Requirement for c-Src Catalytic Activity and the SH3 Domain in Platelet-derived Growth Factor BB and Epidermal Growth Factor Mitogenic Signaling*

(Received for publication, February 29, 1996, and in revised form, April 29, 1996)

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The Src family protein-tyrosine kinases are required for mitogenic signaling from the platelet-derived growth factor (PDGF), colony stimulating factor-1, and epidermal growth factor (EGF) receptor protein-tyrosine kinases (RPTK) (Twamley-Stein, G. M., Pepperkock, R., Ansong, W., and Courtneidge, S. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7696–7700; Roche, S., Koegel, M., Barone, M. V., Roussel, M. F., and Courtneidge, S. A. (1995) Mol. Cell. Biol. 15, 1102-1109). In NIH3T3 fibroblasts, c-Src, Fyn, and c-Yes associate with the activated PDGF receptor, are substrates for receptor phosphorylation, and are themselves activated. Src family catalytic function is required for RPTK mitogenic signaling as evidenced by the SH2-dependent dominant negative phenotype exhibited by kinase-inactive Src and Fyn mutants (Twamley-Stein, G. M., Pepperkock, R., Ansong, W., and Courtneidge, S. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7696–7700). Here, we have generated clonal Src− murine fibroblast cell lines overexpressing various murine c-Src mutants and studied the effect of these mutant Src proteins on PDGF- and EGF-induced mitogenesis. Two c-Src SH3 domain mutants, Y133F and Y138F, each inhibited PDGF BB- and EGF-induced DNA synthesis in quiescent cells. This demonstrates an involvement of the Src SH3 domain in PDGFβ- and EGF receptor mitogenic signaling. Since both Tyr-133 and Tyr-138 are located on the ligand binding surface of the SH3 domain, these results suggest that the c-Src SH3 domain is required for PDGF and EGF mitogenic signaling. The dominant negative effect of either single mutant on PDGF receptor phosphorylation was reversed by a second SH2-inactivating mutation. We conclude that the c-Src SH3 domain function requires the SH2 domain in the case of the PDGF receptor, presumably because binding of c-Src to the receptor via its SH2 domain is a prerequisite for the SH3 domain function. In contrast, SH2 function is apparently not essential for the SH3 function in EGF receptor signaling.

Several studies have established that the Src nonreceptor protein-tyrosine kinase (PTK) family members c-Src, Fyn, and c-Yes are activated and become hyperphosphorylated following PDGF treatment of quiescent fibroblasts (3–5). All three can associate with the activated PDGF receptor protein-tyrosine kinase (RPTK) and form a stable complex. It has been estimated that about 5% of the total cellular population of the receptor and 5% of the Src family molecules associate (5). PDGF causes a shift of ~5% of Src molecules on one- and two-dimensional gels and phosphorylation of N-terminal Tyr and Ser residues (4). Courtneidge and colleagues (6) have analyzed the requirements for Src family kinases in PDGF receptor binding and phosphorylation. In one study, they established that the binding was direct and required the SH2 domain (6). Src family kinases also associate with and are activated by the CSF-1 RPTK, which is in the PDGF RPTK subfamily (7). Src family association with the SKY (Tyr3/BrdTIF) RPTK has also recently been described (8).

The human PDGF β receptor binding site for Src has been identified as Tyr(P)-579/Tyr(P)-581 located in the juxtamembrane region of the activated receptor dimer (9). A requirement for Src family PTKs in PDGF, CSF-1, and EGF-induced mitogenesis has been deduced, based on the ability of microinjected antibodies (cst.1) that recognize c-Src, Fyn, and c-Yes to block PDGF, CSF-1, and EGF mitogenic signaling (1, 2). In addition, kinase-inactive Src and Fyn mutants act as dominant negative inhibitors of PDGF-induced mitogenesis (1). The dominant negative phenotype is SH2-dependent.

We have recently identified Tyr-138, located in the murine c-Src SH3 domain, as the PDGF-induced site of N-terminal c-Src tyrosine phosphorylation. We have shown that phosphorylation of this residue, while not required for PDGF-induced activation, diminishes in vitro SH3 peptide ligand binding ability. This raised the issue of whether phosphorylation of Tyr-138 is needed for some event downstream of c-Src in PDGF signaling. To investigate this we tested whether PDGF and EGF mitogenic signaling was compromised in Src− cells expressing the Y138F c-Src mutant. At the same time we investigated several other c-Src mutants, including the kinase-inactive K297M mutant c-Src. In accordance with Twamley-Stein et al. (1), we found that the kinase-inactive K297M mutant c-Src inhibited PDGF-induced DNA synthesis by ~80%. An N-terminal fragment of c-Src consisting of the first 298 residues, Δ298, also inhibited to the same extent. To determine if Tyr-138 phosphorylation has a necessary role in PDGF and EGF receptor mitogenic signaling, the phosphorylation site

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*This work was supported by United States Public Health Service Grants CA14195 and CA39780. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PTK, protein-tyrosine kinase; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; CSF-1, colony stimulating factor-1; SH2, Src homology 2; SH3, Src homology 3; DMEM, Dulbecco-modified Eagle medium; FCS, fetal calf serum; mAb, monoclonal antibody; BrdU, bromodeoxyuridine; ss, super-selected; wt, wild type; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RPTK, receptor protein-tyrosine kinases.

2 M. A. Broome and T. Hunter, manuscript in preparation.
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EXPERIMENTAL PROCEDURES

In Vitro Mutagenesis and Subcloning—A 1.8-kilobase mouse neuronal c-Src cDNA (10) was cloned into the BamHI site of M13mp18. Single strand DNA template was made, and the neuronal insert of 6 amino acids was removed by deletion of the corresponding 18 nucleotides using a 24-mer mutagenic oligonucleotide CACAGCTCCTCCCT/GGTG TATTGGC synthesized on a Biosearch 8750 DNA synthesizer and the Amersham Corp. oligonucleotide-directed mutagenesis kit (number 1523). This deleted construct, equivalent to wild type mouse c-Src, was then used as a template for further mutagenesis. Additional mutations were made singly and in combination using either the Amersham kit or by the Dut/Ungh method (Bio-Rad) (11). N-terminal tyrosines 92, 94, 133, 138, and 151 were mutated to phenylalanine using CATAGT AACAGAGGGGCC, GTGACTCAAAGTCATAG, TGGGGATGAAACCG GTC, GCGCCAACAGTTGCT, TGTCGAGAAGACCTC mutagenic oligonucleotides, respectively. Lys-297 involved in ATP binding in the catalytic domain was mutated to methionine using the mutagenic oligonucleotide CCTGAGTCTCCCTGCAGGACATCGTGTCATAG. The C-terminal regulatory Tyr-529 to Phe mutation was made in the Y138F single-stranded template using the mutagenic oligonucleotide CTCACCTCTCCCTTCAGGCAGA. This construct was encoded by dideoxy sequencing using Sequenase (U.S. Biochemical Corp.), and the mutant insert was cloned into the 1.8-kilobase mouse neuroblastoma c-Src HindIII fragment, &n263, which contains the first 298 residues of wild type mouse c-Src with the single point mutation, 1296T. The construct encoding this protein was obtained by an in-frame during an attempt to produce the kinase-inactive K297R/M mutation. A frameshift was introduced in a stop codon insertion at residue position 299. Other murine c-Src mutations made included an SH2 mutant, R177K, an SH3 mutant, Y138F, and two SH3 mutants, Y133F and Y138F, and Y133F and Y138F. The SH2-inactivating mutation, R177S, was made in each of the SH3 Y133 and Y138 mutant constructs. The SH2 mutation was introduced, using CATAGTGTCATAG, at the GAC/CCG site in 0.5% FCS/DMEM media for 30–36 h. Quiescent cells were then infected at a multiplicity of 10 pfu/cell, and infected cells were scored for nuclear BrdU incorporation 72 h later. The statistical analysis of the results was as follows: 100% of c-Src expressing cells expressed the protein. For this study, mouse c-Srcs were generated by using a retroviral vector to establish Src immunofluorescence procedures. In brief, c-Src cDNAs were subcloned into the BamHI/HindIII sites of the mammalian retroviral vector pNeoMSV (a generous gift of J. Karn) carrying a G418 resistance selectable marker (12). Orientation was checked by using an internal Sad site in the c-Src cDNA. 

Cell Culture—Primary mouse embryo fibroblasts lacking c-Src, a generous gift from J. Brugge and J. Thomas (13), were derived from Src knockouts (Src-/-) mice (14) and immortalized by infection with SV40 large T expressed from a retroviral vector, ZPTEX with a Neo resistance gene (15). Cells were maintained in DMEM supplemented with 10% FCS (Intergen). Wild type and mutant murine c-Src retrovirus expression constructs were used to generate &epsilon2 retrovirus-producing cell lines (16) by calcium-phosphate transfection (17) followed by G418 selection; the Life Technologies Bio-Rad number 1813–110. A number of resistant &epsilon2 colonies were made for each cell line and propagated in DMEM supplemented with 10% calf serum plus 400 μg/ml G418. &epsilon2 retroviral supernatants (10 ml DMEM, 10% FCS) were harvested after 24-36 h from 30 to 40% confluent 10-cm plates and centrifuged for 10 min at 650 × g. Polybrene was added to the clarified supernatants to 2 μg/ml. Ten milliliters of &epsilon2 retrovirus-producer supernatants were added to 20–30% confluent 100-mm dishes that contained 1 ml of 0.5% FCS/DMEM media for 30–36 h. Quiescent cells were then infected at a multiplicity of 10 pfu/cell, and infected cells were scored for nuclear BrdU incorporation 72 h later. The statistical analysis of the results was as follows: 100% of c-Src expressing cells expressed the protein. For this study, S. Simon, personal communication. 

RESULTS

Generation of Src-/- Clonal Cell Lines Overexpressing Src Proteins—To assess the role of c-Src in PDGF and EGF signaling, cell lines overexpressing wild type and various mutant murine c-Srcs were generated by using a retroviral vector to infect a fibroblast cell line (Src-) isolated from mice engineered to lack c-Src (14). The Src-/- cell line, generated following SV40 large T antigen immortalization of primary fibroblasts from Src-/- mice (13), was found to respond mitogenically to both PDGF and EGF. Presumably, the requirement for Src family members in PDGF- and EGF-induced mitogenesis is supplied by the Fyn and c-Yes present in these cells. Transient expression of c-Src in Src-/- fibroblasts by infection resulted in higher expression than that of the cells expressing the protein. For this study, 

Traysolyl, 50 μM leupeptin, 100 μM NaN3, 1 mM dithiothreitol, and 1 μM phenylmethylsulfonyl fluoride and clarified at 15,000 × g for 10 min at 4 °C. Total cell lysates were prepared by the addition of SDS-PAGE sample buffer to 20 μg of Nonidet P-40 lysate followed by boiling for 5 min. Proteins were resolved by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) by semi-dry electrotransfer followed by blocking in 3% BSA/PBS containing 100 ng/ml 125I-protein A (ICN, 30 μCi/μg) in TBS for 1 h at room temperature followed by 3 washes for 5 min each room temperature with TBS. 125I-protein A was detected by phosphorimaging or autoradiography. Quantitation of the 125I-protein A signal was done using Image Quant software (Molecular Dynamics). Filters were stripped according to enhanced chemiluminescence procedures (Amersham Corp.). 

c-Src Immunofluorescence Staining—Immunofluorescent detection of c-Src proteins expressed in Src fibroblasts was accomplished by established Src immunofluorescence staining procedures. In brief, cells on 15-mm glass coverslips were fixed with 2% formaldehyde in PBS (1.5 mM KH2PO4, 0.8 mM Na2HPO4, 2.5 mM KCl, 136.8 mM NaCl, 0.5 mM CaCl2, and 10 mM NaCl) for 10 min at 4 °C, then washed in PBSAT (PBS with 0.1% BSA and 0.2% Triton X-100). The bound mAb was detected using a biotinylated horse anti-mouse IgG (Vector Labs, number BA2000) at a 1:300 dilution of the 1.5 mg/ml stock or 5 μg/ml final concentration followed by addition of fluorescein-laabeled avidin (Vector Labs, A-2011) at 10 μg/ml. The coverslips were placed in the dark for incubation and washing. Nuclei were stained with 2.5 μg/ml Hoechst 33258. Coverslips were mounted on microscope slides in 1% paraformaldehyde to reduce oxidation, and the edges were sealed with clear nail polish. A Leitz Laborlux S microscope with a 63× objective oil immersion lens was used to view slides at a final 200× magnification. Pictures were taken using Kodak ASA 400 color slide film. 

DNA Synthesis Assays—Clonal cells were plated on 15-mm glass coverslips. To induce quiescence, near-confluent coverslips were placed in 0.5% FCS/DMEM media for ~30–36 h. Quiescent cells were then labeled with 100 μM BrdU (Sigma, B-5002) and treated with 25 ng/ml PDGF BB (Amgen) or 100 ng/ml EGF (Amgen). Eighteen hours later, the cells were washed with PBS, fixed in methanol, and their DNA was acid-denatured according to the supplier's instructions prior to the anti-BrdU FITC-conjugated monoclonal antibody (BMB 1202-693). The detection of incorporated BrdU is a one-step antibody incubation. All incubations were carried out at room temperature according to the supplier's instructions. The final antibody concentration used was 1 μg/ml. The statistical analysis of the results was as follows: 100% of cells in one field were counted at random and scored for nuclear BrdU staining; three separate locations on the same coverslip were counted, and the average percentage of staining cells was calculated for each cell line tested. In addition, triplicate experiments were performed for each cell line on two independent clones. A standard deviation was calculated for each set of results for a given c-Src cell line and is presented in Fig. 3 and Fig. 4.
however, it was necessary to clone uniformly overexpressing cell populations by limiting dilution, since the presence of non-expressing cells in mitogenesis experiments would result in the inability to distinguish dominant negative effects potentially associated with a c-Src mutant. The retrovirus expression vector used has a G418Neo resistance (G418') gene, but these Src- cells already contain two copies of the G418' gene making it difficult to select clones using G418. However, by using a high concentration of G418 clones were obtained and screened for uniform c-Src expression by c-Src immunofluorescence staining using the Src-specific mAb 327 (Fig. 1) (the "Discussion" contains a description of the staining patterns observed). Attempts to select clones using a vector containing a hygromycin resistance gene were unsuccessful, since the cells were too sensitive to hygromycin.

Clones were derived overexpressing wild type (wt) c-Src, kinase-inactive K297M mutant c-Src, an N-terminal 729-residue c-Src fragment, Δ1296T, Y133F SH3 mutant c-Src, Y138F SH3 mutant c-Src, Y138FK297M SH3 kinase-inactive double mutant c-Src, R177K SH2 mutant, Y92FY94F SH3 double mutant c-Src, Y133FR177S SH3/SH2 double mutant c-Src, and Y138FR177S SH3/SH2 double mutant c-Src. Clonal cell lines were designated by an "ss" (super-selected) followed by the specific c-Src point mutation(s) listed. See Fig. 3 for a schematic diagram of each Src protein. Using the a¢st.1 antiserum (5), which is directed against the common C-terminal sequence in c-Src, Fyn, and c-Yes, for immunoblotting, we have measured the levels of endogenous Src family protein expression and the levels of exogenously expressed c-Src mutant proteins in the Y133F and Y138F mutant c-Src expressing cell lines. The Y133F and Y138F c-Src mutants migrated faster than wt c-Src from NIH3T3 cells and Fyn and c-Yes from Src- cells, and this enabled us to calculate the fold overexpression of these mutant proteins relative to the endogenous Fyn and c-Yes in these cell lines. As shown in Fig. 2A, in both cases we found Src levels to be about 4-fold higher than the combined endogenous Fyn and c-Yes levels present. For comparison, we estimated the levels of exogenously expressed Src in these clones to be no more than 5-fold over the level of endogenous c-Src in NIH3T3 cells based on Src-specific mAb 2–17 immunoblotting (Fig. 2B).

The stability of these clonal cell lines varied as did their morphology and growth kinetics. The clonal cell line characteristics are summarized in Table I. The +, ++, + + + designations represent the extent to which each clonal cell line examined compares with the parental Src- cell line for a given parameter. The ssWT c-Src cell line was stable and continued to overexpress c-Src indefinitely. The kinase-inactive ssK297M c-Src cell line, however, lost expression as measured by 35S labeling and immunoprecipitation and by c-Src immunofluorescence staining after 10 passages in 10% whole fetal calf serum-containing media despite continued G418 selection. ssY133F, ssY138F, ssY138FK297M, and ssΔ1296T Src- clonal cell lines also exhibited this phenomenon to a lesser extent. These cell lines were stable for 15 passages. In contrast, the ssR177K SH2 mutant, the ssY133FR177S SH3/SH2 double mutant, the ssY138FR177S SH3/SH2 double mutant, and the ssY92FY94F SH3 double mutant clonal cell lines were all stable. All clonal cell lines that exhibited instability initially showed a 20% incidence of failure to undergo cytokinesis as evidenced by multinucleate cells. Parental Src- cells exhibited right column, and Hoechst nuclear staining of the same field of cells is shown in the left column of each pair of panels. The names of the Src cell lines expressing wt and mutant Src proteins and their passage number are designated on the figure between each pair of panels. The scale bar is 10 μm.
less than a 5% incidence of this phenotype. At higher passages (passage 5 or greater) of these unstable cell populations, only a fraction of cells still expressed dominant negative mutant c-Src proteins, the incidence of cytokinetic failure correlated directly with those cells still expressing the dominant negative Src mutant proteins (Fig. 1). Cell line instability and incidence of cytokinetic failure were always associated with c-Src mutants that exhibited dominant negative effects on PDGF BB and EGF mitogenic signaling as described below. Although extensive characterization of the growth kinetics of the clonal cell lines overexpressing dominant negative c-Src mutants has not been performed, overall doubling times are increased ~1.5-fold in these cell lines as indicated in Table I by a single + in the growth rate column compared with + + + for the parental Src- or + + + for the wild type c-Src cell line, ssWT. In addition, these clonal lines tended to be larger, very flat, and adherent compared with the parental Src- and ssWT cell lines.

Catalytic Activity of c-Src Is Required for PDGF BB Mitogenic Signaling—To assess whether individual mutant Src proteins might have a dominant negative effect on PDGF-induced mitogenesis, clonal Src- cell lines were seeded on glass coverslips and grown to confluence. The cells were serum-starved to induce quiescence and then labeled with bromodeoxyuridine (BrdU) and treated with PDGF BB at 25 ng/ml. After 18 h, the cells were fixed, and incorporated BrdU was detected by staining with a fluorescein-conjugated anti-BrdU monoclonal antibody. The cells that responded to PDGF stimulation and incorporated BrdU into their DNA during S phase were stained specifically. Weakly staining nuclei routinely represented less than 3% of the total stained nuclei and were not counted. To make a quantitative determination of this inhibition, ~100 cells were counted at three locations on the coverslip, and the percentage of stained nuclei for each count was calculated along with the statistical variation. A summary of results obtained from this assay system in separate experiments for each cell line tested is shown in Fig. 3. In addition, two independent clonal cell lines for each Src mutant were examined.

When clonal cells overexpressing wild type c-Src, kinase-inactive K297M c-Src, and ΔI296T were tested, the kinase-inactive K297M mutant c-Src and the N-terminal 298 residue c-Src fragment, ΔI296T c-Src, both showed greatly reduced PDGF BB-induced DNA synthesis. Compared with the parental Src- and the ssWT cell lines, the inhibition of PDGF-induced DNA synthesis by these mutants was ~80%. Overexpression of wt c-Src in Src- cells routinely increased the DNA synthesis response slightly, potentially indicating that total Src family kinase levels are limiting for PDGF responses in the Src- cells. Note that the dominant negative effect of Src mutants we detected in the Src- cells is exerted over endogenous Fyn and c-Yes. Our results in stable cell lines agree with those obtained by Courtneidge and colleagues (1) in transient assays and indicate that our assay system is suitable for assessing dominant negative effects of Src family kinase mutants on PDGF receptor mitogenic signaling.

The c-Src Y133F and Y138F SH3 Mutants Inhibit PDGF BB Mitogenic Signaling Independent of Catalytic Activity—Having confirmed that kinase-inactive K297M mutant c-Src inhibits PDGF receptor mitogenic signaling in the Src- ssK297M clonal cell line, the kinase-active phosphorylation site mutant, Y138F c-Src, was examined. As a control, an adjacent tyrosine mutation, Y133F, which is still phosphorylated at Tyr-138 upon Tyr-138 upon PDGF treatment2 was also examined in this assay system. Clonal cell lines overexpressing Y138F mutant Src and Y133F mutant Src both exhibited a decreased PDGF BB DNA synthesis response indicating that both Y138F c-Src and Y133F c-Src have a dominant negative effect. A kinase-inactive Tyr-138 double mutant c-Src, Y138F K297M, was also examined, and Src- c-Src cell lines overexpressing it were found to have a similarly decreased PDGF BB DNA synthesis response (Fig. 3).

To determine whether other SH3 domain tyrosine mutations affect PDGF BB mitogenic signaling, the c-Src SH3 domain double tyrosine mutation Tyr-92/Tyr-94 to Phe 92/Phe 94 was utilized. Clonal Src- cell lines overexpressing Y92F Y94F were examined for effects on PDGF-induced DNA synthesis. Y92F Y94F mutant c-Src did not have a dominant negative effect

* M. A. Broome, unpublished observations.
To determine if c-Src carrying an SH2-inactivating mutation affects PDGF BB mitogenic signaling, the c-Src SH2 domain conserved Arg-177 (FLVRESE) was changed to Lys and clonal Src cells overexpressing R177K were generated and examined. R177K mutant c-Src did not have a dominant negative effect (Fig. 3).

To address whether the dominant negative effects seen with the Y133F and Y138F c-Src mutants are dependent on the SH2 domain, an SH2-inactivating mutation (R177S) was added to each single SH3 domain mutant. Src cell lines were isolated that overexpress the double mutants, ssY133FR177S and ssY138FR177S. These cell lines remained stable and exhibited growth characteristics and morphology similar to the parental Src and ssWT cell lines (Table I). Both of these cell lines were found to respond normally to PDGF BB indicating that the dominant negative effect of the single SH3 domain Y133F or Y138F mutants requires SH2 domain function.

The c-Src Y133F and Y138F SH3 Mutants Inhibit EGF Mitogenic Signaling—Recent evidence indicates that Src family function is required for EGF-induced mitogenesis in mouse fibroblasts (2). Therefore, we chose a subset of the Src mutant c-Src overexpressing cell lines to test whether they were defective in their EGF mitogenic response. Both ssY133F and ssY138F dominant Src cell lines had a diminished DNA synthesis response to EGF indicating that Y133F and Y138F mutant c-Src proteins had a dominant negative effect (Fig. 4). To address whether the EGF dominant negative effect observed with the Y133F and Y138F c-Src mutants is SH2-dependent, the SH2/SH3 double mutant dominant Src cell lines ssY133FR177S and ssY138FR177S were tested. However, neither double mutant cell line showed an increased response to EGF compared with the corresponding single SH3 domain mutant cell line indicating that SH2 domain function is not required for the dominant negative effect of the SH3 domain mutants on EGF-induced mitogenic signaling. We conclude that c-Src SH3 domain function is required for EGF receptor mitogenic signaling.

**DISCUSSION**

To ask whether Tyr-138 phosphorylation induced by PDGF stimulation is required for RPTK mitogenic signal transduction, dominant Src cell lines overexpressing the phosphorylation site mutant, Y138F murine c-Src, and other murine c-Src mutants were used to assay for inhibition of PDGF BB and EGF mitogenic signaling. Src cells provide a means to assess potential effects that mutant c-Src proteins have on signaling events in the absence of endogenous c-Src, although the Src mutants have to compete with the Fyn and c-Yes in these cells for any general Src family PTK targets. We used c-Src immunofluorescence staining to verify uniform clonal expression in the stable lines, and the c-Src staining patterns were comparable with those reported by others (19, 20). The negative control Src staining of Src cells appeared as a diffuse pattern. The apparent high intensity staining of this control is due to the fact that the pictures in Fig. 1 were taken using automatic light metering, so the actual intensity observed was much less. The strongest c-Src staining was perinuclear with all c-Src mutants examined, except the c-Src N-terminal 298 residue fragment, Δ1296T, which lacks the catalytic domain and was localized to podosome-like structures. Although we have not demonstrated formal co-localization with known focal adhesion components, others have reported a similar subcellular distribution of c-Src when c-Src is activated or when similar catalytic domain truncations are made (21, 22). No changes in c-Src localization were observed following PDGF stimulation.

We have shown that two SH3 domain mutants, Y133F and Y138F, inhibit both PDGF and EGF mitogenic signaling in a...
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The Src- clonal cell lines are listed below the x-axis along with a schematic diagram depicting the Src protein tested in each case. The percentage of anti-BrdU-staining cells from three trials for each cell clone tested with and without EGF is given on the y-axis along with the standard deviation. All clonal Src- cells expressing Src proteins were less than passage 3 following confirmation of clonal status by c-Src immunofluorescence staining.

Fig. 4. Inhibition of EGF-stimulated mitogenic responses. Results of the EGF mitogenic inhibition experiments are shown as histograms. The Src- clonal cell lines are listed below the x-axis along with a schematic diagram depicting the Src protein tested in each case. The percentage of anti-BrdU-staining cells from three trials for each cell clone tested with and without EGF is given on the y-axis along with the standard deviation. All clonal Src- cells expressing Src proteins were less than passage 3 following confirmation of clonal status by c-Src immunofluorescence staining.

dominant negative manner and deduce that the c-Src SH3 domain is required for PDGF and EGF mitogenic signaling independent of catalytic activity. Similar results have been obtained by Erpel et al. (23). We have also duplicated previous results showing that the kinase-inactive c-Src mutant acts as a dominant negative inhibitor of PDGF mitogenic signaling (1) and further demonstrated that an N-terminal 298-residue fragment of c-Src lacking the catalytic domain inhibits. Courtneidge and colleagues (23) used an cellular microinjection technique to assay the effects of Src mutants on mitogenesis, whereas we used clonal Src-expressing cell lines. Our approach enabled us to investigate whether mutant c-Src proteins elicited other cellular phenotypes, in addition to facilitating assays for dominant negative effects on specific mitogens.

Although extensive characterization of Src- clonal cell lines expressing mutant c-Src proteins was not carried out, we consistently found that clonal cell lines expressing PDGF/EGF dominant negative c-Src proteins exhibited slower growth in whole serum than cell lines that did not. The kinase-inactive K297M c-Src mutant had the most severe effect and increased the doubling time almost 1.5 times compared with parental Src- cells. Presumably, other mitogenic pathways that are unaffected by these Src mutants, such as those utilized by lysophosphatidic acid, permit isolation and propagation of Src- cell lines overexpressing these mutants. Lysophosphatidic acid is a major serum mitogen and like bombesin works via a G protein-coupled receptor (24). Neither mitogen is blocked by the Src family antibody αcst.1 indicating that they signal through a Src-independent pathway (2).

A Role for c-Src in Cytokinesis?—An interesting feature of these unstable dominant negative Src cell lines is the incidence of cytokinetic failure, characterized by a single cell having multiple nuclei, which was initially apparent in ~20% of cells in starting clonal populations. In later passages (5–8), when less than half of the cells were expressing dominant negative Src mutants, cytokinetic failure was nearly 100% coincident with the cells still expressing dominant negative Src proteins. Parental Src- cells exhibited less than a 5% incidence of this phenotype as did the wt Src overexpressing cell line, ssWT. A similar phenotype is exhibited by cells overexpressing a truncated T cell protein-tyrosine phosphatase, which has full enzymatic activity, but, unlike the full-length protein, fails to localize to particulate fractions (25). Nuclei within syncytial cells overexpressing this truncated protein-tyrosine phosphatase display asynchronous entry into mitosis. Although the same characterization has not been done with the dominant negative Src- cell lines, the similarity of these phenotypes suggests that mitotic targets of PTKs are important for major cytoskeletal changes to occur such as those required for cytokinesis. Interestingly, a requirement for Src family kinases at the entry into M phase has also recently been defined. The Src, Fyn, and Yes antibody, αcst.1, blocks cells in early prophase as does a GST-Fyn SH2 fusion protein (26). Consistent with these results, the Src SH3/SH2 double mutant cell lines ssY133FR177S and ssY138FR177S which respond to PDGF and EGF also do not display cytokinetic defects. Possibly the G2/M transition is delayed in the cells expressing dominant negative Src mutants, but the cells must ultimately creep past the G2/M boundary in order to grow. Perhaps the most severely retarded cells (10–20%) fail to undergo cytokinesis once they enter M phase, because of a temporal disruption in the normal sequence of mitotic events. However, it is also possible that wild-type c-Src has a direct role in cytokinesis, which needs to be tested.

c-Src SH3 Domain Function—Several reports have implicated the c-Src SH3 domain in addition to the SH2 domain in negative regulation of c-Src catalytic activity, using yeast ex-
pression systems where c-Src expression is toxic unless it is coexpressed with Csk, which results in C-terminal Tyr-527 phosphorylation and negative regulation of c-Src kinase activity (27–29). In this system both the c-Src SH3 and SH2 domains are needed to achieve negative regulation of c-Src kinase activity by Csk-catalyzed Tyr-527 phosphorylation. A recent crystal structure of Lck complexed with a synthetic Tyr(P)-containing peptide corresponding to the Tyr(P)-505 Lck C terminus suggests a way in which this negative regulation might be achieved by the intramolecular interaction of the C-terminal Tyr(P)-505 and the N-terminal SH3/SH2 region (30). However, this interaction involves an Lck dimer in the crystal structure, and no evidence exists to suggest that Lck forms a dimer in solution.

The biochemical characterization of SH3 domain function has led to the discovery of several point mutations that disrupt SH3 domain ligand binding ability. Two highly conserved residues, Trp-118 and Pro-133 (chicken c-Src numbering), when mutated to Ala/Arg and Leu respectively, abrogate SH3 domain ligand binding ability in vitro (31) or in the yeast Src-Csk coexpression system (32). Other conserved residues in the chicken c-Src SH3 domain include Tyr-90, Tyr-92, Arg-95, Trp-119, Tyr-131, Asn 135, and Tyr-136, many of which are found on the SH3 domain binding surface. Some of these residues, as well as others, have been shown to undergo chemical shifts upon peptide ligand binding indicating that they are directly affected by ligand binding (33). Conserved substitution of Tyr-136 (138 in murine c-Src), the PDGF receptor phosphorylation site, by Phe does not disrupt SH3 domain binding to AFAP110 as measured in the yeast two-hybrid system. However, a nonconservative Ala substitution inactivates SH3 function in the yeast Src-Csk coexpression assay system and in GST-fusion protein binding of the phosphatidylinositol 3′-kinase p85α subunit from NIH3T3 cell lysates (32). Actual affinities were not measured in these cases, so the Y136F mutant c-Src SH3 domain could even bind with a higher affinity than the wild type SH3 domain. An example of this effect occurs with the c-Src GST-SH3 R95W substitution, which apparently binds p85α more strongly than wild type (32). This point mutation occurs naturally in v-Src along with G63D and T96I c-Src point mutations. All three point mutations together convert c-Src into a transforming protein (34, 35). Other nonconservative point mutations that disrupt c-Src SH3 domain ligand binding function in the yeast Src-Csk system and in the GST-fusion protein binding system include Y90A, Y92A, W118A, and D99K (32), where the first three are in the c-Src SH3 domain binding groove.

Neither Y133F nor Y138F mouse c-Src transformed NIH3T3 cells (data not shown), in agreement with earlier reports (36), nor was their kinase activity elevated, consistent with the fact that SH3 binding function appears to be normal as assessed in the yeast two-hybrid system. This is in contrast to the effects of other mutations in the SH3 domain of c-Src, which result in activated transforming c-Src proteins (35, 37–39). Most of these other mutations are SH3 domain truncations and insertions, which would be more likely to disrupt SH3 structure significantly. SH3 domain point mutations are less well characterized in terms of biological activity, and an important question is whether nonconservative point mutations at the highly conserved Trp-118, Pro-133, and Tyr-136 c-Src residues correlate with transforming ability. Recent evidence indicates that not all nonconservative SH3 mutations result in transforming c-Src proteins. In fact, both the Y131A and Y136A (Y133 and Y138 in mouse c-Src) mutants that cannot support c-Src intramolecular negative regulation in the yeast Src-Csk system have now been found to be dominant negative inhibitors of PDGF mitogenic signaling (23). Conversely, a positive role for the SH3 domain in mitogenic signaling and transformation is indicated by the fact that fibroblast transformation by the Y527F mutant c-Src is abolished by deletion of the SH3 domain. Our preliminary data showing that the Y133F/Y529F double mutant transforms less efficiently than Y529F c-Src also imply a positive role for the c-Src SH3 domain in transformation.

In general, it appears that conservative SH3 domain mutations do not interfere with the intramolecular interaction that participates with the SH2 domain in the negative regulation of c-Src via Tyr-527 phosphorylation. Some nonconservative mutations can disrupt the interaction required for c-Src repression, but for such mutant c-Srcs to be transforming the mutation must still allow SH3 domain interactions with c-Src target proteins. Some nonconservative c-Src SH3 domain mutations (eg, Y131A and Y136A) that disrupt the SH3 domain binding function are dominant negative inhibitors of PDGF receptor signaling, and these mutations apparently affect as yet unknown c-Src SH3 domain function(s) in the PDGF receptor signaling pathway and in cellular transformation by the activated Y527F mutant c-Src (23). The Y133F and Y138F mutations also appear to affect some other role that the c-Src SH3 domain plays in PDGF receptor signaling, which may involve interactions with downstream targets.

Molecular Models for Dominant Negative Effects on RPTK Signaling—The recent studies by Courtneidge and colleagues (1, 2) have demonstrated a requirement for Src family kinases in PDGF, CSF-1, and EGF mitogenic signaling. The authors proposed a model for inhibitory effects of kinase-inactive Src kinases on PDGF mitogenic signaling in which the exogenously overexpressed kinase-inactive mutant Src kinases compete with the endogenous Src kinases for the PDGF receptor binding site, Tyr(P)-579/Tyr(P)-581 (9). This model predicts that the SH2 domain is essential and requires that the level of exogenously expressed kinase-inactive mutant Src kinase exceeds that of the endogenous wild type Src family kinase levels. It should be noted, however, that in our dominant negative mutant c-Src overexpressing cell lines, we found that the exogenous c-Src was only present at a 3–4-fold higher level than the combined levels of endogenous Fyn and c-Yes (Fig. 2). The effectiveness of dominant negative mutant c-Src under these conditions may indicate that the c-Src SH2 domain of the mutant has a higher affinity for the PDGF receptor than the c-Yes and Fyn SH2 domains, but this remains to be tested. Since Tyr(P)-579 is also a binding site for the adaptor protein, SHC (40), it is also possible that dominant negative Src kinases could prevent SHC or some other crucial effector molecule from associating with the activated receptor. In principle, this model should also apply to the CSF-1 and EGF receptors, although little evidence exists to show that c-Src binds the EGF receptor in vivo (41).

How do SH3 domain mutants act in a dominant negative fashion? Both Tyr-133 and Tyr-138 are located on the SH3 domain binding surface, and their mutation could affect interaction of protein ligands essential for mitogenic signaling with the SH3 domain. Note that the double SH3 tyrosine mutant Y92F/Y94F is not a dominant negative mutant in this assay which is significant, since both Tyr-92 and Tyr-94 are known to be at one end of the ligand binding groove (33). The Y133F and Y138F mutations could in principle increase or decrease binding affinities for individual ligands. Although there are many

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5 M. Brown, personal communication.

6 S. A. Courtneidge, personal communication.
phosphorylation disrupts SH3 binding, whose function is currently unknown. Tyr-138 phosphorylation, whose function is currently unknown. Tyr-138 phosphorylation disrupts SH3 binding in vitro and if this is true in vivo it could facilitate release and exchange of SH3 ligands that may be necessary for productive RPTK signaling to occur.

The SH2-independent inhibition of EGFR mitogenic signaling exhibited by the Y133FR177S and Y138FR177S mutant c-Src proteins is strikingly different from the requirement for SH2 function for the dominant negative effect on PDGF receptor signaling. Although c-Src only binds weakly to the activated EGFR receptor in vitro (41, 42), phosphorylation of the EGFR receptor by c-Src in vitro at sites not autophosphorylated by the receptor creates a binding site for the c-Src SH2 domain (42), and c-Src association with the EGFR receptor can be detected by co-immunoprecipitation following EGFR treatment of some cell lines (42). However, even if c-Src does associate with the activated EGFR receptor via its SH2 domain, the SH2 independence of the predominant negative effect of the Y133F and Y138F c-Src SH3 mutants on EGFR-induced DNA synthesis implies that the essential SH3 domain function of Src family members lies not at the level of the EGFR receptor itself but rather at the level of SH2 targets downstream from the EGFR receptor, which do not require an SH2-dependent interaction. The dominant negative effect could be due to competition between the c-Src SH3 mutant proteins and the endogenous Fyn and c-Yes proteins for Src family SH3 targets. We cannot determine from our results whether c-Src SH2 function is also essential in EGFR receptor signaling, but other studies suggest it may be. For instance, the potentiation of EGFR mitogenic signaling (43) and acetylation of EGFR-induced tyrosine substrate phosphorylation (44) require c-Src myristoylation, the SH2 domain, and catalytic activity (45). However, these studies did not address whether the c-Src SH3 domain is required for enhanced EGFR responsiveness, as our results would suggest.

Role of Cytoplasmic Protein-Tyrosine Kinases in Growth Factor Receptor Protein-Tyrosine Kinase Signal Transduction—RPTKs from more than one subfamily require the Src family for mitogenic signaling. What precise role do the Src family RPTKs play in RPTK mitogenic signaling? Many RPTK effector pathways are known, and some of these, such as phosphatidylinositol 3’-kinase, can also be stimulated by transforming c-Src mutants. However, the dominant negative effects of the relevant Src mutants imply that c-Src is activating a unique pathway not accessible to the RPTKs themselves. In this regard it has recently been shown that the dominant negative effects on PDGF mitogenic signaling exhibited by the kinase-inactive mutant c-Src are reversed by c-Myc overexpression (46), implying that there is a Src-dependent signaling pathway activated by PDGF involving c-Myc function downstream. It is not yet known, however, whether dominant negative effects of the SH3 mutants on PDGF or EGF mitogenic signaling are reversed by c-Myc.

One role for the association of Src family kinases would be to increase the range of RPTK substrates and associated effector molecules that can bind to activated RPTKs. For instance, proteins bound to the SH3 domain could be substrates for the RPTK. However, the requirement for Src catalytic activity indicates that something has to be phosphorylated by Src, and this could be an SH3-binding protein. Alternatively, RPTK-bound c-Src might be responsible for phosphorylating tyrosines in the RPTK itself, which cannot be autophosphorylated, thus providing additional SH2 domain binding sites. The identities of any Src family SH3 domain ligands involved in the PDGF receptor mitogenic pathway are unknown at present, but a number of Src SH3 binding proteins have been identified including p85α, paxillin, Shc, RasGAP, heterogeneous nuclear ribonucleoprotein K, SAM68, clone 10a, and others (31, 47–52).

In conclusion, several questions need to be answered. First and foremost, are the Y133F and Y138F c-Src SH3 domain ligands affinity higher or lower than those of the wild type? What are the physiologically relevant c-Src SH3 domain ligands in the PDGF and EGFR receptor mitogenic signaling pathway that are affected by these two c-Src mutants and are they the same for the two receptors? Is it only the activated receptor bound c-Src population that is required for PDGF receptor mitogenic signaling? The SH2 dependence of both Y133F and Y138F dominant negative mutants seen with PDGF BB mitogenic signaling implies that the PDGF receptor-bound population is important, but this could also be explained by a downstream SH2 domain target.

Acknowledgments—We thank Phil Soriano, Jim Thomas and Jan Brugge for the Src: fibroblasts; Thorsten Erpel and Sara Courtneidge for communicating results prior to publication; Suzy Simon for help and advice on immunofluorescence staining and microscopy; Megan Brown for the yeast two-hybrid analysis of Y136F chicken c-Src.

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