. (**D**) Cst.1 immunoprecipitates were subjected to a standard *in vitro* kinase assay as described in Materials and methods. (**E**) Western blot analysis of SYF cell lysates, littermate controls and lysates derived from $lyn^{-/-}$ and $lyn^{+/+}$ macrophages for expression of Lyn (anti-Lyn N-terminal domain 1:1000). The arrows denote p53 and p56 variants of Lyn.

the defects displayed by the triple mutant embryos are caused, at least in part, by diminished or inappropriate signaling in the absence of the SFKs. To address this possibility, cell lines were generated from two E9.5 mutant embryos and from control embryos. As it is not possible to isolate spontaneously immortalized cell lines at this stage, due to the small size of the embryo and the inevitable loss of cells during multiple passages, we immortalized primary cells by SV40 large T antigen. Immortalized populations of cells were plated at low density and clones were isolated and screened for expression of the PDGFRs by Western blot (Figure 2A, and data not shown). Clonal cell lines expressing comparable levels of both PDGFRs were used for our analysis. To control for clonal differences in response to various stimuli, c-Src was reintroduced into one of the SYF triple mutant cell lines via the retroviral vector pLXSH.

To measure Src expression levels and to determine whether the triple mutant SYF cells contained any SFK activity, cell lysates were tested by Western blot for Src or were immunoprecipitated with an antibody, cst.1, which recognizes Src, Yes and Fyn (Kypta *et al*., 1990). These immunoprecipitates were subjected to *in vitro* kinase assay in the presence of [^{32}P]ATP and the SFK substrate enolase. As expected, Src was detected in control but not SYF mutant lysates by Western blot (Figure 2C). Consistent with these results, cells expressing Src displayed kinase activity towards both Src and enolase. Typically, PDGF induced 2-fold activation of kinase activity in cells expressing endogenous Src when unstimulated controls were compared with cells stimulated with 30 ng/ml PDGF. PDGF-induced activation of kinase activity in the cells re-expressing c-Src was generally lower, probably due to the high level of basal Src activity in these cells. The level of Src expression upon reintroduction to the SYF cells was ~5- to 10-fold that of endogenous Src expressed in the littermate control as determined by both Western blot and kinase assay of cst.1 immunoprecipitates. Importantly, cst.1 immunoprecipitates from the SYF cells did not display any detectable kinase activity, confirming that this cell line is functionally null for Src, Yes and Fyn (Figure 2D, and data not shown).

Although Src, Yes and Fyn are believed to be the only SFKs expressed in fibroblasts, Lyn is also expressed in the early embryo (Nada *et al*., 1993). We therefore checked control and SYF cells for Lyn expression by Western blot but failed to detect immunoreactivity (Figure 2E). As a further precaution against the potential activation of any other expressed SFKs, we overexpressed a kinase-dead (K297R) version of Src in SYF cells. Kinase-dead Src (KD Src) was overexpressed ~5- to 10-fold over levels of endogenous Src, Yes and Fyn from a wild-type control cell lysate (Figure 3A) and, as expected, did not display

catalytic activity in an *in vitro* kinase assay against enolase (Figure 3D). Co-immunoprecipitation of KD Src and the PDGFR was readily detectable upon cst.1 immunoprecipitation and PDGFR Western blot (Figure 3B), suggesting that the KD Src would interfere with the activation of any endogenous SFKs that might be ectopically expressed (Twamley-Stein *et al*., 1993; Roche *et al*., 1995a; Broome and Hunter, 1996).

Cellular responses to PDGF

To determine whether SFK function is required for PDGF-induced DNA synthesis, both SYF cell lines and Src-expressing controls were assayed for DNA synthesis in response to stimulation with PDGF BB. No differences were observed in the ability of the SYF cells to engage in DNA synthesis in response to various concentrations of PDGF BB or serum compared with Src-expressing controls (Figure 4A, and data not shown). Importantly, no defect was observed in the SYF cells which also express KD Src, arguing against mitogenic signal relay via SFKs other than Src, Yes or Fyn (Figure 4A). Consistent with the ability of SYF cells to enter S-phase, *myc* RNA was induced in response to PDGF BB (Figure 4B). Therefore, at least in these cells, SFKs are not required throughout the G_1 phase to mediate a mitogenic response to PDGF.

In some proliferation assays, a modest decrease was observed in the ability of SYF cells, and SYF cells expressing KD Src, to respond to serum (Figure 4A, and data not shown). This suggests that lack of SFK function may reduce mitogenic signaling downstream of other growth factors present in serum. Since lysophosphatidic acid (LPA) is thought to act as the predominant mitogen in serum, we tested the ability of these cells and Src-expressing controls to respond to LPA. Again, no difference was observable in this comparison (data not shown).

Src, Yes and Fyn have also been implicated as playing an important role in mitotic division at the G_2–M transition (Chackalaparampil and Shalloway, 1988; Roche *et al*., 1995a). To investigate whether loss of SFK function at late stages of the cell cycle affects PDGF-driven cell proliferation, cells were cultured in the presence or absence of 30 ng/ml PDGF BB and growth curves were determined. Compared with the SYF cells, cells overexpressing c-Src displayed increased proliferation in the absence of PDGF. However, both the SYF cells and Src-expressing controls displayed increased proliferation in the presence of PDGF (Figure 4C). Our observation that loss of Src, Yes and Fyn does not inhibit PDGF-triggered *myc* expression, DNA synthesis or cell proliferation strongly suggests that SFK function is dispensable throughout the cell cycle for these growth factor-initiated processes.

PDGF-dependent tyrosine phosphorylation and MAP kinase activation

While SFK function may not be required for a PDGF-induced proliferative signal, two recent studies suggest that SFKs contribute to the tyrosine phosphorylation of PDGFR-associated proteins including Shc, RasGAP and SHP-2 (DeMali and Kazlauskas, 1998; Gelderloos *et al*., 1998). To test this role for SFKs, immunoprecipitates of PDGFR-associated proteins from SYF cells were analyzed for phosphotyrosine content following PDGF stimulation. The proteins examined included the PDGFβR, Shc, Ras-

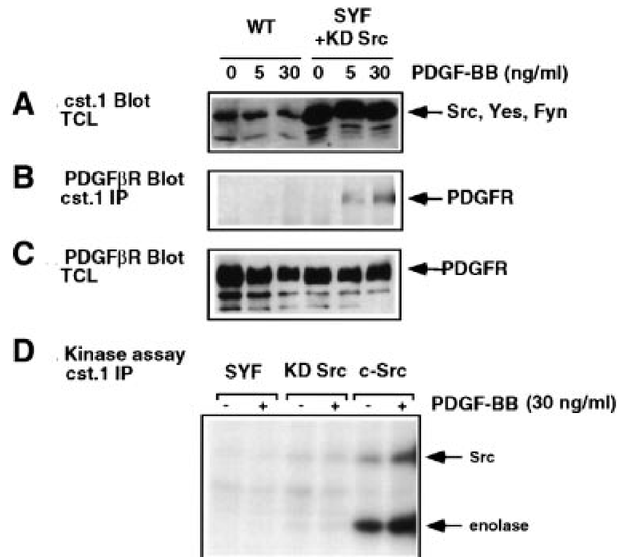

Fig. 3. Kinase-dead Src expression in SYF cells. (A–C) Expression of kinase-dead (K297R) Src (KD Src) in SYF cells and association of KD Src with the PDGFR in response to PDGF. SYF cells expressing KD Src under control of a retroviral LTR, or wild-type controls, were left resting or were stimulated with the indicated concentration of PDGF BB. Cells were lysed and either (**A**) subjected immediately to SDS–PAGE, transferred to Immobilon and probed with cst.1 to compare SFK expression by Western blot or (**B**) immunoprecipitated with cst.1 and blotted with an antibody against the PDGFR (30 A, 1:1000) to detect association with the PDGFR. The level of KD Src expression is 5- to 10-fold greater than that of endogenous Src, Yes and Fyn in wild-type cells. Association of KD Src with the PDGFR is increased relative to the wild-type control, suggesting that it is effective as a dominant-negative molecule. (**C**) Western blot for the PDGFR was also performed to demonstrate that equal levels of receptor were present in the lysates immunoprecipitated with cst.1. (**D**) Kinase assay of cst.1 immunoprecipitates of lysates from SYF cells, or SYF mutants expressing KD Src or c-Src.

GAP, SHP-2 and PLCγ. None of the PDGFR-associated proteins displayed reduced phosphotyrosine content in the absence of Src, Yes and Fyn (Figure 5A). This suggests that the activated PDGFR does not require the SFKs to mediate tyrosine phosphorylation of these substrates.

SFK activity has also been reported to modulate growth factor-induced activation of the Ras-MAP kinase (MAPK) cascade in a variety of systems (Brown and Cooper, 1996; Thomas and Brugge, 1997). Furthermore, it has been shown recently that B cells derived from *lyn*$^{-/-}$ mice exhibit an enhanced MAPK response to BCR engagement (Chan *et al*., 1997). To determine whether a similar effect occurs in the absence of Src, Yes and Fyn, activation of MAPK was assessed by gel shift assay of Erk 1 and Erk 2 following stimulation with PDGF BB. Activation of Erk 1 and Erk 2 was observed in both SYF and Src-expressing cells; however, the profiles of activation consistently differed (Figure 5B). As expected, Erk activation increased upon addition of increasing concentrations of PDGF to cells expressing endogenous Src or to SYF cells re-expressing c-Src. Erk activation in SYF cells, however, was greatest upon exposure to low concentrations of PDGF and decreased upon addition of increasing concentrations of PDGF. This result shows that although SFKs do not appear to be required to execute most PDGFR-mediated events, they do modify certain signaling responses following PDGF stimulation.

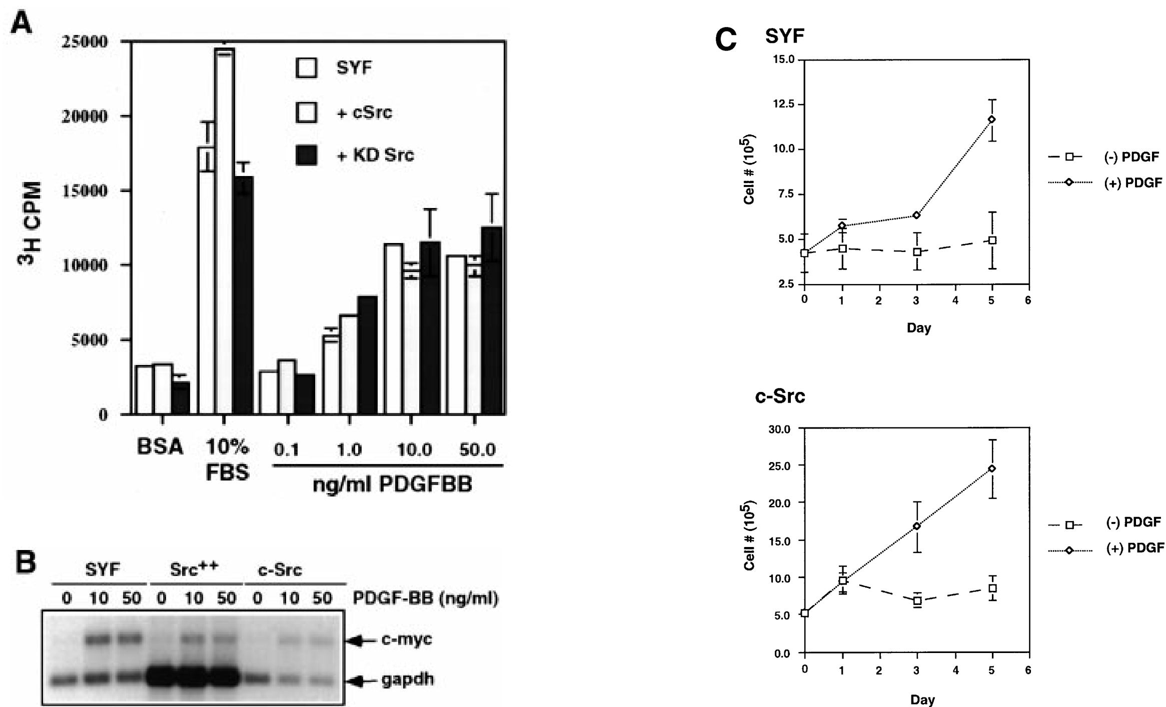

Fig. 4. PDGF-induced proliferation in SYF cells and Src-expressing controls. (**A**) DNA synthesis. SYF cells or SYF cells expressing wild-type c-Src or KD Src were starved to quiescence in DMEM + 0.1% FCS. Buffer, 10% FCS or the indicated concentration of PDGF was added and, after 18 h, cells were pulse labeled with [^{3}H]thymidine and harvested. Data are presented as c.p.m. of incorporated [^{3}H]thymidine. Each experiment was performed four times and the graph shown is representative of these experiments. Each data point was performed in triplicate and the data are the means ± SD. For several data points, the standard deviation was too small to be denoted by error bars. (**B**) Myc induction. SYF cells (SYF), cells expressing endogenous Src (Src^{++}) or SYF cells re-expressing c-Src (c-Src) were rendered quiescent and were either left resting or were stimulated with the indicated concentrations of PDGFBB. RNA was isolated, blotted and probed with a probe specific for *c-myc* or *gapdh* as a control for RNA levels. This assay was repeated three times. (**C**) Cell proliferation. SYF cells or cells re-expressing c-Src were plated in triplicate at 8×10^4 cells per well. Cells were grown in DMEM + 0.1% FCS supplemented with ITS in the presence (+) or absence (–) of 30 ng/ml PDGF BB. Cells were counted on every odd day following addition of PDGF BB. The data are means of triplicate samples ± SD. The graph shown is a representative sample of two assays.

Cell migration

Multiple studies have implicated a role for SFKs in promotion of cell migration (Rodier *et al*., 1995; Cary *et al*., 1996; Boyer *et al*., 1997; LaVallee *et al*., 1998). To determine whether defects in migration may contribute to the *src*, *yes*, *fyn* triple mutant phenotype, SYF cells and Src-expressing controls were first tested for the ability to migrate into a wound introduced by scratching a confluent monolayer of cells. Consistent with a previous observation that fibroblasts derived from *src*$^{-/-}$ mice display a significantly reduced rate of random locomotion (Hall *et al*., 1996), time lapse video showed that SYF cells display an impaired ability to migrate into the wound as compared with Src-expressing controls (Figure 6A). Also, consistent with previous reports that many of the defects observed in Src-deficient cells are restored upon re-expression of KD Src (Kaplan *et al*., 1995; Schwartzberg *et al*., 1997), expression of KD Src in SYF cells partially rescued the ability to fill in the wound (data not shown). Interestingly, the SYF cells appeared to form extensions into the cleared area of the tissue culture plate, but were unable to utilize these extensions for propulsion (Figure 6A, arrows).

Activation of multiple cell surface receptors including the PDGFRs and the integrins has been shown to promote signaling events leading to the cytoskeletal modifications required for cell migration (Lauffenburger and Horwitz, 1996). To define why SYF cells fail to migrate in the wound healing assay, we compared these cells with Src-expressing controls in assays for migration toward PDGF or toward an integrin receptor ligand, FN. While no difference was observed in chemotactic migration toward PDGF (Figure 6B), the SYF cells exhibited reduced migration in the haptotaxis assay toward FN (Figure 6C), suggesting that the reduction in motility may be due to a defect in integrin signaling.

FN-induced tyrosine phosphorylation

Initial events following the activation of integrins by extracellular matrix ligands include recruitment and phosphorylation of proteins such as the FAK, tensin, paxillin, p130^{cas}, Shc and the SFKs (Parsons, 1996). These initial signaling events may play an important role in mediating cytoskeletal rearrangements required for cell motility (Lauffenburger and Horwitz, 1996). To determine the contribution of SFKs to tyrosine phosphorylation of focal adhesion proteins, SYF cells or Src-expressing controls were either left in suspension, grown on tissue culture plates or were plated for the indicated period of time on either FN or poly-L-lysine. Lysates from these samples were analyzed by Western blotting with the anti-phosphotyrosine antibody 4G10. Plating of Src-expressing cells on FN induced tyrosine phosphorylation of proteins which migrate as expected for several focal adhesion proteins including p130^{cas}, FAK, paxillin and Shc (Figure 7A). Overexpression of c-Src in the SYF cells also resulted in constitutive phosphorylation of many of these proteins.

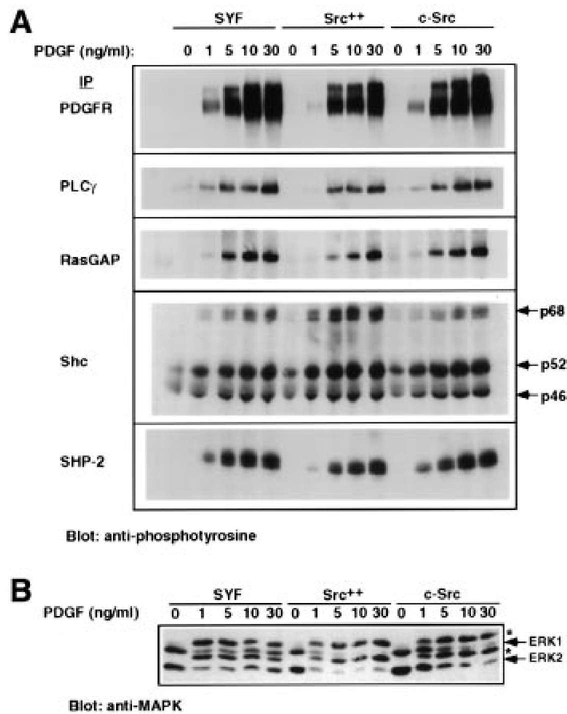

Fig. 5. PDGF-stimulated tyrosine phosphorylation and activation of MAP kinases Erk 1 and Erk 2. SYF cells (SYF), cells expressing endogenous Src (Src^{++}) or SYF cells re-expressing c-Src (c-Src) were rendered quiescent and were either left resting or were stimulated with the indicated concentrations of PDGF BB. (**A**) PDGF-dependent tyrosine phosphorylation. Cells were lysed and were immunoprecipitated with antibodies against the PDGFβR, phospholipase Cγ, RasGAP, Shc or SHP-2. Immunoprecipitates were resolved by SDS–PAGE, transferred to Immobilon and assayed for phosphotyrosine content by Western blotting (4G10, UBI, 1:10 000). All blots were stripped and probed with antibodies against the immunoprecipitated protein to ensure that equal levels had been added to each lane (data not shown). (**B**) Activation of MAP kinases Erk 1 and Erk 2. Cell lysates were resolved by SDS–PAGE, and then subjected to Western blotting with an antibody which recognizes both Erk 1 and Erk 2 (Zymed, 1 μg/ml). The arrows point to the unphosphorylated, rapidly migrating Erk 1 and 2 isoforms. The asterisks denote the phosphorylated, activated forms of Erk 1 and 2. This experiment was repeated at least three times.

In contrast, the SYF cells showed little FN-induced tyrosine phosphorylation, indicating that the SFKs play a prominent role in mediating the phosphorylation events downstream of integrin activation (Figure 7A and B).

This observation raised the possibility that SFKs act upstream of FAK tyrosine phosphorylation and activation. To address specifically the involvement of the SFKs in FN-induced phosphorylation of FAK, cell lysates were immunoprecipitated with anti-FAK antibodies, and then analyzed for phosphotyrosine content by Western blot. Consistent with a role for SFKs upstream of FAK in integrin signaling, tyrosine phosphorylation of FAK in SYF cells was dramatically reduced compared with Src-expressing controls (Figure 7C and D). Similar results were observed with the second Src, Yes and Fyn-deficient cell line, SYF2 (Figure 7E and F). Furthermore, the phosphotyrosine content of two other focal adhesion proteins, $p130^{cas}$ (Figure 7G and H) and paxillin (Figure 7I and J), was severely reduced in both SYF cell lines as compared with Src-expressing controls. These observations indicate that SFKs are required to initiate the majority of integrin-mediated tyrosine phosphorylation in response to adherence to FN.

Focal adhesion formation

Transient modifications of the actin–myosin cytoskeleton involved in cell migration include extension of the leading edge of the cell in the form of lamellipodia and/or filipodia, and attachment of these extensions to underlying matrix proteins via formation of focal adhesions (Lauffenburger and Horwitz, 1996). Since tyrosine phosphorylation of focal adhesion proteins was severely diminished in the SYF cells, and previous studies have shown that focal adhesion formation can be blocked by tyrosine kinase inhibitors (Burridge *et al*., 1992; Romer *et al*., 1992; Ridley and Hall, 1994), we investigated whether a defect in focal adhesion formation may underlie the decreased motility displayed by the SYF cells. To examine focal adhesion formation, cells were plated onto FN-coated coverslips, fixed after 2 h and stained for vinculin, an abundant component of focal adhesions. Cells were also counterstained with phalloidin to visualize the architecture of the actin cytoskeleton. Staining for vinculin (green) demonstrated that both SYF cells and Src-expressing controls formed focal contacts which displayed a classic arrowhead-like appearance and were present in approximately equal numbers (Figure 8). Furthermore, no consistent differences in the actin cytoskeleton were observed (Figure 8). This indicates that focal adhesions form in the absence of tyrosine phosphorylation of FAK, and the motility defect displayed by the SYF cells is not explained by a failure to form contacts. These results are in line with reports that tyrosine phosphorylation plays a greater role in focal adhesion disassembly rather than assembly during cell migration (Crowley and Horwitz, 1995; Ilic *et al*., 1995; Cary *et al*., 1996).

Discussion

We have examined the requirement for ubiquitously expressed SFKs, Src, Yes and Fyn, in mammalian embryogenesis, PDGFR signal transduction, cell motility, FN-induced tyrosine phosphorylation and focal adhesion formation. Our results indicate that Src, Yes and Fyn are essential for early embryonic development as the absence of these kinases leads to severe developmental abnormalities and lethality by E9.5. In cell culture, SFKs are not required for PDGF-induced tyrosine phosphorylation, chemotaxis and cell cycle progression. In contrast, deletion of the SFKs results in reduced cell motility in wound healing and haptotaxis assays which may stem from a defect in linking integrin activation to tyrosine phosphorylation of FAK and subsequent downstream signaling proteins. However, integrin-mediated tyrosine phosphorylation by SFKs is not required for focal adhesion formation.

SFKs are believed to regulate a vast array of cellular functions including cell cycle progression, adhesion and spreading, focal adhesion formation and disassembly, migration, apoptosis, differentiation and gene transcription (Lowell and Soriano, 1996; Thomas and Brugge, 1997). However, initial attempts to examine the function of SFKs by targeted mutation of each individual family member, while fruitful in defining postnatal and perinatal function, previously had not shown a role for SFKs in embryogenesis

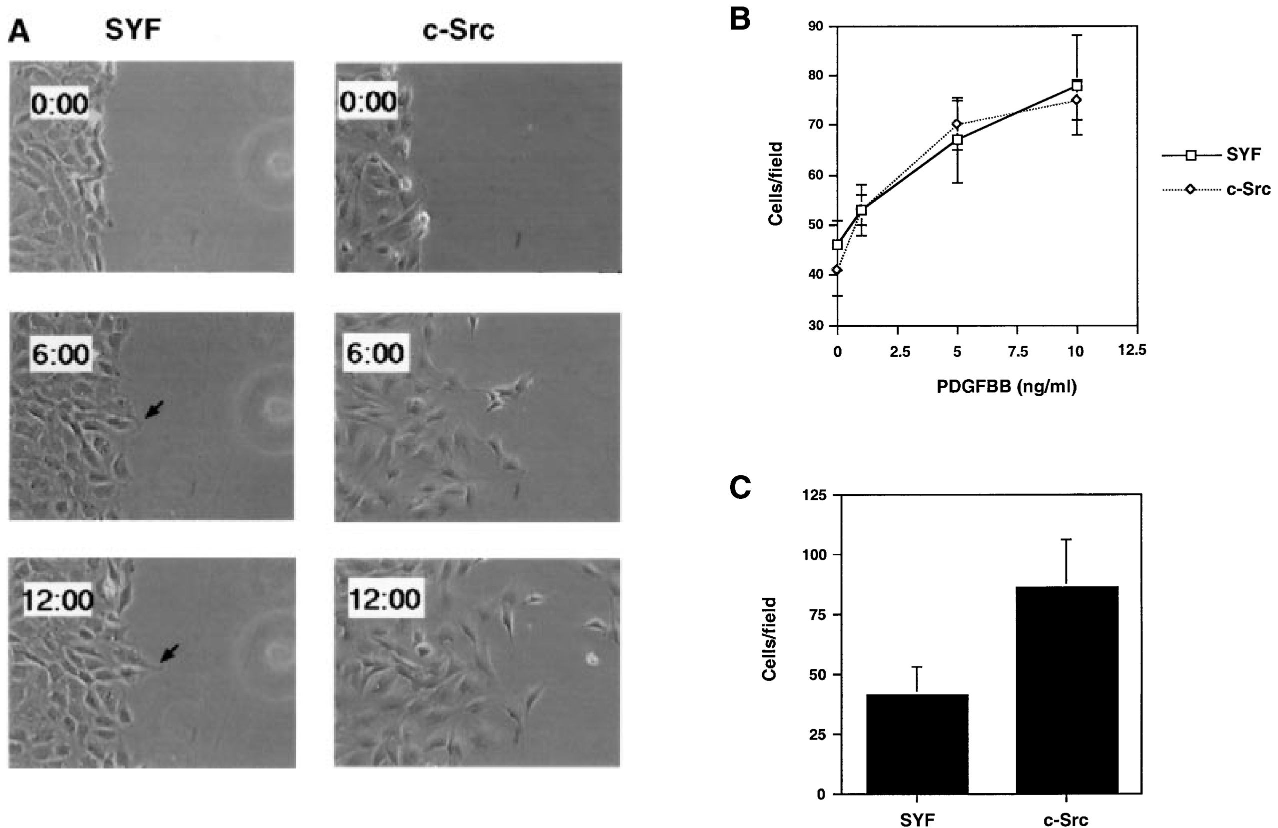

Fig. 6. SYF cells display reduced motility in wound healing and haptotaxis assays, but not in PDGF-induced chemotaxis. (**A**) Wound healing. SYF cells (left column) and cells re-expressing c-Src (right column) were grown to confluence and 'wounded' by introducing a scratch across the plate with a pipet tip. Cell migration was followed by video recording and still images were captured at the denoted times. The arrow indicates the presence of cellular extensions which may represent lamellipodia. This assay was repeated with similar results more than five times. (**B**) Chemotaxis. SYF cells or SYF cells re-expressing c-Src were added to migration chambers and the number of cells migrating towards PDGF at the indicated concentrations was determined after 3 h as described in Materials and methods. (**C**) Haptotaxis. SYF cells or SYF cells re-expressing c-Src were added to migration chambers and the number of cells migrating to immobilized FN (5 µg/ml) was determined after 3 h as described in Materials and methods. Cell migration to 0.5% BSA controls was <0.1%. Each data point in (B) and (C) was performed in triplicate and the data are the means ± SD from three individual experiments.

(Soriano *et al*., 1991; Stein *et al*., 1992, 1994). This is probably due to functional overlap between the SFKs during development (Lowell and Soriano, 1996). Consistent with this possibility, double SFK mutants exhibit more severe postnatal phenotypes and increased perinatal lethality compared with their single mutant counterparts (Stein *et al*., 1994). Furthermore, targeted deletion of Csk, a negative regulator of SFK catalytic activity, leads to embryonic lethality by E9.5 (Imamoto and Soriano, 1993). Our observation that embryos triple mutant for Src, Yes and Fyn exhibit a complex lethal phenotype by E9.5 indicates that SFKs play essential but functionally redundant roles during early development.

Previous biochemical evidence has suggested that SFKs are essential components of PDGFR signaling (Twamley-Stein *et al*., 1993; Roche *et al*., 1995b; Broome and Hunter, 1996; Erpel *et al*., 1996; DeMali and Kazlauskas, 1998). Since targeted deletion of any component of the PDGF–PDGFR system leads to perinatal or embryonic lethality (Leveen *et al*., 1994; Soriano, 1994, 1997; Bostrom *et al*., 1996), we tested whether an impaired cellular response to PDGF underlies the phenotype of SYF mutant embryos. In assays for PDGF-dependent DNA synthesis, induction of *myc* expression, chemotaxis and tyrosine phosphorylation of cellular proteins, no differences were observed between SYF cells and Src-expressing controls. Also, no differences in DNA synthesis were apparent following stimulation with LPA. Furthermore, while basal levels of proliferation were reduced in SYF cells, they were able to proliferate in the presence of PDGF. This suggests that while SFKs may promote cell cycle progression, they are not required for this function in response to growth factor stimulation. These results are consistent with the interpretation that SFK activation is dispensable, at least in some cell types, for PDGF-driven mitogenesis and that the SYF triple mutant phenotype is not likely to be caused by abnormal PDGFR signaling.

These findings contrast with previous reports which demonstrate that proliferative signals downstream of several growth factor receptor tyrosine kinases can be blocked by microinjection of dominant inhibitory reagents, or by overexpression of dominant-negative mutant forms of Src. Several recent reports, however, have shown that PDGFR mutants which do not activate SFKs are fully capable of mediating PDGF-induced *myc* expression and DNA synthesis (DeMali and Kazlauskas, 1998; Gelderloos *et al*., 1998; Hooshmand-Rad *et al*., 1998; Sachsenmaier *et al*., 1999). The concern with this approach is that while the point mutated receptors do not induce the 'initial

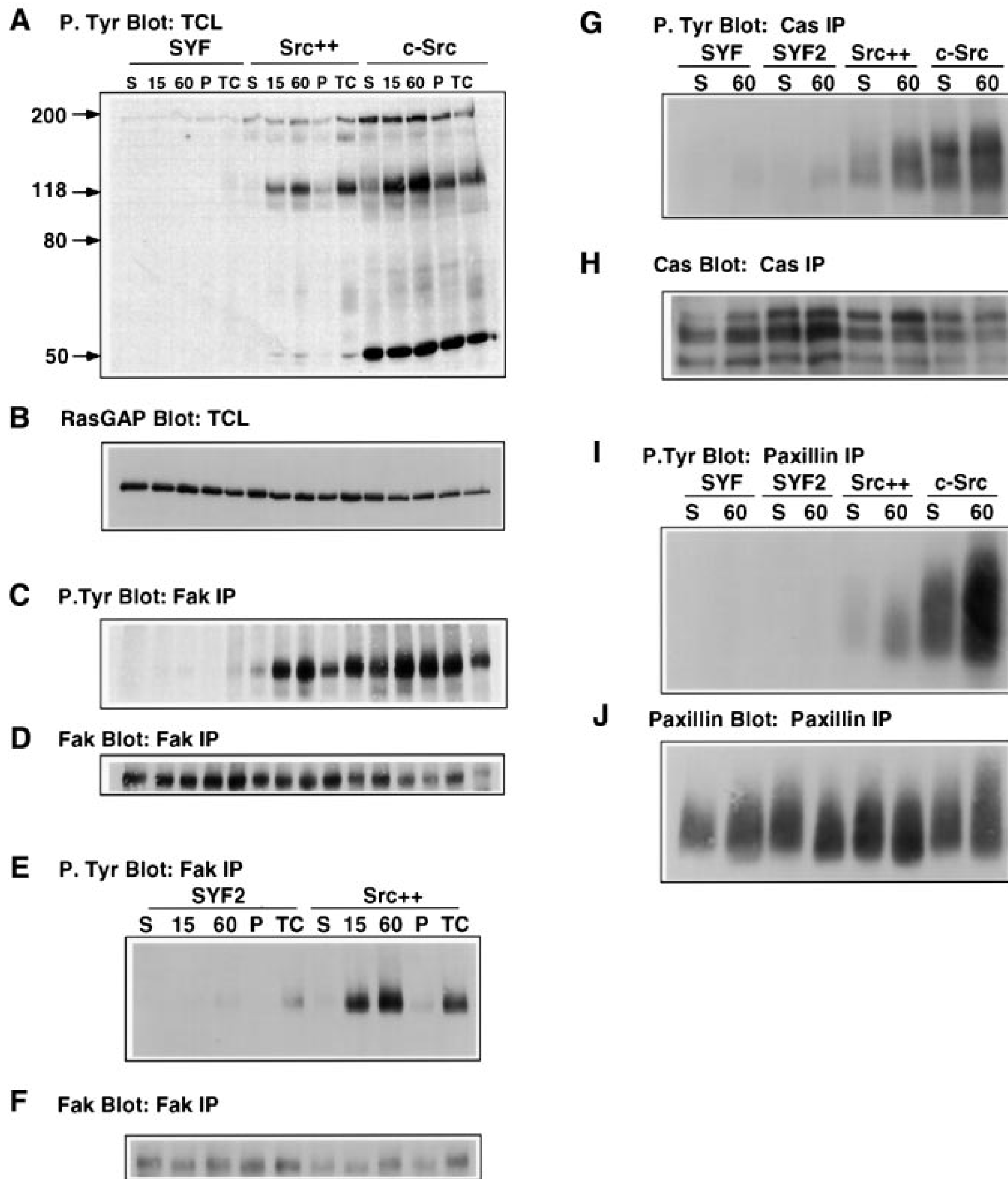

Fig. 7. FN-induced tyrosine phosphorylation. SYF cells, cells expressing endogenous Src and cells re-expressing c-Src were left in suspension (S) or were plated on 5 μg/ml FN for either 15 (15) or 60 min (60), on poly-L-lysine for 60 min (P), or were harvested as growing cultures on tissue culture plates (TC). Cells were lysed in EB^{++}, protein levels were standardized by colorimetric assay, and lysates representing equal levels of protein either were resolved immediately by SDS–PAGE and subjected to Western blot analysis for (**A**) phosphotyrosine (4G10, 1:10 000) or (**B**) RasGAP (70.3, 1:5000) as a control for equal loading of protein, or were immunoprecipitated with an anti-FAK rabbit polyclonal antibody and subjected to Western blot analysis for (**C**) phosphotyrosine or (**D**) FAK (affinity-purified 5592, 1:500). The decrease in phosphotyrosine content of the c-Src sample derived from cells grown on tissue culture plates (TC), relative to that of the sample plated on poly-L-lysine (P) in (C), is likely to be due to a decrease in the amount of precipitated FAK in the TC sample and was not observed routinely. The experiments represented in (C) and (D) were repeated using a second SYF cell line, SYF2, and are shown for (**E**) phosphotyrosine and (**F**) FAK. Similar experiments were performed to examine FN-induced tyrosine phosphorylation of $p130^{cas}$, immunoprecipitated with a mixed polyclonal antisera and subjected to Western blot analysis for (**G**) phosphotyrosine and (**H**) $p130^{cas}$ (antisera B+F, 1:250), and of paxillin, immunoprecipitated with a purified monoclonal antibody and subjected to Western blot analysis for (**I**) phosphotyrosine and (**J**) paxillin (anti-paxillin, 1:1000, Zymed). These experiments were repeated three times each.

burst' of SFK activity, which occurs within minutes of PDGFR activation, late SFK activation may still occur and execute a mitogenic signal. An advantage to the approach presented here is that SFK function is eliminated throughout the entire cell cycle without the addition of exogenous reagents to the cellular environment. However, PDGFRs are known to activate many signaling events, each of which individually can lead to mitogenesis (Valius and Kazlauskas, 1993). Therefore, it is possible that a requirement for SFKs is masked in SYF cells by redundancy with, or up-regulation of other PDGFR-activated signaling molecules such as phosphatidyl inositol 3-kinase

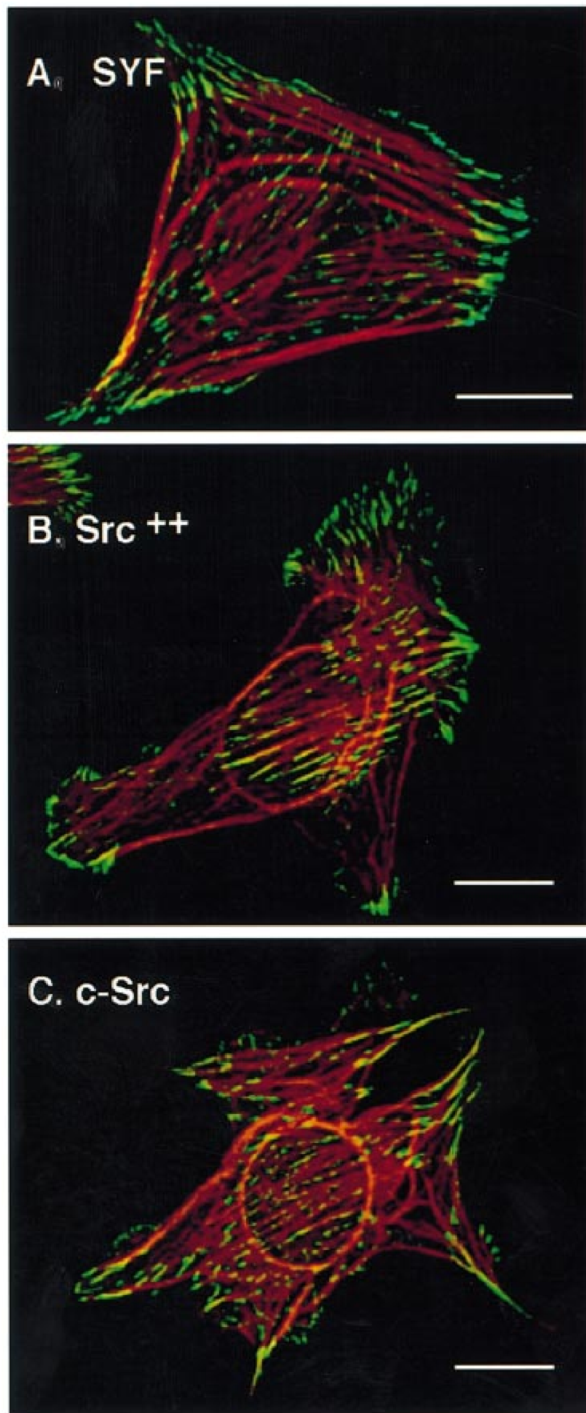

Fig. 8. Focal adhesion formation and cytoskeletal architecture of SYF cells and Src-expressing controls. SYF cells (**A**), cells expressing endogenous Src (**B**) or SYF cells re-expressing c-Src (**C**) were plated onto FN-coated coverslips and incubated at 37°C for 2 h prior to being fixed, permeabilized and stained with anti-vinculin (green) and phalloidin (red) as described in Materials and methods.

(PI3 kinase) or phospholipase Cγ (PLCγ), which mediate mitogenesis. Experiments designed to identify such pathways currently are in process.

Our results prompt the question of the function of the interaction between SFKs and the PDGFR. One possibility is that SFKs are necessary for precise regulation of the Ras/MAPK cascade in response to PDGF stimulation. In contrast to Src-expressing controls which exhibit increased MAPK activation upon exposure to increasing concentrations of PDGF, SYF cells activate MAPK best when exposed to low concentrations of PDGF. The exact role for SFKs in the regulation of the Ras/MAPK cascade has not been elucidated but is particularly intriguing considering the impact that the degree of activation of MAPK has on determining the specificity of the cellular response to growth factors (Traverse *et al*., 1994).

While Src, Yes and Fyn appear to play a modest role in PDGFR signaling, our results indicate that the function of these kinases is of considerable importance to integrin-mediated signaling. Previous studies have demonstrated that adhesion of cells to fibronectin activates integrins, primarily α5β1 dimers, leading to the recruitment and activation of the tyrosine kinase FAK as well as the SFKs (Hanks and Polte, 1997; Parsons and Parsons, 1997). The interaction between FAK and the SFKs upon translocation to the integrins is complex. In a current model, autophosphorylation of FAK at Tyr397 increases FAK kinase activity and creates a high-affinity binding site for the SFKs (Schaller *et al*., 1994). Occupancy of the Src SH2 domain is thought to result in activation of Src, suggesting a role for FAK upstream of Src in FN-induced signal transduction. Formation of this complex may promote further tyrosine phosphorylation of FAK on sites within its catalytic domain, which further enhances FAK catalytic activity, and on sites within regulatory domains which provide additional docking sites for multiple SH2 domain-containing proteins (Schlaepfer *et al*., 1994; Calalb *et al*., 1995). Subsequently, many of these proteins, including paxillin, $p130^{cas}$, Shc and tensin, act as substrates of the FAK–SFK complex (Hanks and Polte, 1997; Parsons and Parsons, 1997). The tight association between SFKs and FAK following integrin activation has precluded elucidation of the precise kinase–substrate relationship within this tyrosine kinase complex, and between the individual components of this complex and downstream substrates. Analyses of cells harboring targeted mutations of either FAK or individual SFKs have shed some light on the division of labor between these kinases. Notably, tyrosine phosphorylation of $p130^{cas}$ is thought to be mediated by Src as this event is inhibited in $Src^{-/-}$ cells (Bockholt and Burridge, 1995; Vuori *et al*., 1996). Furthermore, phosphorylation of FAK is modestly reduced but not eliminated in $Src^{-/-}$ fibroblasts (Schlaepfer *et al*., 1997). Analysis of $FAK^{-/-}$ cells revealed an unexpected increase in phosphotyrosine content of the majority of proteins in total cell lysates compared with wild-type controls (Ilic *et al*., 1995). Whether FAK can mediate the phosphorylation in the absence of Src, Yes and Fyn had not been addressed *in vivo*. Here we show that FN-stimulated tyrosine phosphorylation of multiple proteins is nearly eliminated in SYF cells. Furthermore, the dramatically reduced phosphotyrosine content of FAK immunoprecipitated from SYF cells strongly suggests that phosphorylation of FAK occurs downstream of SFK activation in response to integrin engagement by FN. Therefore, our results indicate that SFKs provide a critical link between integrin activation and the multiple signaling events which emanate from tyrosine-phosphorylated FAK.

The most prominent defect displayed by the SYF cells was a reduction in motility. Based on a previous report

that FAK$^{-/-}$ cells exhibit decreased motility (Ilic *et al*., 1995), and our observation that FAK phosphorylation is diminished in SYF cells, one likely cause for the decreased motility is an inability to phosphorylate FAK. Consistent with this hypothesis, a recent study demonstrated that overexpression of a tyrosine phosphorylation mutant of FAK (Y397F) in CHO cells eliminates FAK phosphorylation and abolishes migration on FN (Cary *et al*., 1996). Overexpression of this mutant did not affect cell adhesion or spreading. Interestingly, this study also showed that a kinase-inactive FAK undergoes tyrosine phosphorylation at Y397, associates with Src and Fyn, and permits migration of cells on FN. Taken together with the data presented here, this suggests that, following tyrosine phosphorylation by SFKs, the primary function of FAK is to act as an adaptor protein which recruits SH2 domain-containing proteins to integrin-based complexes, priming the cell for migration. Reduced tyrosine phosphorylation of other focal adhesion proteins including p130^{cas} and the filamentous actin cross-linking protein cortactin, shown to act as both regulators of cell migration and substrates for Src, may also contribute to the reduced motility of SYF cells (Huang *et al*., 1998; Klemke *et al*., 1998).

While the SYF cells exhibited reduced motility in both wound healing and haptotaxis assays toward FN, chemotaxis toward PDGF was not noticeably affected over the range of concentrations examined. This observation suggests that the PDGFR can circumvent the need for SFK function via activation of alternative signaling molecules capable of modulating rearrangement of the actin–myosin cytoskeleton required for cell movement. Consistent with this possibility, immediate signaling events including PDGF-induced tyrosine phosphorylation of a variety of substrates appear intact in SYF cells. Likely downstream candidates which may promote PDGF-driven migration in the absence of SFKs include PI3 kinase and PLCγ, both of which previously have been shown to play a role in chemotaxis toward PDGF (Kundra *et al*., 1994). Also, it has been reported recently that activation of the Ras/MAPK cascade directly modulates the cellular machinery involved in migration via phosphorylation of myosin light chain kinase, a key regulator of cell motility and contraction (Klemke *et al*., 1997). Although PDGF-induced MAPK activation is modified in SYF cells, it still occurs and, therefore, may help promote PDGF-driven cell migration in an SFK-independent manner. Importantly, our results and the data discussed above suggest that the immediate signaling events initiated by growth factor receptors which culminate in cell movement may be SFK independent and thus distinct from those that are initiated by integrin receptors which require SFK function.

The reduced SYF cell motility suggests that the defects in embryogenesis observed in embryos lacking Src, Yes and Fyn may be due to defective cell migration. Intriguingly, the SYF triple mutant phenotype displays a striking resemblance to that of FAK$^{-/-}$ and FN$^{-/-}$ embryos which also are not turned, have a tuberous allantois, die between E8.5 and E10.5, and exhibit fibroblast abnormalities such as decreased mobility (George *et al*., 1993; Ilic *et al*., 1995). Further experiments currently are underway to define the precise mechanism underlying the SYF triple mutant phenotype.

Materials and methods

Derivation of Src, Yes and Fyn mutant embryos and cell lines

Targeted mutation of Src, Yes and Fyn by homologous recombination in embryonic stem cells, derivation of Src$^{-/+}$, Yes$^{-/-}$, Fyn$^{-/+}$ compound mutant mice and PCR genotyping of yolk sac DNA for Src and Fyn have been described previously (Soriano *et al*., 1991; Stein *et al*., 1992, 1994; Thomas *et al*., 1995). All mice used in this study were hybrid C57BL6J/129Sv. To derive cell lines, E9.5 embryos were dissected in phosphate-buffered saline (PBS), incubated for 2 min in 25% trypsin and 1 mM EDTA at 37°C, and cells were plated on 15 mm gelatinized wells of a 24-well dish containing 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Hyclone) and 2 mM L-glutamine (Gibco-BRL). Cells were immortalized before passage 2 by infection with a retroviral vector transducing the SV40 large T antigen (Brown *et al*., 1986) and maintained in DMEM + 10% FCS by passage at a 1:20 dilution every 3 days. Murine c-Src and K297R KD Src were expressed in the SYF cells via the retroviral vector pLXSH using a standard protocol as described (Miller and Rosman, 1989) and selected in 400 μg/ml hygromycin B (Calbiochem). Expression of Src was tested routinely by Western blot analysis.

It has been reported previously that cell lines harboring the *yes* mutation still generate a catalytically inactive protein that contains the N-terminal half of Yes (Thomas *et al*., 1995), although this is not observed in all tissues in the animal (Stein *et al*., 1994; Lowell and Soriano, 1996). To determine whether the SYF cells expressed this truncated form of Yes, total cell lysates and immunoprecipitates of an antibody which recognizes the unique domain of Yes, Yes 6, were analyzed by Western blot using the mouse monoclonal anti-Yes antibody 2-17. By this analysis, we were unable to detect expression of the truncated Yes protein in either the SYF cells or Src-expressing controls (data not shown).

The cell lines described in this work have been submitted to the American Type Culture Collection (ATCC).

Antibodies

The PDGFR, RasGAP and SHP-2 antisera used in these studies were crude polyclonal antisera (Kazlauskas *et al*., 1991; Valius and Kazlauskas, 1993; Klinghoffer *et al*., 1996) and were the kind gift of Dr Andrius Kazlauskas. The cst.1 antiserum, that was raised against the peptide YQPGENL and recognizes Src, Yes and Fyn by immunoblot and kinase assay (Kypta *et al*., 1990), was generously provided by Dr Sara Courtneidge. The Shc polyclonal antiserum (Lioubin *et al*., 1996) was kindly provided by Dr Larry Rohrschneider. Affinity-purified Lyn polyclonal antibody (Law *et al*., 1993) was the kind gift of Dr Clifford A.Lowell. Affinity-purified FAK polyclonal antibody (Schlaepfer *et al*., 1994) was the generous gift of Dr David Schlaepfer. A mixed polyclonal antiserum (Cas B and F) raised against GST fusion proteins encoding p130^{cas} [residues 318–486 (Cas B) and 670–896 (Cas F)] was the kind gift of Dr Amy Bouton and Dr J.Thomas Parsons (Harte *et al*., 1996). All polyclonal antisera used for immunoprecipitation or Western blotting were used as described in the references above. The 327 anti-Src mouse monoclonal antibody was used for Western blotting as previously described (Lipsich *et al*., 1983) and was kindly provided by Dr Joan Brugge. The anti-phosphotyrosine (4G10) and anti-PLCγ mouse monoclonal antibodies were purchased from Upstate Biotechnology, Inc., and the anti-Erk 1 and 2 and anti-paxillin monoclonal antibodies from Zymed, Inc., and were used according to the manufacturer's specifications.

Western blot analysis

Western blotting was performed as previously described (Kazlauskas *et al*., 1992) with the exception that secondary antibodies were conjugated to horseradish peroxidase instead of to alkaline phosphatase. Blots were developed with Western blot detection reagents (enhanced chemiluminescence; Amersham).

In vitro SFK kinase assay

To assay for Src, Yes and Fyn tyrosine kinase activity, SYF mutant or control cell lines were grown to near confluence, starved overnight in DMEM + 0.1% FCS and were left resting or were stimulated with various concentrations of PDGF. Cells were lysed in a total of 1 ml of EB^{++} [1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 2 mM NaOH, 2 mM Na_3VO_4, 1% aprotinin, 10 mM Tris, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and immunoprecipitated with

1 μl of cst.1 antiserum. Immune complexes were collected and washed as previously described (Kazlauskas *et al*., 1991). Cst.1 immunoprecipitates representing ~4×10^5 cells were subjected to a standard *in vitro* kinase assay by incubation in a total volume of 20 μl of UKB buffer (20 mM PIPES pH 7.0, 10 mM $MnCl_2$, 20 μg/ml aprotinin), in the presence of 1 μCi of [γ-^{32}P]ATP and 0.5 μg of acid-denatured rabbit muscle enolase (Boehringer Mannheim) as the exogenous substrate (Cooper and King, 1986). Samples were incubated at 30°C for 20 min, boiled, resolved by SDS–PAGE and visualized by autoradiography. All gels were stained with Coomassie Blue to ensure that equal amounts of enolase were present in all samples.

[^{3}H]thymidine uptake (DNA synthesis) assay

PDGF-stimulated incorporation of thymidine was assayed as previously described (Kazlauskas *et al*., 1991). Briefly, 6×10^4 cells were plated per well in a 24-well dish containing DMEM + 10% FCS and incubated for 24 h at 37°C. Cells were washed three times in PBS and were rendered quiescent by starving for at least 24 h in DMEM + 0.1% FCS. Vehicle control [10 mM acetic acid, 2 mg/ml bovine serum albumin (BSA)], 10% FCS or growth factor (PDGF or LPA) was added and, after 18 h, cells were incubated for 2 h with 1.0 mCi/ml [^{3}H]thymidine in DMEM + 5% FCS. Radioactivity incorporated into the trichloroacetic acid-insoluble fraction was determined by standard procedures. Each data point was assayed in triplicate and assays were repeated three times.

Cell proliferation

Cells were plated in triplicate at 8×10^4 cells per well of a 6-well plate and were incubated for 24 h at 37°C. Cells were washed three times in PBS and were rendered quiescent by starvation in DMEM + 0.1% FCS supplemented with ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite) for 48 h. The day on which PDGF (30 ng/ml) or vehicle were added to the cultures was considered day 0. Cells were counted periodically using a hemacytometer. Culture medium with or without PDGF was replaced every other day. Each data point was assayed in triplicate and assays were repeated twice.

c-myc RNA isolation and Northern blot analysis

Poly(A)$^+$ RNA isolation and hybridization for c-*myc* was performed as described (Sachsenmaier *et al*., 1994) using a *Cla*I–*Eco*RI fragment of pBS-myc as a probe (Hann *et al*., 1988).

MAPK gel shift assay

Cells were stimulated, lysed in EB^{++}, and proteins were resolved by SDS–PAGE as described above. Proteins were transferred to Immobilon as described (Kazlauskas *et al*., 1992) and blots were probed with a mouse monoclonal antibody against Erk 1 and Erk 2 (Zymed; 1:1000). The faster/slower band ratio was used as a measure of the extent of MAP kinase (Erk) activation.

Cell migration assays

Wound healing. Cells were plated, grown to confluency in DMEM + 10% FCS, and a wound was introduced by scratching the confluent monolayer with a pipet tip. Tissue culture medium was replaced to remove detached cells, and migration was observed by video recording of cells under a 20× objective lens in a 37°C chamber. Still images were captured off of video tape at the indicated times utilizing NIH Image software.

Haptotaxis. Haptotaxis assays were performed as previously described with minor modifications (Klemke *et al*., 1998). Briefly, Falcon cell culture inserts (tissue culture treated, 6.4 mm diameter, 10 μm thickness, 8 μm pores, Falcon; Becton Dickinson and Co.) containing polyethylene terepthalate (PET) track-etched membranes were coated on the underside with 5 μg/ml FN for 2 h at 37°C, rinsed once with PBS, and then placed into the lower chamber containing 500 μl of DMEM + 0.1% FCS supplemented with ITS. Serum-starved cells were harvested by limited trypsin–EDTA treatment. The trypsin was inactivated by soybean trypsin inhibitor (0.5 mg/ml; Sigma) with 0.25% BSA (Fraction V; Sigma) in DMEM, and cells were collected by centrifugation and resuspended DMEM + 0.1% FCS (5×10^5 cells/ml). A total of 50 000 cells were added to the top of each migration chamber and allowed to migrate toward the FN-coated side of the membrane for 3 h. The non-migratory cells on the upper membrane surface were removed with a cotton swab, and the migratory cells were fixed in methanol, stained with Harris hematoxylin, rinsed with dH_2O and exposed briefly to Bluing reagent. The number of migratory cells per membrane was counted with an inverted microscope using a 40× objective lens. Each determination represents the average of three individual wells, and error bars represent the standard deviation. In control experiments, cell migration on BSA was <0.01% of the total cell population.

Chemotaxis. Chemotaxis assays were performed as described above with the following modifications. Cell culture insert membranes were coated on both sides with 5 μg/ml FN to enhance adhesion of the cells to the membrane. DMEM + 0.1% FCS containing the appropriate concentration of PDGF was added to the bottom chamber of each well and cells were allowed to migrate for 3 h before counting.

Cell stimulation with FN

Cells were grown to confluency, starved overnight in DMEM + 0.1% FCS, and harvested by limited trypsin EDTA treatment. The trypsin was inactivated by soybean trypsin inhibitor (0.5 mg/ml; Sigma) with 0.25% BSA (Fraction V; Sigma) in DMEM, and cells were collected by centrifugation and resuspended in serum-free DMEM + 0.1% BSA and held in suspension at 37°C for 1 h. Petri dishes (6 cm in diameter; Falcon) were pre-coated with FN (5 μg/ml from bovine plasma; Sigma) or poly-L-lysine (100 μg/ml; Sigma), diluted in PBS at 4°C overnight, rinsed with PBS and pre-warmed to 37°C for 1 h prior to replating. Cells were either left suspended, or were plated onto ligand-coated dishes (10^6 per dish) and incubated for the indicated amount of time before lysis in EB^{++} buffer. Total cell protein from lysates was measured by colorimetric assay (Bio-Rad laboratories) and standardized prior to SDS–PAGE and Western blotting for PY content.

Immunofluorescence

Cells were prepared for plating onto FN-coated coverslips (5 μg/ml) as described above. Cells were plated for 1 h, fixed with 4% paraformaldehyde (pH 7.4) and permeabilized in 0.4% Triton X-100. Non-specific binding was inhibited by incubation in blocking solution (2% BSA + 0.2% Tween in PBS) for 1 h at room temperature. Focal adhesions were detected by staining with mouse monoclonal anti-vinculin antibody (Sigma; 1:400) followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse secondary antibody (Jackson Labs; 1:400), both for 1 h at room temperature. The actin cytoskeleton was stained with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma; 1:400) which was added along with the anti-mouse secondary antibody. Fluorescence photomicroscopy was conducted on a Zeiss axiophot, and images were captured and processed using a Deltavision microscope and software (Applied Precision, Inc.) (Figure 8). Scale bar is ~20 μm.

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