c-Src enhances the spreading of *src*^{-/-} fibroblasts on fibronectin by a kinase-independent mechanism

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We have explored the role of the tyrosine kinase c-Src in cellular adhesion. Fibroblasts derived from *src*^{-/-} mice (*src*^{-/-} fibroblasts) exhibit a reduced rate of spreading on fibronectin. This defect is rescued by expression of wild-type chicken c-Src. Analyses of mutants suggest that c-Src increases the rate of cell spreading in *src*^{-/-} fibroblasts through a kinase-independent mechanism requiring both the SH3 and SH2 domains. To further address the role of c-Src in adhesion, we examined the activity and subcellular distribution of c-Src during the adhesion of fibroblasts on fibronectin. We observed a transient increase in the specific kinase activity of c-Src accompanied by the partial dephosphorylation of the negative regulatory site Y527. Activation of c-Src is followed by its redistribution to newly formed focal adhesions. These results suggest that the enzymatic activity and subcellular distribution of c-Src are coordinately regulated during cellular adhesion and that c-Src can affect adhesion by a kinase-independent mechanism.

[Key Words: c-Src; cellular adhesion; *src*^{-/-} fibroblasts; fibronectin; focal adhesions; specific kinase activity; subcellular distribution]

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The tyrosine kinase c-Src belongs to a family of cytoplasmic tyrosine kinases whose role in cellular transformation has been well characterized (for review, see Cooper 1989). Despite the potent action of oncogenic forms of Src in deregulating cell growth (e.g., the viral transforming protein v-Src), the normal cellular function of the proto-oncogene c-Src is not understood. In addition, the potential role of c-Src in regulating mitotic events (Chackalaparampil and Shalloway 1988; Morgan et al. 1989; Shenoy et al. 1989) and its high level of expression in terminally differentiated cells suggest that c-Src may influence cellular events unrelated to growth control. Recent genetic studies with mice containing a homozygous disruption of the *c-src* gene implicate c-Src in the physiological function of osteoclasts (Soriano et al. 1991; Boyce et al. 1992, 1993) but have not led to any clear understanding of the cellular function of c-Src.

Recent attempts to understand the function of c-Src have focused on the analysis of protein sequence motifs common to Src family members and believed to mediate protein-protein interactions. Most notable are the conserved SH2 and SH3 domains in the amino-terminal half of c-Src, which are shared with a number of proteins

involved in signal transduction. The SH2 domain interacts with phosphotyrosine-containing sequences (Mayer et al. 1991; Pawson and Gish 1992), whereas the SH3 domain is thought to mediate interactions with proline-rich regions (Ren et al. 1993). The amino terminus of c-Src also contains a "unique" domain (amino acids 2–90), which shares little homology with other Src family members and is involved in membrane attachment (Kaplan et al. 1990).

Whereas the amino-terminal half of c-Src mediates protein/protein interactions, the carboxy-terminal half contains the kinase domain and two major tyrosine phosphorylation sites that regulate kinase activity. Autophosphorylation at tyrosine 416 (Y416) is required for full kinase activity (Piwnicka-Worms et al. 1987; Kmiecik et al. 1988), and phosphorylation at tyrosine 527 (Y527) inhibits activity (Courtneidge 1985; Kmiecik and Shalloway 1987). Normally, Y527 is phosphorylated extensively in the cell by the carboxy-terminal Src kinase (CSK) (Imamoto and Soriano 1993), resulting in an intramolecular interaction between phospho-Y527 and the SH2 domain ("closed" conformation) thought to inhibit kinase activity (MacAuley and Cooper 1989). Deleting Y527 (as in v-Src), mutating it to a phenylalanine (Y527F), and presumably dephosphorylating Y527 in vivo disrupts this interaction and results in a highly active kinase with exposed amino terminal domains ("open" conformation) (Roussel et al. 1991; Liu et al. 1993) that now may interact with target proteins.

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Recently we have shown that the disruption of this intramolecular interaction results in the alteration of the subcellular location of c-Src (Kaplan et al. 1994). Whereas c-Src normally associates with endosomal membranes (David-Pfeuty and Nouvian-Dooghe 1990; Kaplan et al. 1992), decreased phosphorylation at Y527, either by amino acid substitution or expression of c-Src in cells lacking CSK, results in the association of c-Src with focal adhesions (Kaplan et al. 1994). The amino terminus of c-Src, specifically the myristylation site and the SH3 domain, is required for the association of c-Src with focal adhesions. Significantly, the amino-terminal half of c-Src can alter the structure and biochemical properties of focal adhesions even in the absence of c-Src kinase activity (Kaplan et al. 1994). The potential redistribution of c-Src to focal adhesions following Y527 dephosphorylation suggests that c-Src may normally regulate events related to cellular adhesion.

Several lines of evidence have implicated transforming mutants of c-Src in the regulation of cellular adhesion. Biochemical evidence links v-Src with the phosphorylation of integrin receptors in transformed cells and may be partially responsible for the alterations in the adhesive properties of Src-transformed cells (Hirst et al. 1986; Nigg et al. 1986; Pasquale et al. 1986; Aneskievich et al. 1991). The translocation of c-Src to the cytoskeleton during platelet activation is dependent on the integrin α IIb/ β III, arguing for a functional connection between integrins and c-Src (Horvath et al. 1992; Clark and Brugge 1993). In addition, the presence of c-Src in neuronal growth cones (Maness et al. 1988; Sobue and Kanda 1988) and the reduction in the rate of axon growth in Src-deficient neurons implicates c-Src in adhesion events involved in neuronal development (Ignelzi et al. 1994). Finally, the SH2-dependent association of c-Src with the focal adhesion kinase (FAK) during adhesion of NIH-3T3 fibroblasts on fibronectin provides a direct biochemical link between c-Src and a tyrosine kinase thought to be involved in cellular adhesion (Guan and Shalloway 1992; Kornberg et al. 1992; Turner et al. 1993; Cobb et al. 1994; Schaller et al. 1994; Schlaepfer et al. 1994; Eide et al. 1995; Schaller and Parsons 1995).

To assess the role of c-Src in cellular adhesion more directly, we have examined c-*src*-deficient (*src*^{-/-}) fibroblasts, derived from mice homozygous for a c-*src* gene disruption (Soriano et al. 1991), and have shown them to be defective in spreading on fibronectin. The defect in *src*^{-/-} fibroblasts can be rescued by the expression of c-Src. In addition, we observed a transient increase in kinase activity of c-Src during cell attachment to fibronectin, followed by the redistribution of c-Src to newly formed focal adhesions. However, complementation of the adhesion defect in *src*^{-/-} fibroblasts does not require the kinase domain of c-Src, and genetic analysis suggests that the SH2 and SH3 domains are sufficient to positively regulate cellular adhesion. These results argue strongly that the association of c-Src with focal adhesions contributes to the ability of fibroblasts to spread on fibronectin.

Results

src^{-/-} fibroblasts are defective in early cellular adhesion events

To examine the involvement of c-Src in cellular adhesion, fibroblasts lacking c-Src (*src*^{-/-}) were compared with the same fibroblasts expressing chicken c-Src (*src*^{-/-} + c-Src) after plating on fibronectin. We evaluated the behavior of fibroblasts during adhesion on fibronectin at several distinct stages as depicted in Figure 1: (1) the *attachment* of round, refractile cells to fibronectin through cell-surface integrins (Fig. 1A,B; <5 min at 37°C), (2) the *spreading* of cells, characterized by a decrease in cell refractility and the appearance of projections around the cell periphery (Fig. 1E; 10–15 min at 37°C), and, finally, (3) the *flattening* of cells apparent when normal fibroblastic morphology is achieved (Fig. 1F; 45 min at 37°C). As depicted in Figure 1, we observed a striking disparity between the spreading of *src*^{-/-}

Figure 1. Adhesion of *src*^{-/-} and *src*^{-/-} fibroblasts expressing c-Src. *src*^{-/-} fibroblasts and *src*^{-/-} fibroblasts expressing c-Src were plated on fibronectin-coated dishes for 5 min (A,D), 15 min (B,E), and 45 min (C,F) at 37°C and were photographed by phase-contrast microscopy. (B) Open arrowheads indicate examples of attached, refractile cells; (E) solid arrowheads indicate examples of spread cells (scale bar, 15 μm).

fibroblasts and *src*^{-/-} fibroblasts expressing c-*src* on fibronectin (Fig. 1, cf. B and E).

To further explore this observation, we developed a quantitative assay to measure the extent of cell spreading and flattening during cellular adhesion. By incubating cells on fibronectin-coated dishes for various times and washing off attached cells and leaving cells that had either flattened or spread, we were able to distinguish the various stages of adhesion outlined above. The percentage of cells that remained (adherent cells) was determined based on the level of an internal enzymatic marker [β -hexosaminidase (Hall et al. 1990)]. Antibodies to the fibronectin receptor blocked adhesion of all cells efficiently in this assay, demonstrating the specificity of the assay for integrin-mediated adhesion (Fig. 2A). Multiple lines of *src*^{-/-} fibroblasts consistently adhered about threefold less well to fibronectin-coated plates than *src*^{-/+} fibroblasts (Fig. 2A). The difference in cell adhesion required incubation at 37°C, as cells incubated at 4°C exhibited no difference in binding to substrate (data not shown). These results suggest that *src*^{-/-} fibroblasts are defective in some aspect of cellular adhesion following binding of integrins to fibronectin.

Expression of low levels of chicken c-Src in *src*^{-/-} fibroblasts [about three to four times over endogenous levels in *src*^{-/+} cells; data not shown and as described previously (Kaplan et al. 1994)] restored wild-type adhesion (Fig. 2B). The reversible nature of the adhesion defect in *src*^{-/-} fibroblasts suggests that there have been no irreversible changes in the adhesion machinery of the cell in the course of cell line generation. In addition, the increase in adhesion was not attributable to clonal variation between cell lines; multiple independently derived *src*^{-/-} cell lines expressing c-Src exhibited an average increase in adhesion of 2.3-fold when compared with the *src*^{-/-} parental cell line.

The differences in adhesion between *src*^{-/-} and c-*src*-expressing fibroblasts were most apparent after cell attachment but prior to cell flattening, suggesting that a specific stage of cellular adhesion is affected by the absence of c-Src. The initial attachment of cells to substrate (1–2 min at 37°C) was equal for all cells; >95% of cells attached to the substrate but were removed easily during washing (data not shown). The reduction in adhesion exhibited by *src*^{-/-} fibroblasts was most apparent after 5–10 min at 37°C, concomitant with cell spreading. After 25 min at 37°C, *src*^{-/-} and c-*src*-expressing cells could no longer be distinguished (Fig. 2C). During similar incubation times, adhesion on plates blocked with BSA was negligible, ruling out nonspecific binding of cells in the assay. In addition, differences in cellular adhesion were not observed in cells plated on collagen-coated wells (Fig. 2C), suggesting that c-Src may function specifically during fibronectin-mediated cell spreading.

c-Src enhances the ability of fibroblasts to spread on fibronectin

To further characterize the role of c-Src during cellular adhesion, we used phase-contrast microscopy to exam-

Figure 2. *src*^{-/-} fibroblasts exhibit a defect in adhesion on fibronectin. (A) Two independently derived *src*^{-/-} cell lines (open bars) and a *src*^{-/+} cell line (shaded bars) were plated on fibronectin for 5 min at 37°C with [anti-fibronectin receptor antibody (α -FnR); 1/50 dilution] or without anti-fibronectin receptor antibody (untreated), and the percentage of bound cells was determined. (B) A *src*^{-/-} cell line (open bars), *src*^{-/+} (shaded bars), and the same *src*^{-/-} cell line expressing chicken c-Src (solid bars) were analyzed after plating on fibronectin for 5 min. (C) A *src*^{-/-} cell line (broken line) and the same cell line expressing chicken c-Src (solid line) were plated on fibronectin-coated plates ($\circ$), collagen-coated plates ($\bullet$), or BSA-coated plates ($\blacktriangle$) for the indicated times. The percentage of cells that remained bound are displayed as a function of time at 37°C.

ine the rate of cell spreading following plating of *src*^{-/-} fibroblasts on fibronectin-coated plates. A direct comparison of *src*^{-/-} fibroblasts with cells expressing c-Src revealed a dramatic delay in the ability of *src*^{-/-} fibro-

blasts to spread on fibronectin. As expected, little cell spreading was observed in either cell line after 5 min at 37°C, and both cell lines attached to fibronectin-coated plates equally well (data not shown), suggesting that integrin receptors bind to fibronectin normally in the two cell lines. In contrast, after 15 min at 37°C, cells expressing c-Src exhibited a significant amount of cell spreading, whereas little change in cell morphology was observed in *src*^{-/-} fibroblasts (Fig. 3A,B and Fig. 1B,E, respectively). The amount of cell spreading was quantitated by photographing multiple microscope fields and counting the number of spread cells as a percentage of cells in the entire field (see Materials and methods). Results obtained using different cell lines in 11 experiments (Fig. 3C) consistently showed an 8.1-fold increase in cell spreading in fibroblasts expressing c-Src, when compared with the parental *src*^{-/-} cell line. *src*^{-/-} fibroblasts maintained at 37°C for longer time periods (~45 min) eventually spread and became as flat as c-Src-expressing cells, indicating that c-Src expression accelerates the rate of cell spreading but is not absolutely required for cells to flatten on fibronectin (Fig. 1C). These results support a role for c-Src during the spreading of fibroblasts on fibronectin and are consistent with the altered adhesive properties observed in *src*^{-/-} fibroblasts.

Activation of c-Src kinase during cellular adhesion

To address the mechanism by which c-Src influences cell spreading, we examined the biochemical properties of c-Src during attachment of cells to fibronectin. *src*^{-/-} cells expressing chicken c-Src were incubated on fibronectin-coated dishes for indicated times at 37°C to characterize the behavior of c-Src during the major stages of adhesion (discussed above; Fig. 1). After cells had been incubated for indicated times, they were fractionated into detergent-soluble and detergent-insoluble

fractions to assess the relative subcellular distribution of c-Src. Previous studies have suggested that the degree of detergent-insoluble Src correlates with focal adhesion association (Kaplan et al. 1994). Following immunoprecipitation of c-Src, both the relative levels and kinase activity of c-Src were measured in each fraction.

Greater than 95% of cells had attached to fibronectin-coated plates after 5 min at 37°C (Fig. 1A), and detergent fractionation revealed that the majority of c-Src remained in the soluble fraction (Fig. 4B). The amount of c-Src protein was used to calculate the specific kinase activity of c-Src immunoprecipitated from each fraction. Plating on fibronectin induced a 3.4-fold increase in the total kinase activity of c-Src (i.e., combined activities in the soluble and insoluble fractions), and a 5.7-fold increase in the detergent-insoluble fraction, when compared with c-Src isolated from cells plated on BSA-coated wells (Fig. 4C). Following cell spreading at 15 min (15', refer to Fig. 1) the kinase activity of c-Src decreased to control levels. The decrease in kinase activity was accompanied by an increase in the detergent insolubility of c-Src (60% detergent insoluble, Fig. 4B). Prolonged incubation of cells had no permanent effect on relevant regulatory mechanisms, as c-Src was readily activated when cells were replated on fibronectin-coated wells after first flattening on BSA-coated wells (Fig. 4C, 15Fn*). In addition, c-Src isolated from trypsinized cells exhibited a low level of kinase activity, suggesting that c-Src is regulated primarily during adhesion and not during cell rounding (data not shown). In multiple experiments, comparable levels of activation (average $5.3 \pm 1.4 \times$ increase in specific kinase activity of detergent-insoluble c-Src, $n = 12$) and redistribution of c-Src were observed to correlate with the attachment and spreading of cells on fibronectin. The simultaneous regulation of both the kinase activity and subcellular distribution of c-Src suggests that these events may be coordinated through dephosphorylation of Y527.

Figure 3. c-Src enhances the rate of cell spreading of *src*^{-/-} fibroblasts on fibronectin. *src*^{-/-} fibroblasts (A) and *src*^{-/-} fibroblasts expressing chicken c-Src (B) were plated on fibronectin-coated plates for 15 min at 37°C, and individual fields from multiple experiments were photographed by phase-contrast microscopy. (C) Cell spreading was quantitated in selective fields by calculating the percentages of cells that had spread in a single microscope field (five fields per bar, >500 cells/field; error bars indicate the standard deviation from the mean among the five fields of cells counted). Independent experiments were conducted to analyze the percentage of cell spreading in two *src*^{-/-} cell lines and subclones expressing chicken c-Src ($n = 11$, scale bar, 15 μ m).

Figure 4. The kinase activity of c-Src is increased transiently after plating cells on fibronectin. *src*^{-/-} fibroblasts expressing c-Src were plated on fibronectin-coated plates for the indicated times (see Fig. 1) and fractionated in Triton X-100-containing buffer for 1 min. Detergent-soluble (S, open bar) and detergent-insoluble (I, shaded bar) fractions were immunoprecipitated with mAb 327. Cells in lane 15'Fn* were first plated on BSA-coated wells for 45 min, replated for 15 min on fibronectin-coated wells, and analyzed similarly. Immunoprecipitates were analyzed by measuring the incorporation of ³²P into the exogenous substrate enolase (A) and immunoblotting with mAb 327 (B); the upper band is c-Src and the lower band represents the immunoglobulin heavy chain, recognized by the ¹²⁵I-conjugated anti-mouse secondary antibody. The specific kinase activity was calculated by dividing the intensity of the enolase bands by the intensity of the c-Src bands, as determined by PhosphorImager analysis.

Activation of the c-Src kinase is accompanied by the dephosphorylation of tyrosine 527 during cellular adhesion

The role of Y527 during cellular adhesion was addressed by the indirect analysis of c-Src tyrosine phosphorylation *in vivo*. We examined the changes in phosphotyrosine of a mutant c-Src protein containing a single amino acid substitution at the autophosphorylation site (c-SrcY416F). As expected, no tyrosine phosphorylation was observed by antiphosphotyrosine immunoblotting in a mutant c-Src protein containing amino acid substitutions at both Y416 and Y527 (data not shown). Thus, the recognition of c-SrcY416F by anti-phosphotyrosine antibodies depends on the level of phosphorylation of Y527, allowing us to monitor phosphorylation of this site by immunoblotting.

src^{-/-} fibroblasts expressing c-SrcY416F were plated on fibronectin-coated wells for 5, 15, and 45 min at 37°C. Cells were lysed and c-SrcY416F was isolated by immunoprecipitation for analysis by *in vitro* kinase as-

say and anti-phosphotyrosine immunoblotting (Fig. 5). c-SrcY416F from cells incubated at 37°C for 5–15 min showed a 37%–45% decrease in relative levels of phosphotyrosine staining, which then returned to the levels observed in unplate cells (Fig. 5A,B). Consistent with its negative regulatory role, the relative decrease in phosphotyrosine levels was inversely related to the activation of the kinase during cellular adhesion. These data are in agreement with previous studies that have implicated dephosphorylation of Y527 with increases in the kinase activity of c-Src. In addition, the increase in detergent insolubility of c-Src observed during cellular adhesion may result from the dephosphorylation of Y527, as predicted by our previous characterization of the regulation of c-Src distribution in fibroblasts (Kaplan et al. 1994) (Fig. 4B).

Redistribution of c-Src during adhesion on fibronectin

To examine the subcellular distribution of c-Src during adhesion in more detail, we plated fibroblasts on fibronectin-coated coverslips for indicated times and characterized the distribution of c-Src and phosphotyrosine-

Figure 5. Dephosphorylation of Y527 is inversely related to the increase in kinase activity of c-Src after plating cells on fibronectin. *src*^{-/-} cells expressing c-SrcY416F, plated on fibronectin-coated plates for the indicated times, were lysed and immunoprecipitated with mAb 327. Immunoprecipitates were analyzed by measuring the incorporation of ³²P into the exogenous substrate, enolase (data not shown), immunoblotting with mAb 327 (A; α-Src) and anti-phosphotyrosine antibodies (A; α-p-tyr); the upper band is c-Src, and the lower band represents the immunoglobulin heavy chain, recognized by the ¹²⁵I-conjugated anti-mouse secondary antibody. The specific phosphotyrosine/c-Src ratio was calculated by dividing the intensity of the phosphotyrosine bands by the intensity of the c-Src bands (●). The specific kinase activity of c-Src was calculated as in Fig. 6 (○).

containing proteins. Both standard and optical-sectioning immunofluorescence microscopy were used to evaluate the distribution of c-Src during the major stages of cellular adhesion. Fibroblasts expressing c-Src were allowed to attach to fibronectin-coated coverslips for <10 min; under these conditions, very few cells (<1%) had spread. Optical sections at the bottom of the cell did not reveal extensive phosphotyrosine-containing structures, consistent with the lack of focal adhesion formation at this early stage of cellular adhesion (Fig. 6D). Some cells contained a small amount of c-Src at the bottom of the cell, specifically at the leading edge of the attached cell membrane (open arrowhead, Fig. 6C). However, the majority of c-Src was located in optical sections at the top of newly attached cells (open arrow, Fig. 6A) in a perinuclear location that costains with endosomal membrane markers (data not shown). Significantly, this population of c-Src at the top of the cell also costained with phosphotyrosine-containing proteins in the same optical section (arrow; Fig. 6A,B); this particular staining pattern was only observed during cell attachment to fibronectin. The high levels of phosphotyrosine staining may represent either c-Src, which appears to be highly autophosphorylated (K.B. Kaplan, unpubl.), or other protein substrates of active c-Src. In either case, the high levels of phosphotyrosine are consistent with the elevated kinase activity of c-Src following cell attachment.

Fibroblasts begin to spread after ~20 min on fibronectin-coated coverslips (Fig. 6E–H), and optical sections from the bottom of the cell revealed c-Src in a symmetrical pattern of fiber-like structures at the periphery of the adhering cell (Fig. 6G). Costaining of the same cell with anti-phosphotyrosine antibodies revealed similar structures that were also recognized by antibodies against vinculin, another marker of focal adhesions (Fig. 6H; data not shown). A significant proportion of c-Src was also observed at the microtubule-organizing center in optical sections from the middle of the cell (Fig. 6E); costaining experiments with endosomal markers suggest that this staining represents an endosomal membrane compartment (data not shown; Kaplan et al. 1992). Significantly, the population of c-Src associated with endosomal membranes does not costain with anti-phosphotyrosine antibodies, further distinguishing c-Src in the middle and at the bottom of the adhering cell (Fig. 6E,F).

Following the completion of cell spreading, fibroblasts that had flattened on fibronectin maintained a significant proportion of c-Src associated with structures at the bottom of the cell (open arrow, Fig. 7A) that costain with focal adhesion markers (data not shown). In contrast, cells plated on uncoated coverslips exhibit a predominantly endosomal c-Src staining pattern (Fig. 7B). The altered distribution of c-Src in fibroblasts flattened on fibronectin allowed us to analyze these changes by detergent fractionation.

On the basis of previous studies, the shift of c-Src to focal adhesions should also result in an increase in its detergent insolubility (Kaplan et al. 1994). The detergent solubility of c-Src was examined in cells plated on uncoated, BSA-coated, and fibronectin-coated plates. Fol-

Figure 6. c-Src associates with focal adhesions during cell spreading. Cells plated for 10 min (A,B,C,D), or 20 min (E,F,G,H) on fibronectin-coated coverslips were stained with mAb 327 (A,C,E,G) and anti-phosphotyrosine antibodies (B,D,F,H). Indirect immunofluorescence was analyzed by optical sectioning microscopy (see Materials and methods), and sections at the middle of the cell (A,B), at the top of the cell (E,F) and at the bottom of the cell (C,D,G,H) are presented (scale bar, 10 μ m).

lowing flattening (12–16 hr), cells were lysed for various times in Triton X-100 buffer [Fig. 7C–E; (30s) 30 sec, (1') 1 min, 2, 4, 6, 10 min]. On either uncoated or BSA-coated wells, c-Src was predominantly soluble, even at early time points of lysis (30 sec, Fig. 7C,D). In contrast, c-Src in cells flattened on fibronectin-coated wells was predominantly insoluble at the earliest points of lysis (30 sec, 1 min; Fig. 7E), and a significant portion remained insoluble even at later time points (4 min, Fig. 7C). Thus,

Figure 7. c-Src remains associated with focal adhesions in cells adherent on fibronectin. *src*^{-/-} fibroblasts expressing c-Src were plated on fibronectin-coated (A) or uncoated (B) coverslips for 12 hr and stained with mAb 327 (arrow indicates focal adhesions; scale bar, 10 μ m). Cells expressing c-Src plated for 12 hr on uncoated wells (C), BSA-coated wells (D) and fibronectin-coated wells (E) were fractionated in Triton X-100-containing buffer for indicated times and detergent-soluble (S) and detergent-insoluble (I) fractions were immunoprecipitated and immunoblotted with mAb 327. The upper bands in C, D, and E represent the 60-kD c-Src protein and the lower bands represent the immunoglobulin heavy chain, recognized by the anti-mouse secondary antibody.

both detergent fractionation and immunofluorescence analyses indicate that fibronectin-mediated adhesion results in the redistribution of c-Src from endosomal membranes to focal adhesions. This redistribution occurs rapidly (within the first 10 min of adhesion) and is maintained as long as the cells are in contact with fibronectin. Association of c-Src with focal adhesions in cells plated on fibronectin is consistent with the defect in cell spreading exhibited by *src*^{-/-} fibroblasts and argues further that c-Src is involved in the regulation of cellular adhesion.

The enhancement of cell spreading by c-Src is independent of kinase activity and requires the SH2 and SH3 domains

The ability of c-Src to enhance cell spreading may result from the transient increase in its kinase activity, the redistribution of c-Src to focal adhesions, or some combination of these events. To address the importance of the enzymatic activity of c-Src during adhesion, we expressed a kinase-deficient c-Src mutant in *src*^{-/-} fibroblasts; this mutant protein contains a single amino acid change in the ATP-binding site (K295M). Surprisingly, expression of this mutant corrected the adhesion defect in *src*^{-/-} fibroblasts. Thus, the kinase activity of c-Src is not required to complement the cell spreading defect in *src*^{-/-} fibroblasts (Fig. 8A,E). In addition, the kinase-deficient protein redistributed normally during adhe-

Figure 8. c-Src enhances cell spreading in *src*^{-/-} fibroblasts by a kinase-independent mechanism that requires SH2 and SH3. *src*^{-/-} fibroblasts expressing SrcK⁻ (A), Src251 (B), Src251 Δ SH3 (C), and Src251R175L (D) were plated on fibronectin-coated plates for 15 min at 37°C, and individual fields from multiple experiments ($n = 8$) were photographed by phase-contrast microscopy. In E and F, independent experiments were conducted to analyze the percentage of cell spreading in *src*^{-/-} cell lines expressing SrcK⁻, Src251, Src251R175L, and Src251 Δ SH3 after plating on fibronectin and as described in Fig. 3 (scale bar, 15 μ m).

sion, suggesting that the association of c-Src with focal adhesions is sufficient to stimulate cell spreading without an increase in enzymatic activity or without any kinase activity at all (data not shown).

The relationship between focal adhesion association and cell spreading was addressed further by examination of mutant c-Src proteins. A mutant protein lacking the entire carboxy-terminal half of c-Src (Src251) associates with focal adhesions (Kaplan et al. 1994) and was able to induce a 10.6-fold increase in the rate of spreading of *src*^{-/-} fibroblasts (Fig. 8B,E). In general, cells expressing Src251 spread faster (1.4-fold) than cells expressing wild type c-Src (Fig. 8E), perhaps reflecting the greater level of mutant Src protein associated with focal adhesions (Kaplan et al. 1994). These results further support the kinase-independent influence of c-Src on cell spreading and argue for a link between the association of c-Src with focal adhesions and the enhancement of cell spreading.

To identify amino-terminal domains of c-Src required to enhance cell spreading, we expressed additional mutant proteins and determined their effects on the spreading of *src*^{-/-} fibroblasts. A deletion of the SH3 domain (Src251ΔSH3) has been shown previously to abolish the association of Src251 with focal adhesions (Kaplan et al. 1994). Src251ΔSH3 was unable to enhance spreading of *src*^{-/-} fibroblasts (Fig. 8C,F), suggesting that c-Src must associate with focal adhesions to enhance cell spreading.

We examined the importance of the SH2 domain for cell spreading by expressing a protein containing a single amino acid change (R175L) in the conserved region of the SH2 domain. This mutation has been shown to abolish phosphotyrosine binding in vitro (Bibbins et al. 1993) but is still able to associate with focal adhesions (Kaplan et al. 1994). Despite its ability to associate with focal adhesions, Src251R175L did not enhance cell spreading on fibronectin (Fig. 8D,F). Cells expressing Src251R175L showed a consistently reduced ability to spread when compared with the parental *src*^{-/-} fibroblast cell line (Figs. 3A and 8D,F). This result implies that focal adhesion association is required, but not sufficient, for the kinase-independent enhancement of cell spreading by c-Src and that both the SH2 and SH3 domains are involved in regulating cellular adhesion.

Discussion

We have shown that c-Src is regulated and functions in a kinase-independent manner during the early steps of fibronectin-mediated cellular adhesion of fibroblasts. During cell attachment to a fibronectin-coated surface, a reduction in Y527 phosphorylation accompanies an increase in the kinase activity of c-Src and its redistribution to newly formed focal adhesions. Furthermore, we have characterized a phenotype in *src*^{-/-} fibroblasts that has not been observed directly in *src*^{-/-} mice. Although the timing of the defect coincides with the changes in c-Src kinase activity and subcellular distribution during the spreading of fibroblasts on fibronectin,

c-Src is able to enhance the spreading of *src*^{-/-} fibroblasts by a kinase-independent mechanism. In addition, the mechanism by which c-Src enhances cell spreading appears to depend on the redistribution of c-Src to focal adhesions and involves the SH2 and SH3 domains of c-Src. The regulation of c-Src and the phenotype of *src*^{-/-} fibroblasts during adhesion on fibronectin strongly argue that c-Src functions during the early stages of cellular adhesion.

Regulation of c-Src during cellular adhesion on fibronectin

Our previous work showed that the single amino acid substitution Y527F, or expression of c-Src in cells lacking CSK (*csk*^{-/-}), was sufficient to allow the association of c-Src with focal adhesions (Kaplan et al. 1994). We propose that redistribution of c-Src during cellular adhesion represents a biologically relevant regulation of the subcellular distribution of c-Src through Y527 dephosphorylation. These results also represent a novel regulatory scheme for coordinating the activity of a kinase and its subcellular distribution. Our results suggest that c-Src becomes activated through Y527 dephosphorylation while associated with endosomal membranes. Exposed amino-terminal domains then mediate the association of c-Src with focal adhesions, where it becomes deactivated. The redistribution and subsequent inactivation of c-Src in focal adhesions may be required to maintain the dynamic nature of the adhesion process.

Regulation of the enzymatic activity of c-Src is largely ascribed to the phosphorylation state of the negative regulatory site Y527. Mutation of this site results in a highly active kinase (Yaciuk et al. 1988), and a decrease in Y527 phosphorylation correlates with kinase activation during mitosis and platelet activation (Bagrodia et al. 1991; Clark and Brugge 1993). We observed that the extent of Y527 dephosphorylation during adhesion was similar to that seen during mitosis. The portion of c-Src that remains phosphorylated at Y527 may be a subpopulation that is not activated during cellular adhesion; alternatively, the rapid rephosphorylation of this site, presumably by CSK, may occur following activation (Okada et al. 1991). Recent reports that CSK and c-Src simultaneously associate with focal adhesions are consistent with the latter possibility (Howell and Cooper 1994; Sabe et al. 1994).

Although we believe that dephosphorylation of Y527 is the most likely means for regulating c-Src during adhesion, we cannot rule out the possibility that c-Src is regulated through the binding of high affinity ligands to the SH2 or SH3 domains. Such binding might have the effect of opening the conformation of c-Src, resulting in both an increase in the specific kinase activity and a redistribution of c-Src. Although it has been demonstrated that high affinity peptides can increase the kinase activity of c-Src in vitro (Liu et al. 1993), no definitive examples of this type of regulation have been observed in vivo. It is also possible that regulation of c-Src during adhesion involves a combination of Y527 dephosphory-

lation in conjunction with ligand binding to the SH2 or SH3 domains. In this light, it is interesting to note that Y527 appears to be rephosphorylated rapidly while still associated with focal adhesions. One possible explanation for this observation is that high affinity ligands bind to the SH2 or SH3 domains and maintain the association of c-Src with focal adhesions even after Y527 rephosphorylation. Further exploration of the precise mechanism underlying the interaction of c-Src with focal adhesions awaits more detailed studies of signals generated during cellular adhesion and of relevant ligands for the SH2 and SH3 domains.

Although we know little about the signals that regulate the activity of c-Src during adhesion, it is likely that both integrin-dependent and -independent signals are involved. We have attempted to focus our analyses on the effects of integrin-dependent signals in our assays (0.2% serum was used in our assays), but we cannot completely rule out the possibility that serum factors may influence cellular adhesion in our assays. In addition, experiments using antibodies to cross-link fibronectin receptors resulted in only a partial activation of c-Src (K.B. Kaplan, unpubl.). These results are consistent with multiple signals combining to regulate c-Src during cellular adhesion. Multiple signals clearly can play an important role in the regulation of c-Src in platelets, where both thrombin and the integrin GPIIb-IIIa are required to activate c-Src (Clark and Brugge 1993).

Kinase-independent regulation of cellular adhesion

Although the increase in kinase activity of c-Src is transient during cellular adhesion, c-Src remains associated with the detergent-insoluble fraction even after the kinase activity has returned to basal levels. The low kinase activity and the kinetics of phosphorylation of Y527 suggest that high levels of kinase activity may not be important in the regulation of cellular adhesion. Furthermore, the catalytic activity of c-Src is not required to complement the adhesion defect in *src*^{-/-} cells. This is consistent with our previous findings that the amino-terminal half of c-Src (Src251) associates with and modulates the structure of focal adhesions by a kinase-independent mechanism (Kaplan et al. 1994). The function of the kinase domain in cellular adhesion is unclear but may serve to phosphorylate proteins prior to cell spreading or to further amplify integrin-mediated signals. Alternatively, the kinase domain of c-Src may have an inhibitory effect on adhesion, as is the case for transforming mutants of c-Src.

The amino terminus of c-Src is sufficient to enhance cell spreading and mediate the association of c-Src with focal adhesions. Thus, deletion of the SH3 domain probably abrogates the enhancement of cell spreading by preventing Src251 from associating with focal adhesions (Kaplan et al. 1994). Although the SH2 domain is not required for association of c-Src with focal adhesions (Kaplan et al. 1994), it is clearly required for the Src-induced enhancement of cell spreading. This may result from the ability of the Src SH2 domain to mediate the formation

of large protein complexes important in coordinating signal transduction during cellular adhesion. The interaction of the SH2 domain and FAK lends credence to the formation of a signaling complex involved in regulating cellular adhesion (Cobb et al. 1994; Schaller et al. 1994; Schlaepfer et al. 1994). Furthermore, the phosphorylation of integrins in v-Src-transformed cells, as well as in response to a variety of stimuli, may serve to recruit proteins, such as c-Src and FAK, to sites of integrin clustering (Dahl and Gabel 1989; Danilov and Juliano 1989; Falcioni et al. 1989; Freed et al. 1989).

Although the precise biochemical mechanism by which c-Src influences cell spreading is unclear, FAK represents an intriguing candidate for a c-Src target in focal adhesions. FAK has been shown to associate with mutant transforming versions of Src through interactions between the SH2 domain of c-Src and the FAK autophosphorylation site (Schaller et al. 1992; Clark and Brugge 1993; Cobb et al. 1994; Eide et al. 1995) and to be regulated during adhesion of cells on fibronectin (Guan and Shalloway 1992; Kornberg et al. 1992). More recently, the association of FAK and c-Src has been shown to be modulated during cellular adhesion (Schlaepfer et al. 1994). In addition, we have shown that the amino-terminal half of c-Src results in an increase in the levels of phosphotyrosine on FAK in vivo (Kaplan et al. 1994) and that FAK coimmunoprecipitates with the amino-terminal half of c-Src (K.B. Kaplan, unpubl.). If FAK is a primary target of c-Src during adhesion, it may be possible to rescue the adhesion defect in *src*^{-/-} cells with a dominant allele of FAK. Whether FAK is the primary target of c-Src in focal adhesions or just one of many will require more detailed genetic and biochemical analyses of FAK.

Control of cellular adhesion by c-Src

The seemingly paradoxical roles of the oncogenic and the proto-oncogenic forms of c-Src in adhesion raise questions concerning the mechanism by which c-Src affects cellular adhesion. Although c-Src and v-Src are capable of associating with focal adhesions, the consequences for the cell appear to be quite different. Our results indicate that c-Src positively influences cellular adhesion, whereas v-Src results in morphological transformation and an apparent reduction in cellular adhesion. The different levels of kinase activity of the two proteins in focal adhesions may partially explain this paradox. Whereas very few changes in phosphotyrosine levels were observed during adhesion of cells expressing c-Src (K.B. Kaplan, unpubl.), v-Src-transformed cells exhibit a dramatic increase in phosphotyrosine-containing proteins, especially proteins normally associated with focal adhesions (Hirst et al. 1986; Nigg et al. 1986; Pasquale et al. 1986). The cellular consequences of these modifications remain obscure, although it is likely that they alter focal adhesion structure substantially and contribute to the decrease in cell adhesion (Kellie et al. 1986a,b; Nermut et al. 1991; Nakamura et al. 1993).

Our findings demonstrate that fibroblasts from

src^{-/-} mice exhibit a readily detectable phenotype in culture. In general, it may be necessary to use in vitro assays on cultured cells from knockout mice to identify the specific function of the targeted gene. Although the subtle effect of c-Src on adhesion in fibroblasts may preclude a discernible phenotype in vivo, small perturbations in cellular adhesion may help explain the development of osteopetrosis in *src*^{-/-} mice. Specifically, osteoclasts from *src*^{-/-} mice are unable to degrade the bone matrix properly and result in mice with osteopetrosis. c-Src might act to hasten the crucial adhesive interaction between osteoclasts and the bone matrix required for proper osteoclast function (Boyce et al. 1992; Lowe et al. 1993; Hall et al. 1994). The involvement of c-Src in adhesion may have intriguing implications for situations in which the kinase activity of c-Src is known to be regulated, especially in specialized cells like osteoclasts and may illuminate the importance of c-Src in cellular adhesion.

Materials and methods

Cell lines and antibodies

The *src*^{-/-} and *src*^{-/+} cell lines were derived from the spontaneous immortalization of mouse embryo fibroblasts homozygous or heterozygous for a disruption in the *c-src* gene (cells kindly provided by P. Soriano, Fred Hutchinson Cancer Center, Seattle, WA). Three cell lines derived from spontaneous immortalization of mouse embryo fibroblasts were used to control for cell line variation. Alternatively, mouse embryo fibroblasts were immortalized using retrovirus containing the *v-myc* oncogene and used to confirm our results. All cell lines were analyzed for gross differences in growth properties. No obvious differences in doubling rate or mitotic indices were observed in parental *src*^{-/-}, *src*^{-/+}, or *src*^{-/-} cells expressing c-Src (K.B. Kaplan, unpubl.). Interestingly, cells expressing the truncated Src251 construct demonstrated increased doubling times and maintained lower levels of protein expression than analogous cell lines expressing wild-type c-Src. Finally, primary cultures of mouse embryo fibroblasts also were used to confirm our adhesion-related results, suggesting that the phenotype of *src*^{-/-} cells is not specific to immortalized cell lines. Construction of mutant *c-src* alleles and cell lines expressing these proteins were made as described previously (Kaplan et al. 1994).

Monoclonal antibody against c-Src (mAb 327) was kindly provided by J. Brugge (Ariad Pharmaceuticals, Cambridge, MA). Blocking antibodies against the fibronectin receptor (GP140) were kindly provided by C. Damsky (University of California, San Francisco). Polyclonal rabbit sera against phosphotyrosine was purchased from Upstate Biotechnology Inc. (UBI, Lake Placid, NY). Texas Red and fluorescein-labeled secondary anti-rabbit and anti-mouse antibodies were purchased from the Accurate Chemical Co. and used at recommended dilutions.

Immunofluorescence

Cells were plated in six well plates on glass coverslips for various times. Where indicated, coverslips were precoated by placing ~100 μ l of 5 μ g/ml of human fibronectin (Sigma Chemical Co.) and allowing it to dry overnight. Cells plated on fibronectin coverslips took longer to adhere than on fibronectin-coated dishes; thus, times for attachment and flattening are different (10 and 20 min, respectively) than for other experiments. Cells

grown on coverslips were treated for immunofluorescence as described previously (Kaplan et al. 1994).

Three-dimensional optical sectioning microscopy and image processing

Three-dimensional image recording and analysis were conducted as described previously (Kaplan et al. 1994). Data are presented as photographs of digitally recorded images, or in some cases, unprocessed images were recorded directly on Kodak T-Max 400 black-and-white negative film.

Triton X-100 fractionation

Cell lines expressing c-Src were plated on six-well tissue culture plates (~500,000 cells/well), coated either with 5 μ g/ml of fibronectin or 1 μ g/ml of BSA overnight at 4°C. After the indicated incubation at 37°C, cells were chilled on ice for 15–20 min and then washed with ice-cold PBS. Cells were lysed for indicated times (30 sec, 1, 2, 4, 6, 10 min) as described previously (Kaplan et al. 1994). Unfractionated lysates were lysed in 400 μ l of RIPA buffer [50 mM HEPES (pH 7.4), 1% deoxycholate acid, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃(VO)₄, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml of leupeptin, 0.023 units of aprotinin] for 5 min on ice, after which cells were scraped off the dish. Lysates were centrifuged at 16,000g at 4°C for 15 min to clear cell debris and were then transferred to fresh tubes before being immunoprecipitated as described below.

Immunoprecipitation and immunoblotting

For immunoprecipitating proteins from fractionated lysates, each fraction was diluted two times with RIPA buffer. mAb 327 was added (0.5 μ g of mAb 327) and incubated at 4°C for 1–4 hr. Protein A–Sepharose conjugated to a rabbit anti-mouse antibody was added (40 μ l/fraction of 50% slurry; 60 μ l/fraction for samples to be analyzed by kinase assay) and incubated at 4°C for 1–2 hr. Beads were washed two to three times in 1 ml of ice-cold RIPA buffer, boiled in loading buffer, and loaded onto 10% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose in a Bio-Rad electroblot apparatus, and blots were blocked in TBST (10 mM Tris at pH 8.0, 150 mM NaCl, 0.2% Tween 20) plus 2% BSA (Sigma Chemical Co, fraction IV) for 1 hr at 20°C. Blots were incubated first in primary antibody (mAb 327; 0.25 μ g/ml) and diluted in TBST/2% BSA at 20°C for 1–2 hr and in secondary horseradish peroxidase (HRP)-coupled anti-mouse antibody (Boehringer Mannheim; diluted 1/20,000 in TBST/2% BSA) for 1 hr at 20°C. Proteins were detected with the chemiluminescence procedure as described for the Amersham ECL detection system. In most cases, Western blots were also incubated with ¹²⁵I-labeled goat anti-mouse IgG (Amersham; 3000 Ci/mmol) for 1–4 hr at 4°C, after which blots were washed and the bands quantitated on a Molecular Dynamics PhosphorImager.

In vitro kinase assay

Cells were fractionated for 1 min in Triton X-100 lysis buffer, and the remaining cells were lysed in RIPA buffer as described previously (Kaplan et al. 1994). Immunoprecipitations were carried out as described above. After washing beads in RIPA buffer, beads were washed three times in kinase buffer (20 mM Tris-HCl at pH 7.2, 5 mM MnCl₂). Beads were split equally into two tubes: One sample was analyzed by immunoblotting (described above), and the other was analyzed in a kinase assay. Approxi-

mately 30 μ g of rabbit muscle enolase (Sigma Chemical Co.) was denatured in 0.025 M sodium acetate (pH 3.3) at 30°C for 10 min. The denatured enolase was resuspended in 400 μ l of kinase buffer, neutralized with 35 μ l of Tris-HCl (pH 8.8), and adjusted to 10 μ M ATP. Each kinase reaction received 30 μ l of enolase mix and 10 μ Ci of Amersham Redivue- γ -ATP (3000 Ci/mmol) and was incubated at room temperature for 5 min with constant mixing. Reaction products were analyzed on 10% polyacrylamide gels. Gels were prepared for standard autoradiography and quantitated using a Molecular Dynamics PhosphorImager. Specific kinase activity was calculated by dividing the area of the enolase band by the area of the c-Src band determined by immunoblotting.

Cell adhesion and spreading

Cells grown for 24–36 hr were trypsinized in 1 ml 0.05% trypsin and 0.05 M EDTA, and immediately resuspended in SBTI media (DME H-21, 10 mM HEPES at pH 7.2, 0.1 mg/ml of soybean trypsin inhibitor). Cells were adjusted to a final concentration of 300,000 cells/ml and 50 μ l of each cell line (15,000 cells/well) was plated in 96-well tissue culture plates coated with the indicated substrate (5 μ g/ml of human fibronectin, 5 μ g/ml of collagen IV, 1 μ g/ml of BSA). Cells were added to duplicate wells (six wells per data point) containing 50 μ l of adhesion media (DME H-21, 0.2% FCS, 10 mM HEPES at pH 7.2) and spun gently to the bottom of each well (1000 rpm, 30 sec in a clinical centrifuge). The plates were incubated at 37°C for the indicated period of time and then chilled on ice for 15 min. Loosely bound cells were removed by washing five times in 150 μ l of adhesion media. The number of cells left in each well was determined by measuring an internal enzymatic marker, β -hexosaminidase. A fluorometric substrate was added in 60 μ l of buffer [0.25% Triton X-100, 5 mM p-nitrophenyl N-acetyl- β -D-glucosaminide (Sigma Chemical Co.), 40 mM citric acid/80 mM Na₂HPO₄ at pH 4.75] and incubated at 37°C for 3–4 hr, and the reaction was stopped by adding 90 μ l of stop buffer (50 mM glycine/250 mM Na₂CO₃ at pH 10.0, 0.5 mM EDTA). The OD₄₁₀ was determined using a Molecular Devices Microplate Reader. A standard curve for each cell line was used to calculate the number of cells left in each well as a percentage of the total cells plated. The error bars in Figure 2 represent the variation from the mean in the same experiment as recorded over six identically treated wells.

To measure cell spreading, cells were resuspended in SBTI media (see above) and plated in 4 ml of adhesion media on 10-cm tissue-culture dishes coated with fibronectin (5 μ g/ml overnight at 4°C). Plates were incubated at 37°C for 15–20 min and then chilled on ice for 15 min. Five microscope fields from each plate were photographed [magnification, 150 $\times$ (Fig. 2); each field contained between 400 and 1000 cells], and both rounded and spread cells were counted. A spread cell was defined as containing <10% refractility, and the final number of spread cells was calculated as a percentage of the total cells in each field. The error bars in Figures 3 and 8 indicate the range over five fields in a given dish and, therefore, reflect the variation from the mean in cell spreading over the whole dish. The longer incubation at 37°C (i.e., 15–20 min) was required because cells were not spun down onto the plate as was done in the adhesion assay (see above).

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c-Src enhances the spreading of src^{-/-} fibroblasts on fibronectin by a kinase-independent mechanism.

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