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Divergent Roles of c-Src in Controlling Platelet-derived Growth Factor-dependent Signaling in Fibroblasts[D]

Kavita Shah and Fabien Vincent*

Department of Chemistry, Purdue University, West Lafayette, IN 47907

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The vast complexity of platelet-derived growth factor (PDGF)-induced downstream signaling pathways is well known, but the precise roles of critical players still elude us due to our lack of *specific* and *temporal* control over their activities. Accordingly, although Src family members are some of the better characterized effectors of PDGFβ signaling, considerable controversy still surrounds their precise functions. To address these questions and limitations, we applied a chemical–genetic approach to study the role of c-Src at the cellular level, in defined signaling cascades; we also uncovered novel phosphorylation targets and defined its influence on transcriptional events. The spectacular control of c-Src on actin reorganization and chemotaxis was delineated by global substrate labeling and transcriptional analysis, revealing multiple cytoskeletal proteins and chemotaxis promoting genes to be under c-Src control. Additionally, this tool revealed the contrasting roles of c-Src in controlling DNA synthesis, where it transmits conflicting inputs via the phosphatidylinositol 3 kinase and Ras pathways. Finally, this study reveals a mechanism by which Src family kinases may control PDGF-mediated responses both at transcriptional and translational levels.

INTRODUCTION

Platelet-derived growth factor (PDGF) isoforms elicit their effects on target cells by binding to two related tyrosine kinase receptors denoted α- and β-receptors. Exposure of fibroblasts to PDGF-BB leads to a variety of cellular responses, including actin reorganization, chemotaxis, and cell proliferation. These cellular outcomes originate from the simultaneous activation of multiple signaling cascades, including Ras, phosphatidylinositol 3 kinase (PI3K), and phospholipase Cγ (PLCγ) pathways. The numerous players mediating these responses are directly recruited to PDGFβ receptor (PDGFR) as a result of PDGF-BB-induced receptor dimerization and subsequent autophosphorylation at multiple tyrosine residues, which in turn provide recruiting platforms for downstream proteins. These include three Src family kinases expressed in fibroblasts, c-Src, Fyn, and Yes (collectively referred to as SFKs), PI3K, PLCγ, rasGAP, SHP2 as well as the adaptor proteins Nck, Crk, Grb2, Grb7, and Shc. Over the past decade, numerous studies have uncovered the role of most members of the PDGF pathway (Heldin *et al*., 1998; Tallquist and Kazlauskas, 2004). Interestingly, the exact contribution of c-Src (or SFKs), a much studied effector of PDGF-dependent responses, still remains a subject of vigorous debate (DeMali *et al*., 1999; Abram and Courtneidge, 2000; Bromann *et al*., 2004). There is extensive cross-talk between the different pathways activated upon PDGF stimulation, which makes it exceedingly difficult to evaluate the role of c-Src in any individual pathway (Heldin *et al*., 1998). Moreover, whereas the functions of several players of the PDGF pathway have been addressed using a microarray experiment, the exact role of SFKs at the transcriptional level remains to be delineated (Fambrough *et al*., 1999). Finally, the identification of novel c-Src substrates should enable us to better define its contribution in mediating these responses.

Multiple types of receptors are found upstream of SFKs (Brown and Cooper, 1996). Their widespread involvement has generated a vast interest in understanding the mechanisms by which this family of enzymes contributes to signaling cascades. On PDGF stimulation, SFKs associate with PDGFR at Y579 and Y581, leading to activation of its intrinsic tyrosine kinase activity. To evaluate the role of SFKs in PDGF-induced cellular responses, different strategies have been used to date. Both microinjection studies using dominant negative SFK-specific antibodies (Barone and Courtneidge, 1995) and SFK-selective inhibitor SU6656 (Blake *et al*., 2000) suggest that the kinase activity of this family is essential for PDGF-induced S-phase entry through the induction of c-myc. In contrast, experiments using a mutant PDGFR (F579, F581) defective in both binding to and activating SFKs demonstrated no dependence on SFKs for PDGF-dependent cell cycle progression (DeMali and Kazlauskas, 1998). Importantly, this approach does not inhibit the basal activity of SFKs, which may be sufficient for DNA synthesis. Another strategy using SFKs triple Src, Yes, and Fyn knockout (SYF) supported the latter conclusion that the kinase activities of SFKs are not required for PDGF-induced cell cycle progression (Klinghoffer *et al*., 1999). However, the fibroblasts used by Klinghoffer *et al*. (1999) were immortalized with large T antigen, rendering them independent from SFKs activity for PDGF-induced mitogenicity as reported previously (Broome and Courtneidge, 2000). In addition, this report showed that PDGF-induced DNA synthesis requires the inhibition of a p53-dependent pathway that is controlled by c-Src. Finally, a recent study exposing c-Abl as a key effector mediating

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* Present address: Renovis, 2 Corporate Dr., South San Francisco, CA 94080.

Address correspondence to: Kavita Shah (shah23@purdue.edu).

c-Src dependent PDGF-induced DNA synthesis also supports a role of SFKs in this pathway (Furstoss *et al*., 2002). In conclusion, discussion is still ongoing about the exact contribution of SFKs in PDGF-stimulated mitogenicity.

A similar controversy envelops the function of SFKs in controlling actin reorganization and chemotaxis. Microinjection (Mureebe *et al*., 1997) and mutant PDGFR experiments (DeMali *et al*., 1999) support a major role for c-Src in inducing chemotaxis, but knockout studies argue against an involvement of SFKs in this response (Klinghoffer *et al*., 1999). Similarly, although the mutant receptor approach revealed that efficient phosphorylation of PLCγ, rasGAP, Shc, and SHP2 depends on an initial burst of Src kinase activity (DeMali and Kazlauskas, 1998), SYF knockout cells showed no dependence (Klinghoffer *et al*., 1999). Conversely, SU6656 studies revealed Shc, protein kinase C (PKC)δ and c-Cbl as SFKs substrates, but not PLCγ (Blake *et al*., 2000). Although these contradictory results about the role of SFKs in growth factor signaling may be partly attributed to cell types, the experimental approaches chosen may also have contributed significantly to the different outcomes as exemplified previously.

In the present study, we reexamined the role of one of the SFKs, c-Src, in PDGF-stimulated pathways in fibroblasts by using a chemical genetic tool allowing for its temporal control in an absolutely specific manner (Shah *et al*., 1997; Bishop *et al*., 2000; Shah and Shokat, 2002). The combination of a cell-permeable "bumped" inhibitor (1-Na-PP1) with the corresponding sensitized mutant (*c-Src-analog sensitive1*, *c-Src-as1*) revealed a major role for c-Src in controlling actin reorganization and chemotaxis, but a relatively minor role in DNA synthesis. Furthermore, the specific and temporal control afforded by this tool enabled us to decouple different pathways and to demonstrate that c-Src both positively and negatively regulates DNA synthesis at different times after PDGF stimulation. These studies led to the identification of a dual role for c-Src in down-regulating the Ras-MAPK pathway and up-regulating the PI3K and PLCγ pathways. In addition, we used c-Src-as1 with a complementary γ-^{32}P-labeled nucleotide analog (A*TP) to identify novel substrates of Src upon PDGF stimulation. This association provides a unique handle by which the direct substrates of any particular kinase can be traced in presence of other protein kinases. Accordingly, a broad screen for phosphorylation targets of c-Src upon PDGF stimulation using [γ-^{32}P]N^6(benzyl)ATP ([γ-^{32}P]A*TP) uncovered several novel direct substrates. These are known to be involved in cytoskeletal reorganization, suggesting that the dramatic role of c-Src in controlling actin reorganization may be mediated to a significant extent through direct phosphorylation of key proteins. Finally, a gene expression analysis using *c-Src-as1* cells and specific inhibitor 1-Na-PP1 revealed a distinctive role for Src in mediating differential gene regulation initiated by PDGF. Our microarray data strongly support a role for c-Src in promoting chemotaxis at the transcriptional level. Furthermore, c-Src likely plays another important function by preventing apoptosis and supporting cell growth by controlling the induction of multiple key genes after PDGF stimulation.

MATERIALS AND METHODS

Materials

PDGF-BB was obtained from Austral Biologicals (San Ramon, CA), prolong antifade kit was from Molecular Probes (Eugene, OR), Diff-Quik Stain was from VWR (West Chester, PA), and rat tail collagen was from Roche Diagnostics (Indianapolis, IN). Antibodies against Cortactin (H191), rasGAP (B4F8), Dok (A3), Fak (A-17), Actin (C2), Fyn (sc434), Yes (sc8403), and Vimentin (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (4G10), H-Ras antibody (Ras10), and RBD-agarose were from Upstate Biotechnology (Lake Placid, NY). Antibodies against Paxillin (P13520), PI3K (P13020), SHP2 (P54420), and PLCγ (P12220) were from BD Transduction Laboratories (Lexington, KY); phospho-Erk1/2 (9101) was from Cell Signaling Technology (Beverly, MA); and monoclonal antibody 327 was from Calbiochem (San Diego, CA).

Expression Plasmids

Wild-type *c-Src* was cloned into the pBabe puro vector at BamHI and Hind III sites. The *T338G c-Src* mutant (*c-Src-as1*) was produced by QuikChange (Stratagene, La Jolla, CA).

Transfection and Retroviral Infection

Both *c-Src* and *c-Src-as1* pBabe puro plasmids were transiently transfected into Bosc 23 cells. The viruses were harvested and used to infect Src-negative fibroblast (SNF) cells as reported previously (Shah and Shokat, 2002). After selection, cells were replated at low density, grown for several weeks, and individual colonies were isolated. Multiple clones were screened and the individual colonies selected were those expressing endogenous levels of c-Src comparable with that of NIH3T3 cells.

Kinase Assays and Two-Dimensional (2D) Gel Electrophoresis of c-Src and c-Src-as1 NIH3T3 Cells

c-Src and *c-Src-as1* cells were serum starved in 0.5% bovine calf serum (BCS) for 48 h, followed by stimulation with PDGF (50 ng/ml) for 8 min. Cells were rinsed twice with chilled phosphate-buffered saline (PBS) and lysed in NP-40 lysis buffer (1% NP-40, 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and cleared by centrifugation at 10,000 rpm for 10 min at 4°C. Cleared lysates were mixed with PDGFR antibody and protein A-Sepharose beads (Sigma-Aldrich, St. Louis, MO) and incubated at 4°C for 4 h. Immune complexes were washed twice with NP-40 lysis buffer and once with kinase buffer (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 10 mM $MnCl_2$). Synthesis of [γ-^{32}P]A*TP, kinase assays, electrophoresis, and visualization were conducted as reported previously (Shah and Shokat, 2002).

Immunoprecipitation and Immunoblotting of Different Substrates from c-Src and c-Src-as1 SNF Cells

With the exception of FAK and paxillin experiments (7 ng/ml), serum-starved cells were stimulated with 50 ng/ml PDGF for 8 min as described above. Unless otherwise specified, the experiments were conducted as reported previously (Shah and Shokat, 2002).

SFK Activation Assay

Both *c-Src* and *c-Src-as1* SNF were serum starved, stimulated with PDGF (50 ng/ml), lysed, and subjected to immunoprecipitation with c-Src, Fyn, and Yes antibodies as described above. Immune complexes were subjected to in vitro kinase assays using [γ-^{32}P]ATP and 5 μg of EIYGEF-GFP (substrate) for 10 min at room temperature. For specific inhibition experiments (Figure 1D), 1-Na-PP1 (500 nM) was included in the kinase reaction.

Ras Binding Domain (RBD) Binding Assay

c-Src and *c-Src-as1* SNF were serum starved, stimulated with PDGF (50 ng/ml) in the absence or presence of 1-Na-PP1 (1 μM), and lysed in 1% NP-40 buffer. The lysates were subjected to immunoprecipitation with RBD-agarose for 30 min at 4°C and the beads were then washed three times with wash buffer (20 mM Tris, pH 8.0, 50 mM NaCl, and 10 mM $MgCl_2$), boiled in SDS loading buffer, and separated by electrophoresis. After transfer, the polyvinylidene difluoride (PVDF) membrane was probed with anti-H-Ras and the amount of H-Ras bound to the RBD agarose beads was visualized using anti-rabbit horseradish peroxidase in conjunction with West Pico (Pierce Chemical, Rockford, IL).

Chemotaxis Assay

Both *c-Src* and *c-Src-as1* cells were serum starved in 0.2% fetal bovine serum (FBS) for 16 h and isolated by limited trypsin digestion. Cell suspensions (10^5 cells/300 μl DMEM/0.5% bovine serum albumin [BSA]) were added to the top compartment of rat collagen-coated Boyden chambers, and 400 μl of growth media (migration media with 50 ng/ml PDGF) was added to the bottom compartment. After incubating for 4 h at 37°C, chambers were taken out and the inner surface of the membrane was wiped with a cotton applicator. Chambers were washed once with PBS and were then stained using Diff-Quik. Cells were counted under a phase contrast microscope in 10 random fields at 100× magnification. The assays were performed in triplicate. For PP1 and 1-Na-PP1 experiments, inhibitors were added to both sides of the filter at 500 nM concentration.

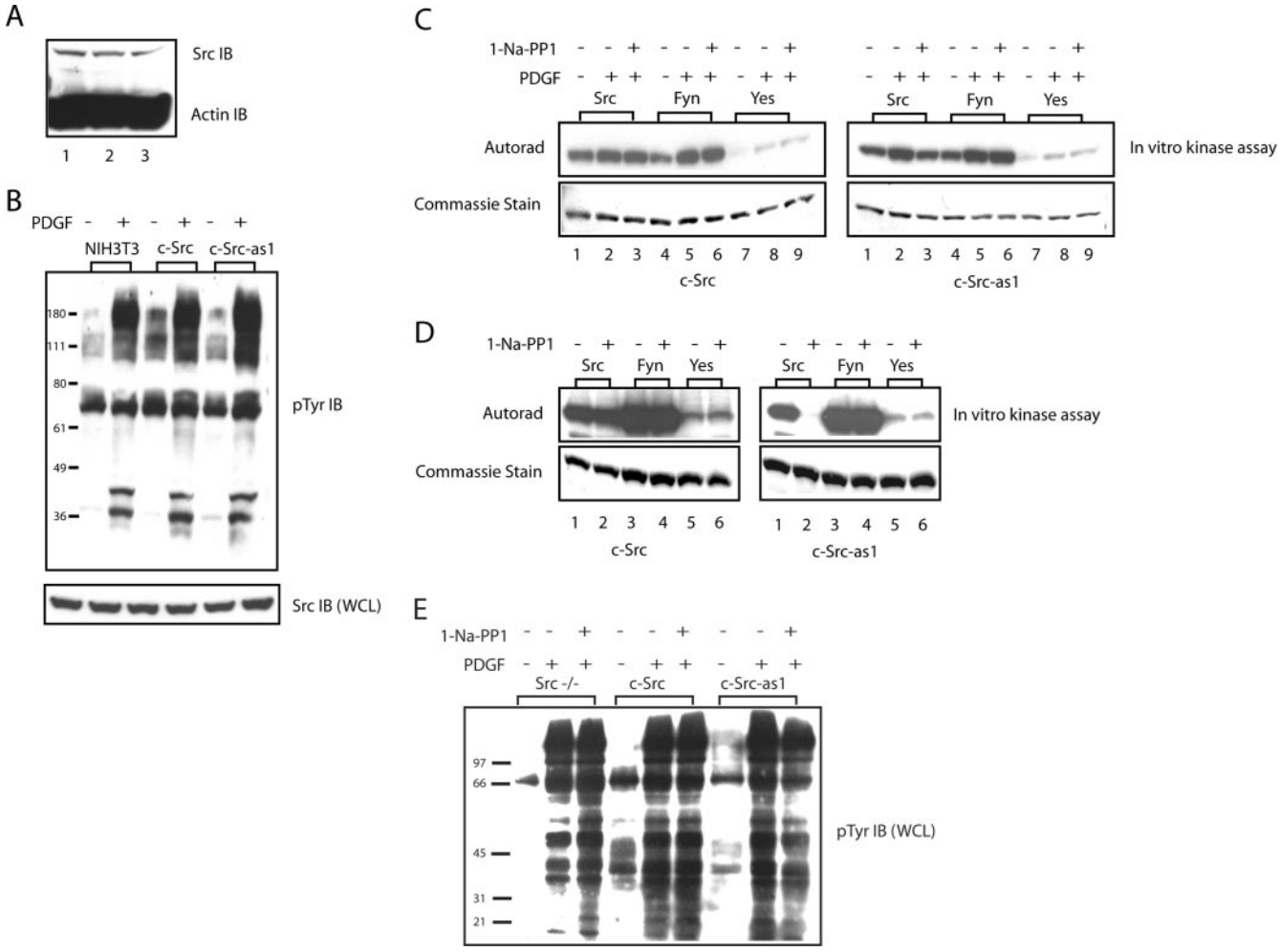

Figure 1. (A) Expression levels of c-Src and c-Src-as1 in *c-Src* and *c-Src-as1* SNF cells compared with NIH3T3 cells. Whole cell lysate from NIH3T3, *c-Src*, and *c-Src-as1* cells (lanes 1–3, respectively) were resolved by electrophoresis, transferred, and probed by Src antibody. Actin immunoblot was used as a loading control. (B) PDGF stimulation of NIH3T3, *c-Src*, and *c-Src-as1* SNF cells. The cells were treated with PDGF (25 ng/ml) for 5 min. PDGFR immune complexes were resolved by electrophoresis, transferred, and probed using pTyr antibody. To confirm equal loading, 10 μl of the beads was loaded on a separate gel, and the membrane was probed by Src antibody (bottom). (C) Activation of c-Src, Fyn, and Yes in *c-Src* and *c-Src-as1* SNF upon PDGF stimulation. Immune complexes were isolated using SFKs antibodies from unstimulated, PDGF-stimulated and 1-Na-PP1 (1 μM)-treated PGDF-stimulated cells. In vitro kinase assays were carried out with [γ-^{32}P]ATP and EIYGEF-GFP. PVDF membrane was Coomassie stained to confirm equal loading of EIYGEF-GFP substrate (bottom). (D) Specific inhibition of c-Src-as1 by 1-Na-PP1. Immune complexes were isolated using SFKs antibodies and were subjected to an in vitro kinase assay with [γ-^{32}P]ATP and EIYGEF-GFP (substrate) with or without 1-Na-PP1 (500 nM). PVDF membrane was Coomassie stained to confirm equal loading of EIYGEF-GFP substrate (bottom). (E) PDGF stimulation of *c-Src−/−*, *c-Src*, and *c-Src-as1* SNF cells in the presence or absence of 1-Na-PP1. The cells were treated with PDGF (50 ng/ml) for 10 min. Whole cell lysates were resolved by electrophoresis, transferred, and probed using pTyr antibody. Experiments shown in Figure 1, A–E, were repeated multiple times, and representative blots are shown.

Immunofluorescence

Cells were grown on coverslips until 50–60% confluence, followed by serum starvation in 0.5% BCS for 24 h. Dimethyl sulfoxide (DMSO), PP1 (1 μM), or 1-Na-PP1 (1 μM) was added 15 min before the addition of PDGF (50 ng/ml). After incubating the cells for 15 min at 37°C, coverslips were washed three times with chilled PBS, fixed in 3.7% formalin/phosphate-buffered saline for 10 min, permeabilized with 0.2% Triton in PBS for 5 min, washed twice with PBS, and blocked in 5% BSA/PBS for 2 h at 25°C. Cells were labeled with primary antibodies (anti-vimentin for 1 h at 1–100 dilution in 1% BSA/PBS, followed by incubation with fluorescein isothiocyanate (FITC)-phalloidin (800 ng/ml), Hoechst (1–5000), or tetramethylrhodamine B isothiocyanate-conjugated secondary antibody. Cells were counterstained with prolong antifade and visualized with DeltaVision Optical Sectioning Microscope Olympus IX-70 (API, Issaquah, WA). Images were captured using 40× objective and were deconvolved using DeltaVision software SoftWoRx version 2.5. Retention of actin stress fibers or loss of stress fibers and ruffle formation were scored using a Nikon TE2000 microscope, equipped with an X-Cite 120-arc lamp, an FITC filter cube, and a Cascade 512B camera (Roper Scientific, Trenton, NJ). A 10× (0.3 numerical aperture) objective was used for imaging.

[^{3}H]Thymidine DNA Synthesis Assay

Both *c-Src* and *c-Src-as1* cells were seeded at a concentration of 4×10^4 cells per well in a 24-well plate. After 24 h, cells were serum starved in 0.2% fetal calf serum (FCS)/BSA (2 mg/ml) for 48 h. The cells were stimulated with either with 50 ng/ml PDGF or 10% FCS. PDGF buffer (10 mM acetic acid and 2 mg/ml BSA) and DMSO were added as control. After 18 h, the cells were pulsed with 1 μCi of [^{3}H]thymidine and 5% FCS (DeMali and Kazlauskas, 1998) along with the inhibitors. After 4 h, cells were washed once with chilled PBS, twice with chilled 5% trichloroacetic acid, and lysed in 0.25 N NaOH (300 μl/well). Then, 250 μl of this solution was directly added to the scintillation vial containing 50 μl of 6 N HCl. The solution was mixed with scintillation fluid and counted.

In Vitro PI3K Assay

c-Src-as1 cells were serum starved for 16 h in 0.5% FBS. DMSO, PP1 (1 μM), or 1-Na-PP1 (1 μM) was added 15 min before the addition of PDGF (10 ng/ml). After incubating them for 15 min at 37°C, the cells were harvested and PDGFRβ complexes were immunoprecipitated as described above. Im-

mune complexes were washed with the following buffers: PBS with 1% NP-40, PBS, 100 mM Tris-HCl, pH 7.5, with 500 mM LiCl, distilled water, and finally 25 mM HEPES, pH 7.5, 100 mM NaCl, and 1 mM EDTA. The beads were preincubated for 10 min in 50 μl of reaction buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EGTA, and 0.2 μg/μl phosphatidylinositol) before adding 20 μCi of [γ-^{32}P]ATP, 2 μM ATP, and 10 mM $MgCl_2$. Allowed to proceed for 10 min at room temperature, the reaction was stopped by the addition of 100 μl of 1 M HCl. Subsequent extraction, thin layer chromatography (TLC) separation, and visualization of the lipids were carried out as described previously (Jones *et al*., 1999).

Inositol Turnover: PLCγ Activity Assay

c-Src-as1 cells plated in triplicate were incubated in inositol-free media for 30 min before being labeled for 24 h in inositol-free media containing 10 μCi of [^{3}H]inositol/ml. Cells were treated with LiCl (20 mM) 45 min before PDGF stimulation. Additionally, DMSO, PP1 (1 μM), or 1-Na-PP1 (1 μM) was added 15 min before the addition of PDGF (10 ng/ml). After incubating them for 5 min at 37°C, the cells were rinsed with chilled PBS containing 100 μM Na_3VO_4 and lysed in 1 M HCl (400 μl) and methanol (400 μl). After scraping, the cell lysate was transferred to Microfuge tubes, and two successive extractions were carried out using 400 μl of chloroform. The organic layers were combined, and the total uptake of radioactivity in cellular phosphoinositides was determined by scintillation counting. The aqueous layer, containing inositol, glycerophosphoinositol, and inositol phosphates, was fractionated using high-performance liquid chromatography (HPLC) as described previously (DeMali *et al*., 1997). Scintillation counting of the fractions containing inositol phosphates allowed us to monitor inositol turnover when normalized to the total uptake of label into cellular phosphoinositides. Statistical significance was determined by *t* test using Excel (Microsoft, Redmond, WA) on the results obtained from four independent experiments.

Genome-Wide Expression Analysis

To prepare total RNA for microarray analyses, *c-Src* and *c-Src-as1* cells were either stimulated with PDGF (40 ng/ml) or with 0.1% of a 10% acetic acid/water solution (control) for 30 min, 2 or 4 h, washed with ice-cold PBS and then quickly frozen at −80°C. To monitor changes in expression caused by specific inhibition of c-Src kinase, both cell types were incubated with 1-Na-PP1 (1 μM) for 15 min before the addition of PDGF (for the control cells, 0.002% DMSO was added).

Total RNA, cDNA, and subsequent cRNA preparations were performed as described previously (Fambrough *et al*., 1999). Fifteen micrograms of cRNA was added to Affymetrix MG_U74Av2 chips with 1× MES hybridization buffer using manufacturer's protocol. After washings, arrays were scanned with a laser scanner (Agilent Technologies, Wilmington, DE). The output files from the scanner were uploaded into the GNF chip informatics server (www.gnf.org), and expression profiles were analyzed using a built-in analysis program, analysis of variance.

Mass Spectral Analysis

Gel spots were manually excised and automatically processed for peptide mapping experiments using a Micromass MassPREP Station in conjunction with manufacturer-specified protocols. Matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained using a MALDI-R time-of-flight mass spectrometer (Micromass, Manchester, United Kingdom). Protein identification searches were performed using MASCOT (Matrix Science, Boston, MA).

RESULTS

We created a functionally silent mutation in the active site of c-Src kinase (*c-Src-as1*), which rendered it sensitive to an orthogonal ATP analog (Shah *et al*., 1997; Liu *et al*., 1998) or an orthogonal inhibitor (Bishop *et al*., 2000) that are not accepted by any wild-type kinase. The design strategy encompasses the engineering of both a "pocket" in the ATP binding site of c-Src and of ATP analogs or cell-permeable inhibitors containing substituents that complement it. The pocket is created by the replacement of threonine 338 with a glycine in the active site, and compounds complementing it are generated by attaching bulky substituents to ATP or SFK inhibitor PP1. The T338G mutation creating the sensitized allele has been shown to have no effect on the substrate specificity of the kinase (Witucki *et al*., 2002). We and others have applied this strategy for the identification of direct substrates of several kinases (Habelhah *et al*., 2001; Shah and Shokat, 2002; Eblen *et al*., 2003; Ubersax *et al*., 2003). Additionally, the combination of a cell permeable bumped inhibitor with the corresponding sensitized mutant (*c-Src-as1*) of a particular kinase is a powerful instrument for dissecting the role of any kinase of interest in vivo (Bishop *et al*., 2000). Because our previous studies with v-Src and c-Src showed 1-Na-PP1 to be an extremely potent inhibitor of the corresponding as1 mutant kinases (IC_{50} of ~1.5 nM), with no effect on wild-type kinases even at millimolar concentrations, 1-Na-PP1 was the orthogonal inhibitor of choice for the present studies (Bishop *et al*., 2000).

Generation and Characterization of c-Src and c-Src-as1 SNF Clonal Cells

Both wild-type *c-Src* and *c-Src-as1* were expressed in SNFs that were spontaneously immortalized and thus lack any large T antigen. Multiple clones were screened and individual colonies expressing endogenous levels of c-Src comparable with those of NIH3T3 cells were selected (Figure 1A). Because this is the first study using these cells, both *c-Src* and *c-Src-as1* cells were subjected to multiple functional assays to evaluate their responses in comparison with NIH3T3 cells. We first examined their tyrosine phosphorylation levels and patterns in response to different stimuli. As shown in Figure 1B, these cells were identical to NIH3T3 cells in their response to PDGF.

Because all three SFKs (c-Src, Yes, and Fyn) expressed in fibroblasts are activated upon PDGF stimulation, their activations were then examined. SFKs were immunoprecipitated after PDGF stimulation, and their catalytic activities were measured using a green fluorescent protein (GFP)-tagged Src substrate (Yang *et al*., 1999). As shown in Figure 1C (compare lanes 2, 5, and 8 with lanes 1, 4, and 7, respectively), exposure to PDGF resulted in a marked increase in c-Src, Fyn, and Yes kinase activities in both cell lines. Most importantly, the relative basal activities of SFKs and their relative degree of activation (2- to 5-fold) upon PDGF treatment were similar to results reported for NIH3T3 cells (Kypta *et al*., 1990). Interestingly, when SFKs activations were measured in 1-Na-PP1-treated PDGF-stimulated *c-Src* and *c-Src-as1* cells, a modest decrease in activity was specifically observed for c-Src-as1 kinase activity (Figure 1C, compare lanes 2 and 3 in c-Src-as1 panel). This result indicates that specific inhibition of c-Src activity during PDGF stimulation reduces its own degree of activation. The catalytic activities of SFKs immune complexes in the presence or absence of 1-Na-PP1 were then investigated using vitro kinase assays. Inhibition of c-Src-as1 with 1-NaPP1 abolished the phosphorylation of the GFP-tagged Src substrate, but it had no effect on Fyn or Yes kinase activities (Figure 1D, c-Src-as1 panel, lane 2). PDGF-induced global tyrosine phosphorylation was next compared in *Src−/−* SNF cells versus *c-Src* and *c-Src-as1* cells. Similar phosphorylation patterns were observed in all three cases, presumably because of the presence of Fyn and Yes in *Src−/−* cells (Figure 1E). In addition, usage of 1-Na-PP1 specifically affected the global phosphorylation of *c-Src-as1* cells upon PDGF stimulation, but it showed no role in *Src−/−* or *c-Src* SNF cells (Figure 1E). Together, these results confirm that all three SFKs are activated in a similar manner in *c-Src* and *c-Src-as1* SNF and in fibroblasts upon PDGF stimulation and that 1-Na-PP1 inhibits c-Src-as1 kinase with absolute specificity. With this chemical–genetic tool in hand, we set out in this study to address the precise role of c-Src in PDGF-stimulated pathways.

Role of c-Src in Controlling Actin Reorganization

When exposed to PDGF, loss of actin stress fibers (shown in green) and formation of membrane ruffling was observed as

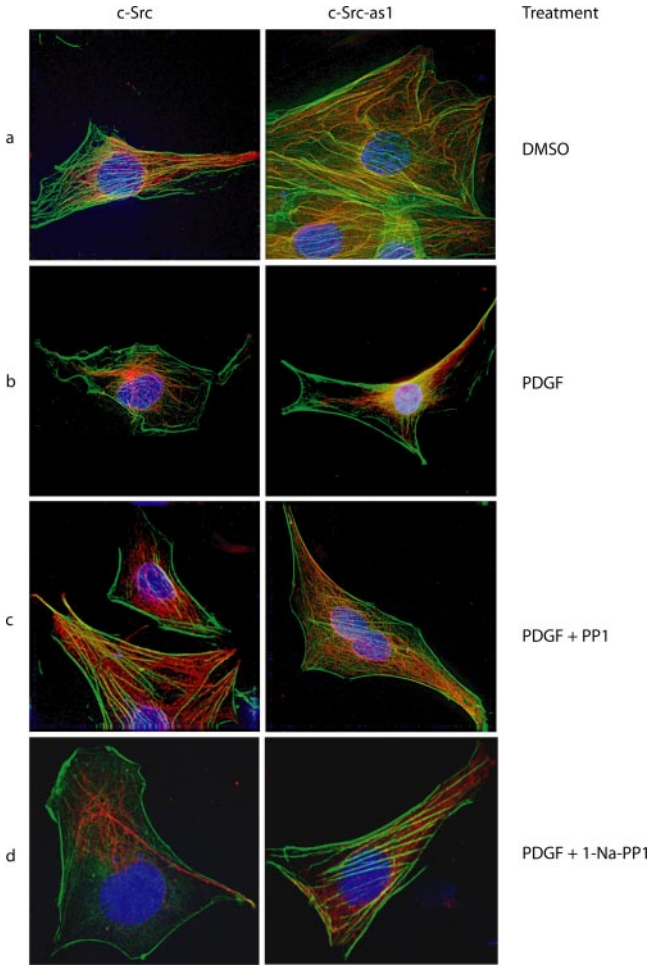

Figure 2. Effect of c-Src inhibition on PDGF-stimulated actin and vimentin reorganization in fibroblasts expressing c-Src or c-Src-as1. Cells were treated with DMSO (a), PDGF + DMSO (b), PDGF + PP1 (1 μM) (c), and PDGF + 1-Na-PP1 (1 μM) (d) before being labeled with anti-vimentin (red), FITC-phalloidin for actin (green), and Hoechst (blue) and were visualized using deconvolution microscopy. Bar, ~25 μm. These experiments were conducted in triplicate on three different occasions using each of the conditions described in Figure 2 in every experiment. In each instance, 200 random cells were scored, and >80–90% of cells for each indicated treatment displayed the phenotypes that are represented in this figure. A *t* test analysis between c-Src and c-Src-as1 cells treated with PDGF + 1-NaPP1 for scoring actin reorganization revealed a $p < 0.001$.

expected (Figure 2B). Importantly, addition of 1-Na-PP1 to *c-Src-as1* SNFs conferred strong resistance to actin reorganization upon PDGF stimulation (Figure 2D), whereas it had no effect when added to *c-Src* SNFs (control). This result confirms a critical role of c-Src in promoting actin reorganization upon PDGF stimulation. In addition, when the general SFK-specific inhibitor PP1 was used, both *c-Src* and *c-Src-as1* cells failed to undergo PDGF-mediated actin reorganization, further supporting a positive role of SFKs in this pathway (Figure 2C). Because PP1 is also an inhibitor of PDGFR (Waltenberger *et al*., 1999) and Bcr-Abl and c-Kit (Tatton *et al*., 2003), the latter result was rather predictable and was included as a positive control.

Role of c-Src in PDGF-induced Chemotaxis

We next determined the contribution of c-Src kinase activity in PDGF-dependent chemotaxis. SNF-expressing c-Src-as1

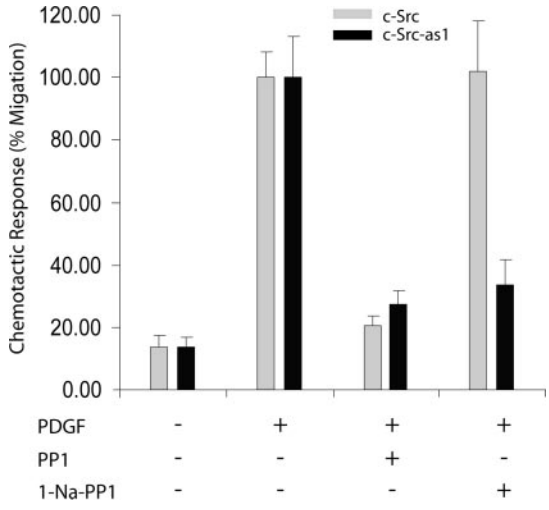

Figure 3. Effect of c-Src inhibition on PDGF-induced chemotaxis in fibroblasts expressing c-Src and c-Src-as1. The migrating abilities of cells stimulated by PDGF were determined in a Boyden chamber in the presence of the inhibitors PP1 (500 nM) or 1-Na-PP1 (500 nM).

treated with 1-Na-PP1 were dramatically impaired in their response to PDGF stimulation, whereas DMSO-treated control cells readily migrated (Figure 3). Because 1-Na-PP1 only inhibits c-Src-as1, whereas Fyn and Yes are still functional in these cells, the observed 70% decrease in chemotactic response can be solely attributed to c-Src inhibition. Treatment with PP1 inhibited *c-Src-as1* cells migration to a similar extent, underscoring a critical role for c-Src in controlling chemotaxis. As a control, when a similar experiment was performed on wild-type c-Src cells, 75% inhibition of cell migration was observed in the presence of PP1, whereas 1-Na-PP1 treatment had no effect.

Role of c-Src in PDGF-induced DNA Synthesis

Our next goal was to evaluate the responsibility of c-Src in mediating PDGF-stimulated DNA synthesis. Both *c-Src* and *c-Src-as1* cells were treated with either DMSO, 1-Na-PP1, or PP1 before PDGF stimulation. After 18 h, the cells were pulsed with [^{3}H]thymidine for 4 h, and DNA synthesis was measured. Specific inhibition of c-Src-as1 led at most to a very modest (~5–10%) decrease in PDGF-stimulated DNA synthesis (Figure 4). In contrast, substantial inhibition was observed when PP1 was used. This result indicates that although c-Src clearly controls chemotaxis, other PP1-sensitive kinases seem to have a greater influence on DNA synthesis. Importantly, the role of c-Src-as1 was investigated using 1 μM 1-Na-PP1 and identical results were obtained when 500 nM of 1-Na-PP1 was used, confirming that c-Src-as1 kinase is completely inhibited at this concentration (Figure 1D).

Chemical Genetic Dissection of Different Pathways Controlled by c-Src

To further deconvolute the molecular mechanism of c-Src's role in the PDGF pathway, we set out to identify the signaling molecules regulated by c-Src. To this end, we stimulated *c-Src* and *c-Src-as1* SNFs in the absence or presence of 1-Na-PP1. Signaling complexes were isolated by PDGFR immunoprecipitation and subjected to an in vitro kinase assay. As shown in Figure 5A, *c-Src* and *c-Src-as1* cells displayed similar patterns of overall phosphorylation upon PDGF stimu-

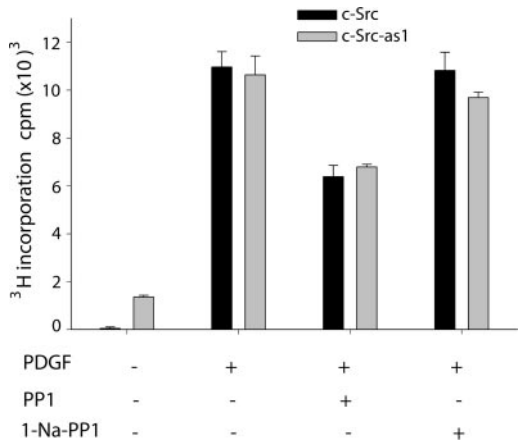

Figure 4. Effect of c-Src inhibition on PDGF-stimulated DNA synthesis in fibroblasts expressing c-Src or c-Src-as1. [^{3}H]Thymidine incorporated in cells stimulated by PDGF in the presence of the inhibitors PP1 (1 μM) or 1-Na-PP1 (1 μM) was quantitated to determine the amount of newly synthesized DNA.

lation (lanes 2 and 6). The similarity in total cellular phosphoprotein content observed is in agreement with previous studies showing that as1 mutant kinases and the corresponding wild-type kinases exhibit the same phosphoacceptor specificity, kinase activity, and regulatory properties (Witucki *et al*., 2002). Whereas 1-Na-PP1 treatment had no effect on *c-Src* expressing fibroblasts (lanes 3 and 4), *c-Src-as1*-expressing cells showed a marked decrease in the phosphorylation levels of several proteins (lanes 7 and 8). Accordingly, c-Src either directly phosphorylates some of the proteins present in the complex or promotes the recruitment of downstream targets by controlling the phosphorylation of the receptor itself. Alternatively, it may activate secondary kinases that subsequently phosphorylate these substrates.

To distinguish between these possibilities, we stimulated *c-Src-as1* cells in the absence or presence of 1-Na-PP1 and investigated signaling proteins known to be phosphorylated upon PDGF stimulation (Heldin *et al*., 1998). Phosphotyrosine immunoblots showed many of the chosen proteins to be phosphorylated in a c-Src-dependent manner (Figure 5B). When a similar experiment was performed on *c-Src* cells, 1-Na-PP1 treatment had no effect on any phosphorylation event. Interestingly, PDGFR was one of the main targets of c-Src dependent tyrosine phosphorylation (Figure 5B). Additionally, a low level of PDGFR phosphorylation was observed in the presence of 1-Na-PP1 in *c-Src-as1* cells, but not *c-Src* cells, in an in vitro kinase assay (Figure 5A, compare lanes 5 and 6 with lanes 7 and 8). Current models of PDGF signaling emphasize that the receptor autophosphorylates at multiple sites upon ligand binding, resulting in the recruitment of downstream proteins. However, our results suggest that c-Src contributes significantly to the phosphorylation of PDGFR and thus may also control PDGF-dependent signaling at the recruitment level.

Consequently, we studied the recruitment of several known downstream proteins in the presence or absence of c-Src kinase activity. PDGFR-associated proteins were isolated using PDGFR antisera and were probed for PLCγ, rasGAP, PI3K, SHP2, and Dok-1. As shown in Figure 5C, association of PLCγ, rasGAP, Dok-1, and PI3K with the receptor depended on c-Src kinase activity to a significant extent. On the other hand, SHP2 recruitment to PDGFR was independent of c-Src.

Specific Role of c-Src in PI3K, PLCγ, and Ras Pathways

Because PDGF-dependent responses are mediated by the simultaneous activations of PI3K, PLCγ, and Ras pathways, we proceeded to determine the exact contributions of c-Src kinase activity in these cascades.

PI3K Pathway Is Controlled by c-Src

PDGF signaling models place the role of SFKs in controlling the actin cytoskeleton morphology downstream of the PI3K pathway, which includes the Rho family of GTPases (Hall, 1998; Jimenez *et al*., 2000). However, our results suggest a possible role for c-Src in controlling PI3K activity through the modulation of its recruitment (Figure 5C). To test this hypothesis, we carried out in vitro PI3K kinase assays on PDGFR complexes isolated after PDGF stimulation in the presence or absence of inhibitors. As shown in Figure 6A, specific c-Src inhibition by 1-Na-PP1 results in a significant decrease (~30%) in PI3K activity, a level consistent with its reduced recruitment. Accordingly, c-Src control of cytoskeletal changes via the PI3K pathway could encompass both PI3K recruitment and direct interactions with its downstream effectors.

Role of c-Src in the PLCγ Pathway

We next investigated the role of c-Src in PLCγ pathway upon PDGF stimulation. Monitoring of the intracellular levels of inositol phosphates in *c-Src-as1* SNF cells revealed a moderate (~30%), but statistically significant, decrease in PLCγ activity when c-Src was inhibited (Figure 6B). In contrast, treatment with PP1 resulted in a pronounced ~70% inhibition of inositol phosphate production, most likely reflecting the inhibition of PDGFR itself as well as c-Src, Fyn, and Yes in addition to other PP1 sensitive kinases.

Although it has been shown that tyrosine phosphorylation of PLCγ is essential for its activation, this event depends in turn on PLCγ recruitment to the PDGFR. The recruitment of PLCγ to the membrane was demonstrated to be mediated by PI3K (Falasca *et al*., 1998). Because specific inhibition of c-Src kinase activity leads to reduced PI3K recruitment and results in a subsequent decrease in PI3K activity, we postulated that it may also down-regulate PLCγ recruitment. We observed substantially less association of PLCγ with PDGFR in the presence of 1-Na-PP1 for *c-Src-as1* cells (Figure 5C, compare lanes 2 and 3). In addition, our results revealed significantly less phosphorylation of PLCγ was observed when c-Src kinase activity was inhibited (Figure 5B). Because both PDGF-mediated recruitment and phosphorylation are responsible for PLCγ activation (Falasca *et al*., 1998), these results provide a possible mechanism by which c-Src plays an important role in up-regulating the PLCγ pathway.

Role of c-Src in the Ras Pathway

To determine the role of c-Src in the Ras pathway, we monitored the phosphorylation levels of extracellular signal-regulated kinase (Erk)1/Erk2. Previous studies using SYF cells have revealed that SFKs up-regulate the Ras pathway upon integrin, but not PDGF, stimulation (Klinghoffer *et al*., 1999). However, due to the presence of large T antigen, the need for SFKs was most likely abrogated in the cells used. Because Erk1/Erk2 phosphorylation is an early event after PDGF stimulation (up to 40–45 min), a time-course study was carried out to monitor their phosphorylation in our cellular system. Surprisingly, strong phosphorylation was observed for Erk1/Erk2 in the presence of specific c-Src inhibition (Figure 7A). Although Kazlauskas *et al*. have reported a negative role for SFKs in c-Cbl-mediated degrada-

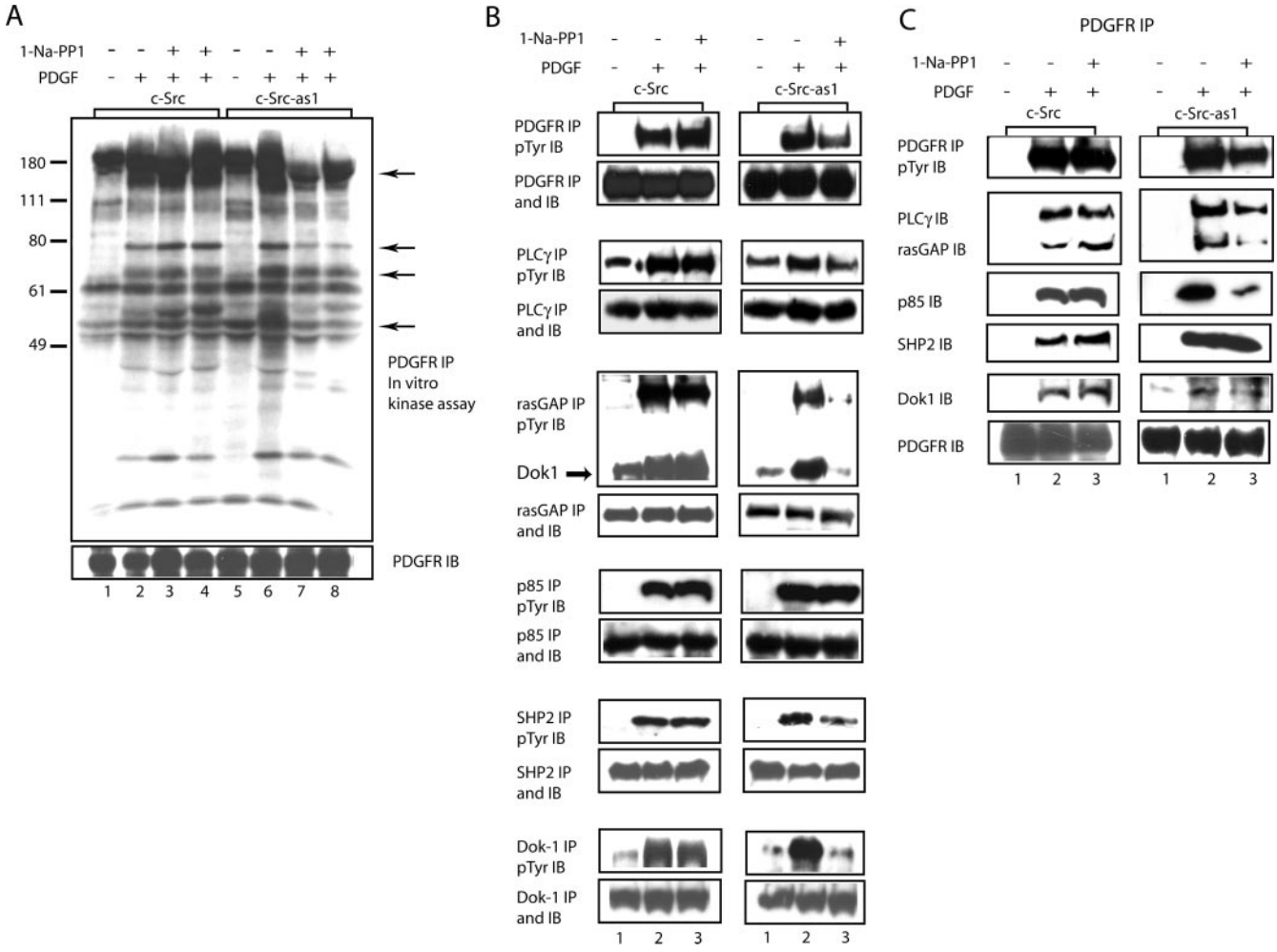

Figure 5. (A) Effect of c-Src inhibition on the kinase activity of PDGFR immune complexes. *c-Src* and *c-Src-as1* cells were treated in the following manner: lanes 1 and 5, DMSO; lanes 2 and 6, PDGF + DMSO; lanes 3 and 7, PDGF + 1-Na-PP1 (500 nM); and lanes 4 and 8, PDGF + 1-Na-PP1 (1 μM) before immunoprecipitation of PDGFR. After an in vitro kinase assay, the samples were resolved, transferred, and visualized by autoradiography. To confirm equal loading, 10 μl of the beads were loaded on a separate gel before the kinase assay, and the membrane was probed by PDGFR antibody (bottom). (B) Effect of c-Src inhibition on the phosphorylation of PLCγ, rasGAP, PI3K, SHP2, and Dok1 to PDGFR. *c-Src* and *c-Src-as1* cells were treated with platelet-derived growth factor and 1-Na-PP1 (1 μM) before immunoprecipitation with the indicated antibodies. Membranes were probed by a pTyr antibody. (C) Effect of c-Src inhibition on the recruitment of PLCγ, rasGAP, PI3K, SHP2, and Dok1 to PDGFR. *c-Src* and *c-Src-as1* cells were treated with PDGF and 1-Na-PP1 (1 μM) as indicated before immunoprecipitating PDGFR. The samples were probed by the indicated antibodies.

tion of αPDGFR (Rosenkranz *et al*., 2000), this result was unexpected, because all the current models suggest either no role or a positive role for c-Src activity on the Ras pathway in PDGFβ receptor signaling cascades. Interestingly, when c-Src-as1 activity was inhibited during integrin stimulation, Erk1/Erk2 phosphorylation was completely inhibited, in agreement with the previously published study using SYF cells (our unpublished data).

To further confirm the effect of c-Src inhibition on Ras activation, we used the glutathione *S*-transferase (GST)-tagged RBD of Raf1 kinase to quantify Ras activation (de Rooij and Bos 1997). PDGF stimulation of *c-Src* and *c-Src-as1* cells revealed increased Ras-RBD binding as reported earlier for fibroblasts (Figure 7B) (Bondeva *et al*., 2002). Specific c-Src-as1 inhibition led to greatly enhanced Ras-RBD binding, in agreement with our results showing that Src inhibition leads to activation of the Ras pathway upon PDGF stimulation. Importantly, the time courses of Ras activation and Erk1/Erk2 phosphorylation coincided with each other: maximal activation was achieved after 5 min and lasted for at least 30 min after stimulation (Figure 7C). Together, these observations confirm that c-Src kinase activity negatively regulates the Ras pathway upon PDGF stimulation. Because only one of the SFKs is inhibited in this approach, while Fyn and Yes are still active, this result is especially noteworthy and reveals a key novel role of c-Src in the Ras pathway.

Divergent Roles of c-Src in Controlling DNA Synthesis: Decoupling the Ras and PI3K Pathways

PDGF-stimulated DNA synthesis depends on both Ras and PI3K pathways (Jones and Kazlauskas, 2001). Although Ras contribution is essential in the early phase (~30–60 min), the first wave of PI3K activity (~15–30 min) does not contribute to DNA synthesis but instead is mainly responsible for the cytoskeletal changes associated with PDGF stimulation. It is the second phase of PI3K activity (~6–8 h after stimulation) that is absolutely essential for DNA synthesis. Because our

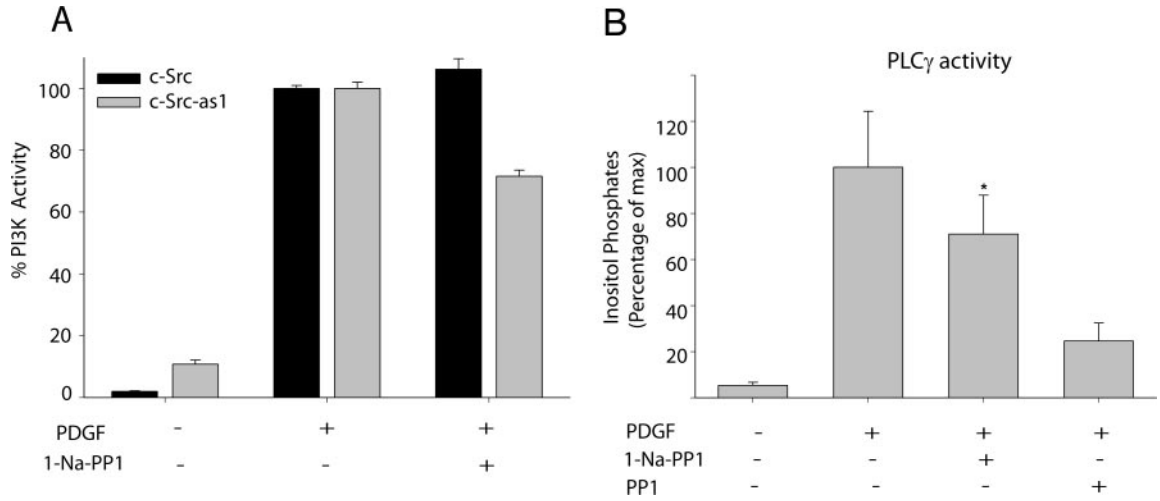

Figure 6. (A) Effect of c-Src inhibition on PI3K kinase activity. *c-Src* and *c-Src-as1* cells were treated with PDGF and 1-Na-PP1 (1 μM) as indicated before immunoprecipitation of PDGFR. After being subjected to an in vitro PI3K kinase assay, the samples were resolved by TLC, and the amount of phosphatidylinositol 3-phosphate (PI3P) was quantitated by autoradiography. (B) Effect of c-Src inhibition on inositol turnover. *c-Src-as1* cells were treated with PDGF, 1-Na-PP1 (1 μM), and PP1 (1 μM) as indicated before cell lysis and separation of the cellular contents in aqueous and organic phases. The aqueous phase of each sample was fractionated by HPLC, and the amount of inositol phosphates was quantitated. The results of four separate experiments conducted in triplicate were used for statistical analysis. *t test, <0.05.

results suggest that c-Src positively regulates the PI3K pathway and down-regulates the Ras pathway, we postulated that inhibiting c-Src after the first wave of Ras activity subsides would have a more dramatic effect on DNA synthesis. Accordingly, specific c-Src inhibition was carried out both before and after PDGF stimulation. Time-course studies confirmed that early c-Src inhibition leads only to a very moderate decrease in DNA synthesis (Figure 7C). In contrast, if c-Src inhibition is carried out after the initial wave of Ras activity, a much more pronounced decrease is detected, likely reflecting the inhibition of late phase PI3K activity necessary for DNA synthesis. Moreover, PDGF induced c-Src activation was found to persist until later time points (measured up to 45 min), supporting a key role of c-Src kinase activity in DNA synthesis when inhibited at later times (Figure 7, D and E).

Global Screen for Direct Substrates of c-Src upon PDGF Stimulation

To further deconvolute the role of c-Src in the PDGF pathway, we next aimed at identifying the direct phosphorylation targets of c-Src, likely responsible for mediating PDGF-dependent effects. We carried out an analysis of *direct* c-Src substrates using *c-Src-as1* SNF cells and [γ-^{32}P]A*TP. The use of [γ-^{32}P]A*TP allows the exclusive labeling of c-Src-as1 substrates, because it can only be used efficiently by c-Src-as1 and not by wild-type protein kinases (Shah and Shokat, 2002). In vitro kinase assays using immune PDGFR complexes isolated from both *c-Src* and *c-Src-as1* SNF cells with [γ-^{32}P]ATP showed indistinguishable patterns of phosphoproteins (Figure 8A, lanes 1 and 3 vs. lanes 5 and 7), confirming the similar substrate specificities of wild-type and mutant c-Src. Labeling of PDGFR complexes isolated from *c-Src-as1* SNF cells with [γ-^{32}P]A*TP displayed a different phosphorylation pattern, as only the *direct* substrates of c-Src-as1 were radiolabeled (lane 8). As a control, identical kinase assays with PDGFR complexes from *c-Src* SNF cells were also performed with [γ-^{32}P]A*TP. Only minimal labeling occurred in this case, confirming the specific use of [γ-^{32}P]A*TP by c-Src-as1 (lanes 2 and 4).

To further resolve the direct c-Src substrates from other proteins, 2D gel electrophoresis was carried out on PDGFR complexes isolated from *c-Src-as1* SNF cells phosphorylated with either [γ-^{32}P]ATP or [γ-^{32}P]A*TP. More than 20 proteins phosphorylated with [γ-^{32}P]ATP were not detected by autoradiography in the presence of [γ-^{32}P]A*TP (Figure 7, B and C). This result was expected as all cellular kinases used [γ-^{32}P]ATP, and thus Figure 8B displays the composite labeling of numerous kinase substrates. On the other hand, Figure 8C reveals only the kinase activity of c-Src-as1. Importantly, a high concentration of nonradiolabeled ATP was included in all reactions to ensure a similar overall stoichiometry of phosphorylation and thus conservation of protein mobility.

Mass spectral identification of radiolabeled c-Src-as1 substrates revealed several direct targets of c-Src upon PDGF stimulation: PDGFR (from one-dimensional gel), vimentin, tubulin-β5, β- and γ-actin, and myosin light chain 2-A. It is noteworthy that most of the substrates successfully identified were abundant cytoskeletal proteins. Unfortunately, the technical limitations of mass spectrometry prevented the identification of several intensely radiolabeled spots displaying little or no Coomassie staining.

Identification of tubulin-β5 as a substrate of c-Src was not surprising, because it is also a known v-Src substrate (Shah and Shokat, 2002). Another c-Src substrate, vimentin, also displayed increased phosphorylation after stimulation in the absence of 1-Na-PP1 (Figure 8D). Importantly, vimentin has been shown to undergo dramatic cytoskeletal reorganization in PAE cells upon PDGF stimulation, an event requiring PI3K, but not Src, activity (Valgeirsdottir *et al.*, 1998). Accordingly, we investigated PDGF-mediated vimentin reorganization in *c-Src* and *c-Src-as1* SNF in the presence or absence of 1-Na-PP1. Because 1-Na-PP1 treatment blocked vimentin reorganization in *c-Src-as1* but not *c-Src* cells (Figure 2D), this event seems to be c-Src-mediated in fibroblasts, in contrast to the results obtained in PAE cells. Finally, similar experiments monitoring the phosphorylation levels of actin and myosin light chain

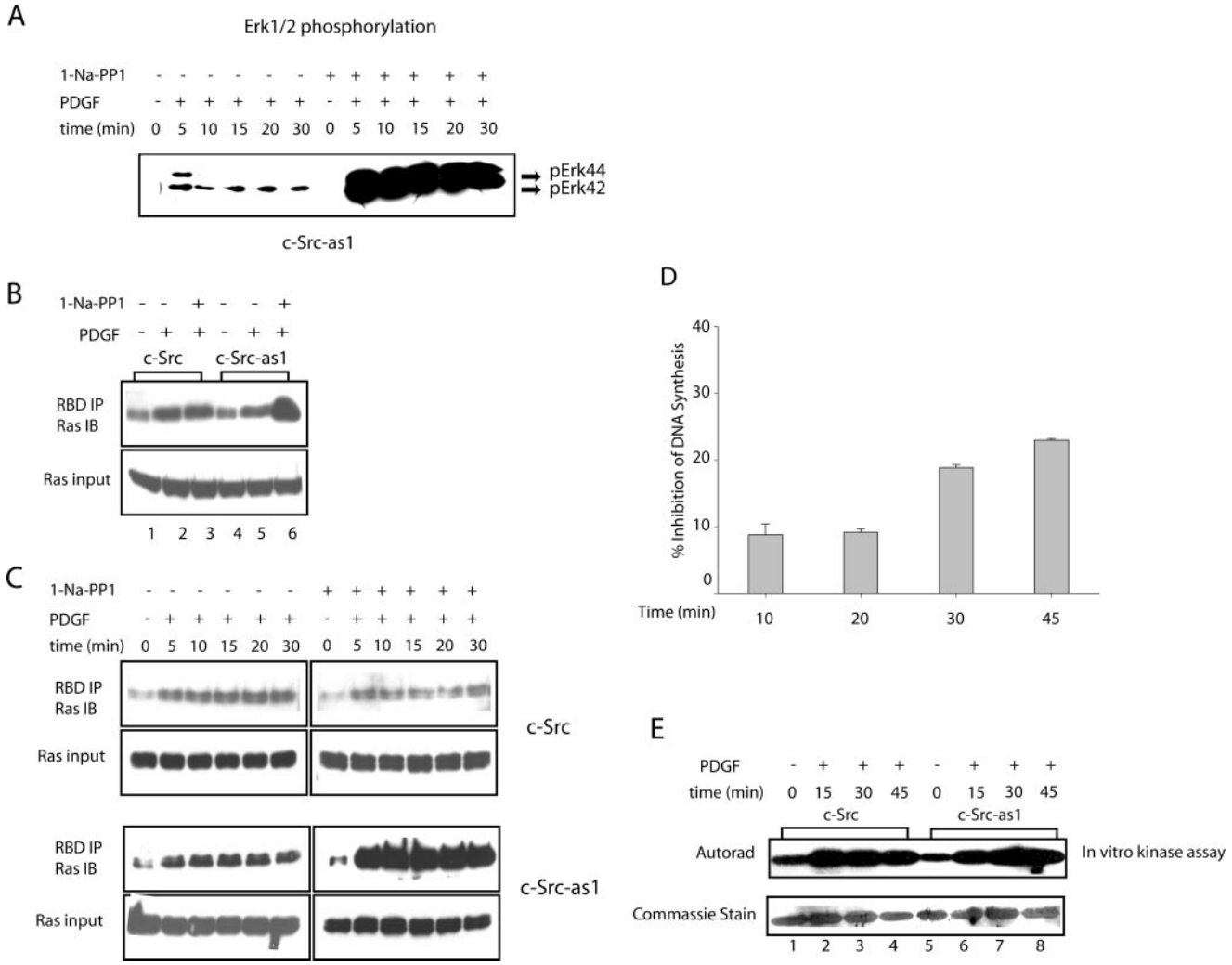

Figure 7. (A) Effect of c-Src inhibition on Erk1/Erk2 phosphorylation after PDGF factor stimulation. *c-Src-as1* cells were treated with PDGF and then with 1-Na-PP1 (1 μM) in a time-dependent manner as indicated. Samples were probed by anti-phosphoErk1/Erk2 antibody. (B) Effect of c-Src inhibition on Ras activation. *c-Src* and *c-Src-as1* cells were treated with PDGF and 1-Na-PP1 (1 μM) as indicated. After immunoprecipitating active Ras using RBD agarose beads, the samples were probed by Ras antibody. To confirm equal amounts of cell lysates were subjected to RBD immunoprecipitation, 5% of cell lysates was separated by electrophoresis and probed by Ras antibody (bottom). (C) Time course of Ras activation after c-Src inhibition. *c-Src* and *c-Src-as1* cells were treated with PDGF and 1-Na-PP1 (1 μM) in a time-dependent manner as indicated. The samples were probed by Ras antibody. To confirm equal amounts of cell lysates were subjected to RBD immunoprecipatation, 5% of cell lysates was separated by electrophoresis and probed by Ras antibody (bottom). (D) Effect of c-Src inhibition on DNA synthesis. *c-Src-as1* cells were treated with PDGF and then, at the indicated time, with 1-Na-PP1 (1 μM). [^{3}H]Thymidine incorporated in newly synthesized DNA was quantitated and normalized to the amount obtained in the absence of 1-Na-PP1 to calculate the inhibition percentage. (E) Activation of c-Src in *c-Src* and *c-Src-as1* SNF upon PDGF factor stimulation. Immune complexes were isolated using c-Src specific antibody (antibody 327) from unstimulated and PDGF-treated cells after 15, 30, and 45 min of stimulation. In vitro kinase assays were carried out with [γ-^{32}P]ATP and EIYGEF-GFP. PVDF membrane was Coomassie stained to confirm equal loading of EIYGEF-GFP substrate (bottom).

2-A revealed their absolute dependency on c-Src kinase activity, confirming them as direct substrates of c-Src (Figure 8D).

c-Src-mediated Phosphorylation of Several Proteins upon PDGF Stimulation

Because the global screen for direct c-Src substrates revealed only highly abundant cytoskeletal proteins, we carried out a more focused screen to identify low-abundance proteins that may mediate c-Src-dependent signaling cascades upon PDGF stimulation. Because c-Src plays a spectacular role in controlling actin reorganization and chemotaxis, we investigated its potential phosphorylation of less abundant cytoskeletal proteins known to play key roles in these events. Accordingly, cortactin, focal adhesion kinase (FAK), and paxillin were chosen as putative targets. Although some basal tyrosine phosphorylation of cortactin and FAK was present under quiescent conditions, we observed a robust increase after PDGF stimulation, increase which was c-Src-dependent (Figure 8E). PDGF-mediated phosphorylation of paxillin depends on PI3K activity and cytoskeleton integrity (Abedi *et al.*, 1995). However, neither the functional consequences nor the kinase responsible for this event have been identified. We observed that paxillin is yet another cytoskel-

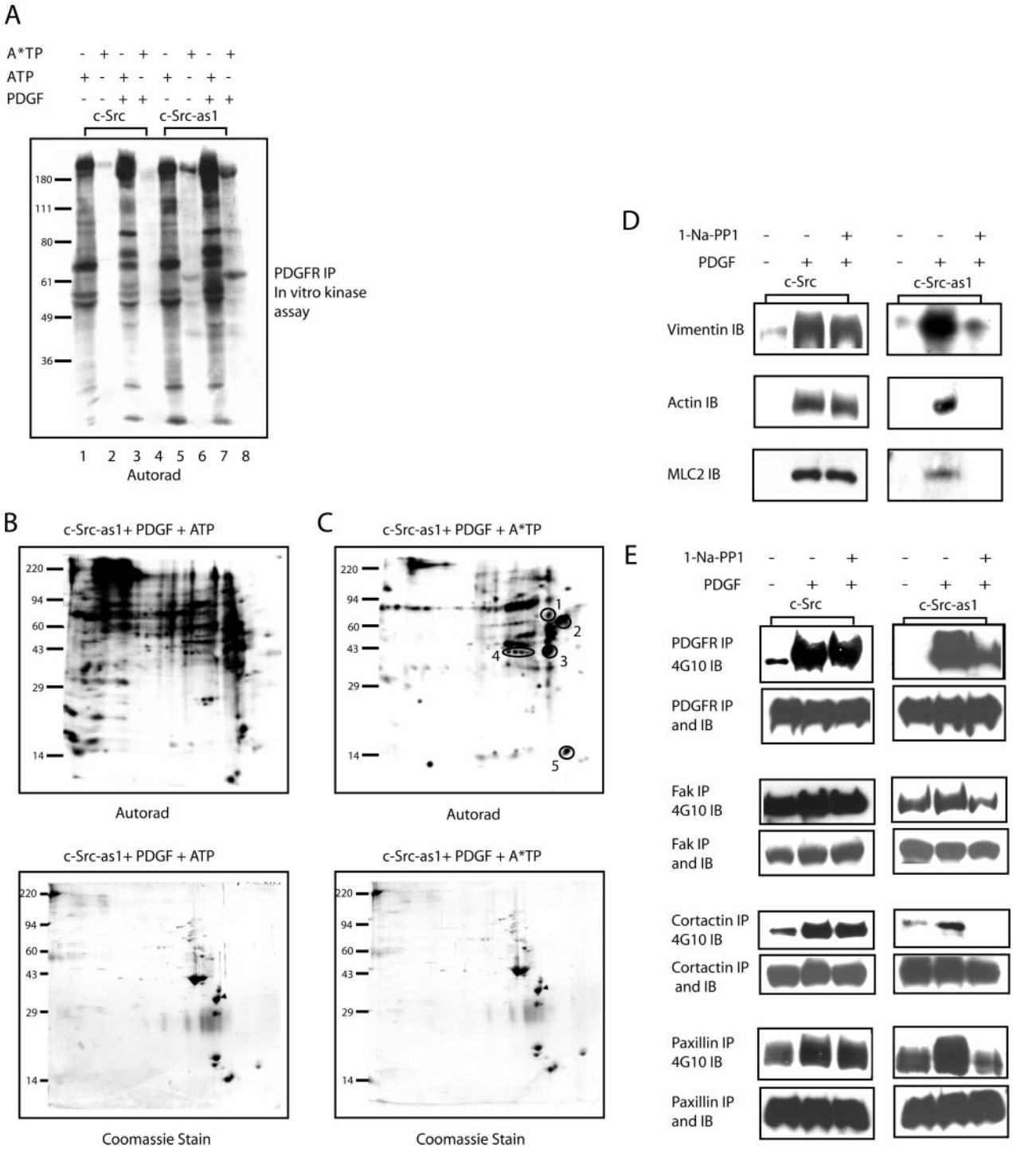

Figure 8. (A) Global screen for direct substrates of c-Src upon PDGF stimulation. *c-Src* and *c-Src-as1* cells were treated with PDGF as indicated, and PDGFR immune complexes were isolated. After being subjected to an in vitro kinase assay with either [γ-^{32}P]ATP or [γ-^{32}P]A*TP, the samples were resolved by electrophoresis, transferred to a PVDF membrane, and visualized by autoradiography. (B) 2D gel electrophoresis of the kinase reaction using [γ-^{32}P]ATP of the PDGFR immune complex isolated from *c-Src-as1* SNF after PDGF stimulation. The gel was Coomassie stained (bottom), dried, and visualized by autoradiography (top). (C) 2D gel electrophoresis of the kinase reaction using [γ-^{32}P]A*TP of PDGFR immune complex isolated from *c-Src-as1* SNF after PDGF stimulation. The gel was Coomassie stained (bottom), dried, and visualized by autoradiography (top). The spots circled in black on the autoradiograph indicate the labeled proteins successfully identified by mass spectrometry (1, vimentin; 2, tubulin-β5; 3, γ-actin; 4, β-actin; 5, myosin light chain 2-A). (D) Effect of c-Src inhibition on the phosphorylation of substrates identified by 2D gel after PDGF stimulation. *c-Src* and *c-Src-as1* cells were treated with PGDF and 1-Na-PP1 (1 μM) as indicated. Phosphotyrosine containing proteins were immunoprecipitated with PY20 antibody, and the membranes were probed with the indicated antibodies. (E) Effect of c-Src inhibition on the phosphorylation of several proteins after PDGF stimulation. *c-Src* and *c-Src-as1* cells were treated with PDGF and 1-Na-PP1 (1 μM) as indicated. The indicated proteins were immunoprecipitated, resolved, and transferred, and the membranes were probed with the anti-phosphotyrosine antibody 4G10.

etal protein whose phosphorylation is enhanced by c-Src upon PDGF stimulation (Figure 8E).

Finally, we wished to determine whether the phosphorylation of rasGAP, Dok1, and SHP2, members of the PDGFR complex, was affected by c-Src. Using *c-Src-as1* cells, both rasGAP and Dok1 showed strong c-Src dependent phosphorylation after PDGF stimulation, as addition of 1-Na-PP1 substantially decreased their phosphorylation level. On the other hand, SHP2 phosphorylation was only moderately affected by 1-Na-PP1 treatment. Because SHP2 recruitment to PDGFR is not affected by c-Src inhibition (Figure 5B), this result suggests that its phosphorylation partially depends on c-Src kinase activity (Figure 8E).

DNA Microarray Data Analysis

The last part of our study was directed at analyzing the role of c-Src in PDGF-BB-mediated gene regulation. A similar analysis was conducted to assess the transcriptional contributions of different signaling pathways in PDGF-stimulated fibroblasts (Fambrough *et al*., 1999). This study was carried out by taking advantage of known Y→ F mutations on PDGFRβ, which enabled the selective inhibition of signaling cascades. The diverse signaling pathways induced by PDGF resulted in broadly overlapping, instead of independent, sets of genes. Importantly, the role of c-Src kinase was not addressed in this study because Y→ F mutation of Src binding sites (579 and 581) on PDGFRβ has been shown to compromise the receptor's kinase activity (DeMali *et al*., 1997).

Consequently, we coupled genome-wide expression analysis tools with the chemical genetic approach to isolate the genes regulated by c-Src's kinase activity after 30 min and 2 and 4 h (Table 1 and Supplemental Material). PDGF stimulation led to only 33 transcripts being up- or down-regulated after 30 min, but >120 after 2 and 4 h in both cell types. Significantly, comparison of expression profiles between wild-type and *c-Src-as1* SNF revealed only 10 genes to be different out of 270 PDGF-regulated genes, suggesting that these cell lines are virtually identical.

Specific c-Src-as1 inhibition by 1-Na-PP1 revealed that the expression of five genes among these 270 was affected more than twofold after 30 min, whereas 18 and 13 genes behaved differently after 2 and 4 h, respectively (Table 1). Treatment of *c-Src* cells with 1-Na-PP1 showed virtually no effect of the inhibitor (Supplemental Material). Overall, c-Src kinase activity seems to control the expression of only a fraction of the 270 genes regulated by PDGF stimulation.

DISCUSSION

Previous studies using genetic and chemical approaches revealed conflicting roles for SFKs in chemotaxis, myc induction, and DNA synthesis. Similarly, although the mutant PDGFR approach exposed the phosphorylation of rasGAP, PLCγ, SHP2, and Shc to be Src dependent, SYF knockout studies argue against it. Furthermore, studies with SFK-specific inhibitor SU6656 indicate the phosphorylation of c-Cbl, PKCδ, and Shc to be SFK dependent but that of PLCγ to be Src independent. All of these studies have contributed a great deal to our current understanding of SFK functions; however, some of the contradictory results obtained seem to be contingent upon the approach used. Lack of temporal control, immortalization with large T antigen (for SYF knockout cells) and presence of basal SFKs activity (mutant PDGFR studies) are some of the limitations of genetic approaches. Use of SU6656 offers temporal control and allows for the inhibition of both the basal and activated kinase activities of SFKs; however, it is unlikely to be absolutely specific given the diversity of protein kinases (Blake *et al*., 2000).

c-Src Controls Actin Reorganization and Chemotaxis at Both Transcriptional and Posttranslational Levels

In the present study, we reexamined the role of one of the SFKs, c-Src, in PDGF-stimulated events at the cellular and molecular levels using a chemical genetic approach that provides absolute specificity and temporal control over its kinase activity. This approach confirmed a major role for c-Src kinase in controlling actin reorganization. We further explored a possible mechanism for this potent control with global substrate labeling experiments, revealing multiple cytoskeletal proteins to be directly phosphorylated by c-Src (Figure 8, A–E).

Cortactin has been identified as a c-Src substrate in multiple signaling cascades, including FGF, EGF, syndecan 3, H_2O_2, and integrin stimulated pathways. However, it is thought to be a substrate of the tyrosine kinase Fer in the PDGF pathway (Weed and Parsons, 2001). Our results suggest that, in addition to Fer, c-Src also plays a significant part in cortactin phosphorylation. Recent reports have revealed a key role of FAK in cell migration upon PDGF stimulation (Sieg *et al*., 2000). Because FAK kinase activity is dispensable for chemotaxis, direct recruitment of c-Src to FAK might be the key event responsible for FAK-mediated cell migration. Indeed, Sieg *et al*. showed that PDGF treatment prompts the docking of c-Src to FAK at Y397, resulting in elevated c-Src kinase activity. Because FAK phosphorylation leads to the recruitment of downstream effectors, its phosphorylation by c-Src suggests a possible mechanism by which c-Src may direct chemotaxis. Our results clearly show c-Src-dependent phosphorylation and reorganization of vimentin filaments in fibroblasts upon PDGF stimulation (Figure 1). Whereas other cytoskeletal proteins such as tubulin-β5, paxillin, myosin light chain 2-A, and β- and γ-actin were also phosphorylated in a c-Src-dependent manner, the role of these phosphorylation events in actin reorganization is unclear. Together, we propose that c-Src's strong hold on the actin cytoskeleton reorganization may arise, in part, from phosphorylation of key cytoskeletal proteins.

Although multiple reports have established c-Src kinase as a downstream effector of PI3K, our results suggest c-Src may also be an upstream regulator of the PI3K pathway. This intricate relationship between c-Src and PI3K offers yet another explanation for c-Src's important role in cytoskeletal changes and chemotaxis. Additionally, PLCγ activity is known to promote chemotaxis upon PDGF stimulation in fibroblasts. Along these lines, our data also demonstrate that c-Src controls PLCγ activity to a certain extent by influencing both its phosphorylation and its recruitment (Figures 5, B and C, and 6B). This further underscores the participation of c-Src in PDGF-induced cell migration.

Finally, the strongest support regarding the spectacular role of c-Src in chemotaxis was revealed by the DNA microarray analysis. Several genes whose protein products promote chemotaxis were strongly induced in a c-Src-dependent manner. These are HB-EGF (Elenius *et al*., 1997), CXCR3 (Bonacchi *et al*., 2001), PAI-1 (Blasi, 1997), GPI anchor attachment protein 1 (Sendo and Araki, 1999), and uPAR (Aligayer *et al*., 2002) (Table 1 and Supplemental Material). Likewise, the expression levels of Pscd2 (Jackson *et al*., 2000), and *Drosophila* frizzled homolog 1 (Fzd1) (Winter *et al*., 2001), which both regulate actin reorganization, were up-regulated by c-Src. Thus, the gene microarray data are in agreement with our functional studies and confirm a

Table 1. Genes affected by c-Src inhibition after PDGF stimulation in fibroblasts

Encoded protein	RefSeq accession no.	Molecular function	Biological process	Functional classification	Peak time point	Fold induction at peak	
						PDGF	% Inhibition by 1-Na-PP1
ACSL1	NM_008553	Ligand binding or carrier	Developmental processes	Transcription factor	30 min	+7.4	108
GADD45a	NM_007836		Mitogenesis	Nuclear	30 min	−5.3	94
MEF2C	NM_025282		Mitogenesis	Transcription factor	30 min	+5.0	47
Plk	NM_011121	Enzyme	Mitogenesis	Nuclear	30 min	−2.8	80
RPS25	XM_112396			Cytoplasmic regulatory	30 min	−3.6	81
BPGM	NM_007563	Enzyme	Cell growth and or maintenance	Cytoplasmic metabolic	2 h	+2.7	101
CYP1B1	NM_009994	Enzyme	Cell growth and or maintenance	Cytoplasmic metabolic	2 h	+13.2	136
FZD1	NM_021457		Actin reorganization	Transmembrane	2 h	+9.0	100
Grcc9	NM_013539			Unknown	2 h	+2.4	94
GLI	NM_010296	Nucleic acid binding, protein binding	Cell growth and/or maintenance; cell communication; development	Transcriptional	2 h	−3.0	98
GADD45b	NM_008655		Mitogenesis	Nuclear	2 h	+9.4	24
HB-EGF	NM_010415		Chemotaxis, mitogenesis	Secreted	2 h	+5.9	37
Itm3	NM_022417	Ligand binding		Transmembrane	2 h	−3.8	69
IFI30	NM_023065	Enzyme	Cell communication	Cytoplasmic regulatory	2 h	−5.2	39
IDH2	NM_008322	Enzyme	Cell growth and or maintenance	Mitochondrial	2 h	−7.1	43
MGLAP	NM_008597	Ligand binding	Development	Matrix	2 h	−26.5	91
MLL*	L17069	Enzyme, ligand binding	Cell growth and or maintenance	Transcription factor	2 h	−2.2	96
Resp18	NM_009049			Secreted	2 h	+7.5	79
PAI-1	NM_008871	Enzyme regulator	Chemotaxis, cell motility	Secreted	2 h	+8.5	46
TNFα3	NM_009397	Ligand binding	Death	Cytoplasmic regulatory	2 h	−3.7	131
Ubce8	NM_019949			Cytoplasmic regulatory	2 h	−5.1	83
Ube2h	NM_009459	Enzyme	Cell growth and or maintenance	Cytoplasmic regulatory	2 h	−17.1	94
uPAR	NM_011113		Chemotaxis Cell motility	Extracellular receptor	2 h	+13.3	98
Ccr4	XM_130865		Rhythmic behavior	Transmembrane	4 h	+7.2	33
CXCR3	NM_009910	Signal transducer, ligand binding	Chemotaxis	Transmembrane	4 h	+6.0	84
CYP1B1	NM_009994	Enzyme	Cell growth and or maintenance	Cytoplasmic metabolic	4 h	−5.5	99
Gata4	NM_008092	Transcription regulator, ligand binding	Cell growth and or maintenance	Transcription factor	4 h	+2.8	103
GPAA1	NM_010331	Enzyme	Cell growth and or maintenance	Transmembrane	4 h	+3.4	100
Hsf1	XM_128055			Transcription factor	4 h	+2.5	95
Hoxd4	NM_010469	Transcription regulator, ligand binding	Cell growth and or maintenance Development	Transcription factor	4 h	−3.8	78
Mst1r	NM_009074	Enzyme, ligand binding	Cell growth and or maintenance	Transmembrane	4 h	−5.1	99
Mrpl45	NM_025927			Mitochondrial	4 h	−10.3	68
Pscd2	NM_011181		Actin reorganization	Cytoplasmic regulatory	4 h	−2.2	118
uPAR	NM_011113		Chemotaxis Cell motility	Extracellular receptor	4 h	+8.6	38
MafG	NM_010756		Stress response	Transcription factor	4 h	−6.1	115
Zic2	NM_009574	Ligand binding	Development	Transcription factor	4 h	−3.5	112

Genes affected by c-Src inhibition after PDGF stimulation (40 ng/ml) of quiescent *c-Src-as1*-3T3 cells, listed by time of observed peak induction. The listed accession number is the RefSeq entry matching the probe sequence. *GenBank accession number.

pivotal role of c-Src in mediating cellular migration upon PDGF stimulation.

Dual Role of c-Src in Controlling DNA Synthesis

One of the most controversial questions in c-Src signaling concerns its exact contribution to growth factor-promoted DNA synthesis. Elegant work by Jones and Kazlauskas (2001) revealed inputs from both the Ras and PI3K pathways in controlling PDGF-mediated DNA synthesis. Our results show that c-Src negatively regulates the Ras pathway and positively controls the PI3K pathway. Although the overall positive effect on DNA synthesis is modest, c-Src's individual contributions to each of these pathways are very significant. Notably, both enhanced Ras activation and spectacular up-regulation of Erk1/2 phosphorylation upon c-Src inhibition were observed. Src-dependent recruitment and phosphorylation of cellular proteins identified in this study may provide some insight about the mechanism for this up-regulation. A role of Dok1 in negatively regulating Erk1/2 activation is well established (Zhao *et al*., 2001; Shinohara *et al*., 2004); however, the precise mechanism leading to this event is unclear. Importantly, Dok1 has 14 tyrosine residues whose phosphorylation provides recruiting platforms for downstream proteins. These are likely to orchestrate Dok1-mediated negative regulation of Erk1/2 phosphorylation. We observed a significant inhibition of both Dok1 recruitment to PDGFR and of its phosphorylation upon c-Src inhibition, both events potentially leading to enhanced Erk1/2 phosphorylation. Enhanced Erk1/2 phosphorylation could also be a consequence of the increased Ras activation observed upon c-Src inhibition (Figure 7A), which itself may potentially be explained by the reduced recruitment of rasGAP to PDGFR (Figure 7, B and C). Additionally, because PI3K competes with rasGAP for binding to the effector region of activated Ras, a decrease in rasGAP levels may lead to more PI3K binding to Ras and enhanced Ras activation (Feig and Schaffhausen, 1994). Increased Ras activation may also stem from SHP2 whose recruitment is unaffected by c-Src inhibition (Figure 5B) and thereby may contribute to lessened rasGAP recruitment through dephosphorylation of PDGFR (Zhao and Zhao, 1999).

Most importantly, it should be noted that we observed Ras activation and Erk1/2 phosphorylation upon PDGF stimulation in the *presence* of c-Src, Fyn, and Yes kinase activities (Figure 7, A–C). However, if c-Src is specifically inhibited, Ras/Erk signaling is amplified. It is only this amplification of the signal, controlled by c-Src, which is likely to influence the overall response of PDGF-mediated events. Once all the signaling proteins are recruited to the receptor, Ras contributes to the activation of PI3K kinase, implying that c-Src-mediated Ras down-regulation should have a negative effect on PI3K activity, despite c-Src playing a positive role in PI3K activation. Similarly, PI3K activation also contributes to Ras activation, in addition to its normal role in up-regulating the Akt pathway. As a result of these conflicting actions, c-Src plays only a modest but definite positive role in PDGF-induced DNA synthesis.

Overall, the modest control of c-Src on DNA synthesis supports the results obtained using a mutant receptor approach, where the basal activities of SFKs are retained and no effect on DNA synthesis is apparent. Thus, it seems that if the total basal activity of SFKs is above a certain threshold, it may be enough to drive DNA synthesis. Complete inhibition of c-Src kinase activity using 1-Na-PP1, while Fyn and Yes are still active, may bring it just below the threshold; thus, the small decrease in DNA synthesis.

Interestingly, c-Src did not control the expression of mitogenic genes such as epiregulin, thymilidate synthase, Jun B, or GRO1 oncogene, all of which were strongly up-regulated upon PDGF stimulation (2 h: 57.7-, 22.2-, and 12-fold, respectively). Similarly, VEGF, which is dramatically up-regulated after 2 h (106-fold), showed no dependence on c-Src kinase activity. However, c-Src does regulate several genes that assist cell proliferation and inhibit apoptosis, such as HB-EGF (Elenius *et al*., 1997), MEF2C (Zhao *et al*., 2002), GADD45a (Kettenhofen *et al*., 2001), and ribosomal protein S25 (Adilakshmi and Laine, 2002) (Table 1 and Supplemental Material). Likewise, tumor necrosis factor α-induced protein 3, another apoptosis inducing gene, was specifically down-regulated by c-Src. In contrast, we also witnessed a down-regulation of the Plk transcript, suggesting a possible negative regulation of cell proliferation by c-Src (Liu and Erikson, 2002). Importantly, induction of mitosis-promoting and apoptosis-inhibiting transcripts does not necessary correlate with cellular proliferation because the resulting proteins may be regulated posttranslationally. Nevertheless, the regulation of a majority of the c-Src-dependent transcripts supports a positive role for c-Src in cell proliferation. Because the major mitogenic genes are not regulated by c-Src, a modest net contribution of this kinase to cell cycle events at the transcriptional level seems likely, in agreement with the results obtained in the DNA synthesis experiment. It should be noted, however, that Fyn and Yes are still functional in the presence of 1-Na-PP1, suggesting that c-Src/Fyn/Yes together may play a more important role in initiating mitogenesis.

In addition to specific regulation of different signaling pathways initiated by a particular stimulant, there are also global down-regulation mechanisms that are equally critical for eliciting appropriate physiological responses by maintaining the right amplitude and duration of any signal. In the case of PDGFR, these down-regulation mechanisms include ligand-induced internalization, ubiquitin-mediated proteolysis and dephosphorylation (Chiarugi *et al*., 2002). Our microarray data revealed strong down-regulation of two different ubiquitination-promoting enzymes, Ubce8 and Ube2h, after 2 h, suggesting that PDGFR may maintain the duration of its signaling by controlling the negative regulators of its pathway. More importantly, both events are mediated by c-Src kinase, suggesting a positive regulation by Src of PDGF-induced signaling events at the transcriptional level.

It should be noted, however, that the list of c-Src controlled genes after PDGF stimulation is by no means exhaustive, most likely due to the technical limitations often associated with DNA microarray experiments. For example, c-myc, which is known to be induced upon growth factor treatment, was never observed in our experiments. We think that this failure was most likely due to the quality of the c-myc probe on MG_U74Av2 arrays, because our positive controls also failed to show any c-myc expression, unless it was highly overexpressed (Shah, unpublished observations).

A combination of immunofluorescence studies, cell motility experiments, and the identification of novel c-Src cytoskeletal substrates confirmed its major role in actin reorganization and cell migration. The spectacular role played by c-Src in promoting chemotaxis was strongly supported by the microarray data analysis, revealing robust up-regulation of multiple cell motility-promoting transcripts upon PDGF stimulation. Furthermore, specific inhibition of c-Src during PDGF treatment revealed the actions of c-Src on different components of the PDGFR complex. Although

PDGFR, PLCγ, rasGAP, Dok1, SHP2, cortactin, FAK, and paxillin displayed c-Src-dependent phosphorylation, PI3K was unaffected. This result implies that p85 recruitment is not necessary for its tyrosine phosphorylation. In addition, our study reveals a role of c-Src in the recruitment of several key players of the Ras, PI3K, and PLCγ pathways to PDGFR. We notably dissected the role of c-Src in DNA synthesis and found its contribution to be the sum of its down-regulation of the Ras pathway and its activation of the PI3K pathway. This is also the first report demonstrating that c-Src down-regulates the Ras pathway upon PDGF-BB stimulation, a result obtained through the specific decoupling of the PI3K and Ras pathways. Although the overall effect on DNA synthesis is modest, c-Src's individual contributions to each of these pathways are much more pronounced. c-Src-mediated recruitment and phosphorylation of different effectors of PDGF pathway provide a possible mechanism by which SFKs may control PDGF-mediated responses. In addition, we isolated 34 genes *specifically* dependent upon c-Src's kinase activity out of the 270 transcripts whose expression changes after PDGF stimulation.

In summary, we defined the roles of c-Src at multiple levels in signaling cascades induced by PDGF stimulation in fibroblasts. These levels include its role in overall physiological responses, its specific contribution to individual pathways, identification of its direct substrates and downstream effectors, and its role at the transcriptional level. We think that extending these combined approaches to other kinases of interest will further help us reveal the molecular mechanisms of given signaling pathways and also better understand the fundamentals of signal transduction networks as a whole.

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