The Src SH3 Domain Is Required for DNA Synthesis Induced by Platelet-derived Growth Factor and Epidermal Growth Factor*

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The Src family of protein tyrosine kinases has been implicated in the response of cells to platelet-derived growth factor (PDGF) or epidermal growth factor (EGF). We recently described a microinjection approach that we used to demonstrate that kinase activity of Src family members is required for PDGF- and EGF-induced S-phase entry of fibroblasts. We have now used this approach to ask whether a functional SH3 domain of Src is required to transduce the mitogenic signal upon PDGF or EGF stimulation. Microinjection of plasmids encoding Src mutants lacking the SH3 domain (SrcΔSH3) or point-mutated within the ligand binding surface of the SH3 domain, but with intact kinase domains, inhibited the mitogenic effect of PDGF and EGF in fibroblasts. SrcΔSH3 could still associate with the PDGF receptor, suggesting that the inhibitory effect of the Src SH3 mutants was brought about by a failure of the PDGF receptor-SrcΔSH3 complex to relay the mitogenic signal further downstream. Chimeric molecules in which the Src SH3 domain was replaced with that of spectrin or Lck also blocked PDGF-induced DNA synthesis, whereas a chimera containing the Fyn SH3 domain did not. These data suggest that the Src or Fyn SH3 domain is required either for correct substrate selection or to recruit other proteins to the PDGF receptor.

Stimulation of quiescent fibroblasts with growth factors produces a series of changes that culminate in DNA synthesis and cell division. The receptors for some of these growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), possess intrinsic tyrosine kinase activity. Binding of these ligands to their cognate receptors induces dimerization, activation of the tyrosine kinase activity, and trans-phosphorylation at several tyrosine residues in the intracellular domain of the receptor. These phosphorylated tyrosines can either influence the activity of the kinase or serve as docking sites for binding of downstream signal transduction molecules, via their Src homology (SH) 2 domains. Different SH2-containing proteins have been shown to interact with activated growth factor receptors, including the GTPase-activating protein of Ras, phospholipase Cγ1, phosphatidylinositol 3-kinase, three members of the Src family of tyrosine kinases (Src, Fyn, and Yes), a phototyrosine phosphatase (Syp), and several adaptor proteins (which lack catalytic activity but contain SH2 and/or SH3 domains), including Grb-2, Shc, and Nck (1, 2).

The Src family of tyrosine kinases is composed of nine members (3). Topographically the N-terminal half of the Src family kinases consists of membrane targeting sequences, a unique domain, an SH3 domain, and an SH2 domain. The C-terminal half contains the catalytic domain followed by a short sequence of amino acids, the tail, involved in the regulation of the kinase activity (3). Src tyrosine kinase activity is down-regulated by Csk, which phosphorylates Src on Tyr-527 in the C-terminal tail (4, 5). Several studies have shown that both the SH2 and the SH3 domain are required for full regulation of Src kinase activity (6–8). In the phosphorylated and down-regulated conformation, the tail is thought to be bound intramolecularly to the SH2 domain. Deletion of the Src SH3 domain prevents or destabilizes the association of the SH2 domain with the phosphorylated tail (6). Point mutations in the ligand binding surface of the Src SH3 domain also result in a constitutively active kinase in a heterologous Schizosaccharomyces pombe expression system, even though the tail is still phosphorylated by Csk (9). Transfection of NIH-3T3 cells with these SH3 mutants of Src gave rise to morphologically normal cells, even though all mutants had high intrinsic kinase activity (9). Furthermore, the efficiency of transformation of NIH-3T3 cells by an activated form of Src (Y527F) is also diminished when the SH3 domain is deleted (2). Collectively these data suggest that the SH3 domain of Src is required for transformation (9).

In fibroblasts three members of the Src family are expressed, Src, Fyn, and Yes. Stimulation of NIH-3T3 cells with PDGF induces a transient increase of the kinase activity of Src, Fyn, and Yes (10–12), as well as a direct association of these proteins with the activated PDGF receptor (11, 13). It is the SH2 domains of the Src family kinases that are involved in the association with the PDGF receptor (14). Since the binding specificities of Src family SH2 domains are similar (15, 16), Src, Fyn, and Yes all interact with the receptor at the predominantly used site, Tyr-579 (17). Upon association with the PDGF receptor, Src family tyrosine kinases become phosphorylated by the receptor (10, 12, 14). These phosphorylation sites have recently been mapped in both Src and Fyn. In the case of Src, the phosphorylation site is in the SH3 domain in an area that

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The abbreviations used are: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; DMEM, Dulbecco’s modified Eagle’s medium; BrdU, bromodeoxyuridine; phosphate-buffered saline; wt, wild type.

2 A. Heber, T. Erpel, and S. A. Courtneidge, unpublished observations.

3 M. A. Broome and T. Hunter, manuscript in preparation.

4 K. Hansen, G. Alonso, S. A. Courtneidge, L. Rönnstrand, and C.-H. Heldin, manuscript in preparation.
is part of the contact region for protein-protein interactions (20, 21). This phosphorylation is therefore likely to influence the affinity of the Src SH3 domain for other proteins.

It has also been observed that overexpression of Src augments the mitogenic response of fibroblasts to EGF, whereas it is reduced by expression of kinase-inactive Src (22, 23). Furthermore, microinjection of constructs that express kinase-inactive Src or Fyn, or a neutralizing antibody against Src, Fyn, and Yes, dramatically inhibits the mitogenic response to EGF, demonstrating a requirement for Src family kinases for EGF-induced DNA synthesis (24). However, activation of Src kinases by EGF has been detected only in cells that express very high levels of the EGF receptor (25, 26). Direct association of the EGF receptor and Src has been reported in vitro (27), but convincing evidence for a direct interaction in vivo is still lacking.

Here we ask whether the SH3 domain is also required for Src function in normal cells. For this purpose we applied the microinjection approach that we have recently used to show the requirement for tyrosine kinase activity of Src in normal cells. For this purpose we applied the microinjection approach that we have recently used to show the requirement for tyrosine kinase activity of Src family kinases for S-phase entry of fibroblasts in response to PDGF, EGF, and other growth factors (24, 28).

MATERIALS AND METHODS

Constructs—All constructs used in this study have been described previously (9). For expression in NIH-3T3 cells some of the mutant c-Src cDNAs were subcloned from the S. pombe expression vector pSSG (6) into the mammalian expression vector pSG5. This vector is a derivative of pSG5 (Stratagene) that contains a different polylinker, as described previously (9).

Cell Culture Techniques and Microinjection of Cells—NIH-3T3 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics under an atmosphere of 10% CO₂. 48 h before microinjection cells were seeded onto glass coverslips and grown to approximately 70% confluency. The medium was replaced 24 h before microinjection with DMEM containing 0.5% fetal calf serum, transferrin (5 μg/ml), insulin (5 μg/ml), and antibiotics. DNA (25 μg/ml) was microinjected into the nucleus using an automated microinjection system (Zeiss, A1S) as described (28). Six hours later PDGF (human recombinant homodimer BB, Upstate Biotechnology) was added at 25 ng/ml or EGF (Upstate Biotechnology) at 1 ng/ml. The needles for microinjection were pulled from capillaries (Clark Electro-medical Instruments) with a Flaming/Brown micropipette puller. Concomitant with growth factor addition, BrdU (final concentration 100 μM, Sigma) was added to monitor DNA synthesis. The cells were incubated at 37 °C for further 18 h and then fixed for immunostaining.

Immunofluorescence—Coverslips were washed once with phosphate-buffered saline (PBS) and fixed for 5 min with −20 °C methanol. Cells expressing different Src alleles were stained by incubating the coverslips with affinity-purified cost-1 antibody (1:50 in PBS), washing three times in PBS, and then incubating with fluorescein-conjugated goat anti-rabbit antibody. To analyze DNA synthesis, cells were incubated for 30 min in 1.5 μHCl, washed three times in PBS, and stained with monoclonal anti-BrdU antibody (1:10 in PBS; Boehringer Mannheim). Subsequently the cells were washed again and incubated with a Texas Red-conjugated anti-mouse antibody (Dianova). All coverslips were finally incubated in PBS containing Hoechst 33258 (final concentration 1 μg/ml, Sigma), washed with PBS, rinsed in ethanol, and mounted in Moviol (Hoechst) on glass slides. Slides were viewed with an Axiopt fluorescence microscope.

Cell Lines—NIH-3T3 cells were transfected by the calcium-phosphate method (9). Briefly, 5 × 10⁵ cells were seeded in 6-cm dishes and transfected with 5 μg of the different Src constructs (c-Src or SrcΔSH3) together with 200 ng of pS2neo plasmid, to supply G418 resistance. After 48 h, the cells were trypsinized and placed in three 10-cm dishes. The selection was started by adding 1 mg/ml G418 (Life Technologies, Inc.) to the culture medium. The medium was replaced every 2–3 days until foci arose (10–15 days). Colonies were picked and expanded, and the expression of the different Src alleles was determined by immunoblotting. Confluent dishes were incubated for 40 h in DMEM containing 0.5% fetal calf serum, transferrin (5 μg/ml), insulin (5 μg/ml), and antibiotics prior to stimulation with PDGF (25 ng/ml for 5 min at 37 °C).

Biochemical Analyses—Methods for immunoprecipitation of proteins, SDS-polyacrylamide gel electrophoresis, and immunoblotting have all been described before. Briefly, cells were rinsed twice with cold TBS (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 100 μM sodium orthovanadate) and then lysed by scraping in LB (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% aprotinin, 20 μM leupeptin, 1 mM dithiothreitol, 100 μM sodium orthovanadate, 10 mM NaF). Lysates were transferred to Microfuge tubes, vortexed, incubated 10 min, and centrifuged for 10 min at 10,000 × g to remove insoluble material. Lysates were incubated with anti- src for 60 min, centrifuged before transfer to tubes containing 10 μl of protein A-Sepharose, and incubated for 30 min. All incubations and centrifugations were carried out at 4 °C. Immunoprecipitates were washed four times with LB and once with TBS. For immunoblotting experiments, transfer of proteins to nitrocellulose (BA85, Schleicher & Shuell) was performed using a semi-dry apparatus according to the manufacturer's instructions (Millipore). Following two rinses in PBS, the membrane was incubated in blocking solution (3% bovine serum albumin, fraction V (ICN) in PBS) for 1 h. The incubation with the antibodies was carried out for 1 h in blocking solution, followed by horseradish peroxidase-coupled antibodies, and detected using the ECL detection reagent (Amersham Corp.).

RESULTS

Src Lacking the SH3 Domain Blocks the Mitogenic Response to PDGF—We have previously reported that Src family tyrosine kinases are required for transmission of the mitogenic response after PDGF stimulation (24, 28). To analyze further this requirement we tested whether the SH3 domain of Src was necessary for the proliferative response to PDGF. Since we had observed that NIH-3T3 cell lines expressing Src with a deleted SH3 domain (SrcΔSH3) or with a defect in the ATP-binding (SrcK-) site could not be maintained in culture at high expression levels, we expressed the constructs transiently using an automated microinjection system as described previously (data not shown and Ref. 28). The percentage of BrdU-positive cells from several coverslips each with several hundred microinjected cells was calculated and analyzed statistically as described in the legend to Fig. 1. Unstimulated and non-microinjected cells showed only a background level of BrdU incorporation, whereas approximately 65% of PDGF-stimulated cells non-microinjected cells entered S-phase (Fig. 1). As previously reported (28) microinjection of a kinase-inactive form of Src (SrcK-) reduced the number of cells that went into S-phase by 70%, whereas microinjection of wt Src did not affect the mitogenic response to PDGF (Fig. 1). Microinjection of a
plasmid expressing an Src mutant lacking the SH3 domain, but wild type in its tyrosine kinase function (SrcΔSH3), resulted in a 80% reduction in the number of cells that incorporated BrdU. This suggests that the involvement of Src tyrosine kinases in the transduction of the PDGF-induced mitogenic response not only requires an active tyrosine kinase domain but also the SH3 domain.

Mutations in the Ligand Binding Surface of the Src SH3 Domain Inhibit PDGF-induced DNA Synthesis—We next tested whether the ligand binding surface of the Src SH3 domain was required for the mitogenic response to PDGF. We had previously shown that certain mutations in the ligand binding surface, such as Trp-118 → Ala and Tyr-136 → Ala, abolished the ability of the Src SH3 domain both to participate in intramolecular regulation and to bind to heterologous binding partners like Sam68 and p85 in vitro (9). Microinjection of plasmids encoding SrcW118A or SrcY136A inhibited the mitogenic response to PDGF to the same degree as SrcΔSH3 (Fig. 2 and 3). Two other Src molecules with mutations in the SH3 domain showed only moderate (Y131A) or no (K104E) effect in an in vitro binding assay with heterologous proteins (9). One of these, SrcY131A, reduced the number of cells entering S-phase after PDGF stimulation significantly (Fig. 3). The other, SrcK104E, did not inhibit PDGF-induced DNA synthesis to a statistically significant degree (p = 0.32). These results suggest that a functional ligand binding surface of the SH3 domain is required for PDGF-induced mitogenesis.

SrcΔSH3 Can Associate with the PDGF Receptor in Vivo—Src family tyrosine kinases become associated with the PDGF receptor upon stimulation with PDGF. To test whether the dominant negative effect of SrcΔSH3 could be brought about by binding of SrcΔSH3 to the activated PDGF receptor, thereby displacing wild type Src family tyrosine kinases, we created stable cell lines expressing wt Src or SrcΔSH3 as described previously (9), and examined the association of these molecules with the activated PDGF receptor. In PDGF-stimulated cells that were transfected with wt Src or SrcΔSH3, we detected a band at 180-kDa comigrating with the PDGF receptor (Fig. 4A), whereas we could not detect this band at this exposure time in Src immunoprecipitates from mock transfected cells.
Stripping and reprobing of the blot with an antibody specific for Src family tyrosine kinases (anti-cst.1) showed that wt Src and SrcΔSH3 were expressed to approximately the same level (Fig. 4B). These results show that deletion of the SH3 domain does not affect the capacity of Src to bind to the PDGF receptor and is consistent with the hypothesis that inhibition of PDGF-induced DNA synthesis by SrcΔSH3 is due to a signaling defect of the PDGF receptor-SrcΔSH3 complex.

Chimeric Src Molecules in Which the SH3 Domain of Src Is Replaced with That of Spectrin or Lck Inhibit PDGF-induced DNA Synthesis—To test whether any SH3 domain could restore function to SrcΔSH3, we microinjected chimeric Src molecules in which the Src SH3 domain was replaced with SH3 domains from other proteins. Microinjection of plasmids encoding a chimeric Src molecule containing the SH3 domain of spectrin reduced the number of cells going into S-phase by about 70% compared with PDGF-stimulated non-microinjected cells, whereas cells microinjected with wild type Src were not inhibited in their PDGF response (Fig. 5). Interestingly a Src kinase-inactive form of Src, inhibited the EGF-induced mitogenic response (24). To test whether any SH3 domain could restore function to SrcΔSH3, we microinjected chimeric Src molecules in which the Src SH3 domain was replaced with SH3 domains from other proteins. Microinjection of plasmids encoding a chimeric Src molecule containing the SH3 domain of spectrin reduced the number of cells going into S-phase by about 70% compared with PDGF-stimulated non-microinjected cells, whereas cells microinjected with wild type Src were not inhibited in their PDGF response (Fig. 5). Interestingly a Src kinase-inactive form of Src, inhibited the EGF-induced mitogenic response (24). To test whether any SH3 domain could restore function to SrcΔSH3, we microinjected chimeric Src molecules in which the Src SH3 domain was replaced with SH3 domains from other proteins. Microinjection of plasmids encoding a chimeric Src molecule containing the SH3 domain of spectrin reduced the number of cells going into S-phase by about 70% compared with PDGF-stimulated non-microinjected cells, whereas cells microinjected with wild type Src were not inhibited in their PDGF response (Fig. 5). Interestingly a Src kinase-inactive form of Src, inhibited the EGF-induced mitogenic response (24).

SrcΔSH3 Also Inhibits the Mitogenic Response to EGF—We have previously shown that microinjection of an antibody specific for Src family tyrosine kinases, or a plasmid coding for a kinase-inactive form of Src, inhibited the EGF-induced mitogenic response (24). To test whether the mitogenic signal from the EGF receptor also required the Src SH3 domain, we microinjected a plasmid encoding SrcΔSH3 into NIH-3T3 fibroblasts and stimulated them with EGF (Fig. 6). Approximately 40% of non-microinjected cells entered S-phase when stimulated with EGF. Microinjection of a plasmid encoding SrcΔSH3 6 h prior to EGF stimulation reduced the number of cells incorporating BrdU by half (approximately 50%), suggesting an important role for the Src SH3 domain in EGF mitogenic signaling.

SrcΔSH3 Does Not Nonspecifically Inhibit All Signaling Pathways Originating from the PDGF Receptor—One potential concern about experiments such as these is that the SrcΔSH3 we are expressing has an exposed SH2 domain (6), which might compete nonspecifically for binding to several sites on activated receptors. To address this, we took advantage of our recent observations that ablation of the Src pathway does not inhibit fos induction in response to PDGF (29) but that other SH2-driven interactions with the receptor are required.5 We therefore examined transcriptional activation of the fos promoter in a cell line engineered to express β-galactosidase under the control of the fos promoter. As shown in Fig. 7, expression of β-galactosidase was inhibited by expression of a dominant negative form of Ras (N17Ras), as expected. However, neither kinase-inactive Src nor SrcΔSH3 had any inhibitory effect on the fos promoter. We can therefore rule out that SrcΔSH3 is having a nonspecific effect.

**DISCUSSION**

We analyzed the role of the Src SH3 domain in the mitogenic response to PDGF and EGF. SrcΔSH3 inhibited both PDGF- and EGF-induced DNA synthesis in NIH-3T3 cells. Since these

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**Fig. 5.** Inhibition of PDGF-induced DNA synthesis by chimeric Src molecules. Chimeric Src molecules where the SH3 domain was replaced with that of α-spectrin, Lck, or Fyn (Src(specSH3), Src(LckSH3), Src(FynSH3)) were microinjected into quiescent NIH-3T3 cells, and 6 h later PDGF (25 ng/ml) and BrdU were added. After further incubation for 18 h the cells were fixed, stained, and analyzed for BrdU incorporation with immunofluorescence as described under "Materials and Methods." In every experiment a few hundred microinjected cells were analyzed for each of the indicated constructs, and the extent of DNA synthesis on every coverslip was calculated according to the formula in the legend to Fig. 1. Shown are the mean of several experiments and standard errors.

**Fig. 6.** SrcΔSH3 blocks EGF-induced DNA synthesis. Quiescent NIH-3T3 cells were microinjected with the indicated constructs and either left quiescent or stimulated with EGF 6 h later. Microinjected cells were processed for immunofluorescence as described under "Materials and Methods," and the percentage of cells that incorporated BrdU was calculated as in Fig. 1.

Cells also express endogenous levels of Src family tyrosine kinases, but fail to go into S-phase. SrcΔSH3 is likely to be acting in a dominant negative manner. Three point mutations (W118A, Y136A, Y131A) in the ligand binding surface of the Src SH3 domain that inhibit PDGF- and EGF-induced DNA synthesis were previously shown to disrupt (W118A, Y136A) or weaken (Y131A) the binding to heterologous proteins (9), including Sam68 (30–32) and p85, the regulatory subunit of phosphatidylinositol 3-kinase (33). These point mutants specifically disrupt the ligand binding surface but very likely leave the overall structure of the SH3 domain intact (9). Removal of the SH3 domain does not disturb other functional domains of Src such as the SH2 domain, since the capacity of SrcΔSH3 to bind the PDGF receptor was not impaired compared with that of wild type Src. Moreover, we could rule out a model in which the exposed SH2 domain in SrcΔSH3 bound nonspecifically to other SH2-domain binding sites on the activated PDGF receptor, by showing that SrcΔSH3, like SrcK− (29), did not impair transcriptional activation of the fos promoter. Taken together, it therefore seems most likely that the SH3 domain is required for PDGF signaling in order to recruit molecules to the Src-PDGF receptor complex.

Broome and Hunter (34), using a different approach, could also show a requirement for the Src SH3 domain in the PDGF- and EGF-elicited mitogenic responses. They generated clonal Src− mouse fibroblasts cell lines and expressed mutants similar to those reported here. When made quiescent these cell lines were inhibited in their mitogenic response to both PDGF and EGF (34).

Mutations in an SH3 domain that disrupt a signaling pathway have been previously described. Signal transduction by the Let-23 receptor protein tyrosine kinase is abrogated by SH3 domain mutations in the Sem-5 adaptor protein of C. elegans, which results in impaired vulval development (35). Experiments with the Sem-5 homolog in vertebrates, Grb-2, have shown that the guanine nucleotide exchange factor SOS binds the Grb-2 SH3 domain via a proline-rich region in its C-terminal region. Activation of the EGF receptor recruits this complex to the receptor and thereby to the cytoplasmic face of the plasma membrane where SOS can activate Ras (36–40). The results described here are thus a second example where an SH3 domain mutation can impair a signaling pathway.

One of the inhibitory SH3 domain mutants we describe here, Y136A, is of particular note, since Tyr-136 has recently been shown by Broome and Hunter to be phosphorylated by the activated PDGF receptor. The ligand binding area of an SH3 domain is made up predominantly of aromatic and hydrophobic residues that form a relatively flat surface (41). Structural studies with SH3 domains and proline-rich peptides have revealed that the proline residues are the main contributors to van der Waals interactions with the ligand binding surface of the Src SH3 domain and also determine the secondary structure (polyproline helix type II) of the peptide (20). Tyr-136 makes decisive contacts with these proline residues (20), and although a functional role for this phosphorylation has not yet been determined, it is quite likely phosphorylation of Tyr-136 in the ligand binding site would alter the binding properties of the Src SH3 domain. However, it is not possible at this point to ascribe the effect of the Y136A mutation on PDGF-induced DNA synthesis to the lack of phosphorylation by the PDGF receptor, since it has been shown that the mutation also affects the binding to heterologous proteins in vitro (9). To clarify the

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**Fig. 7.** SrcΔSH3 does not inhibit transcriptional activation of the fos promoter. LIA cells, which constitutively express β-galactosidase under the control of the fos promoter (19), were microinjected with the constructs shown, stimulated with PDGF as shown, and then β-galactosidase expression assessed with a specific antibody. Shown are the means from 3 to 4 experiments and the standard deviations.

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**Materials and Methods:** The percentage of cells that incorporated BrdU was calculated as in Fig. 1. Further incubation for 18 h the cells were fixed, stained, and analyzed for BrdU incorporation. In every experiment a few hundred microinjected cells were analyzed for each of the indicated constructs, and the extent of DNA synthesis on every coverslip was calculated according to the formula in the legend to Fig. 1. Shown are the mean of several experiments and standard errors.
function of the phosphorylation of Tyr-136 on Src by the PDGF receptor we will have to await the identification of proteins that specifically bind to the Src SH3 domain phosphorylated on Tyr-136.

How does an Src molecule lacking its SH3 domain, but with intact tyrosine kinase and SH2 domains, inhibit PDGF- and EGF-induced DNA synthesis? The simplest explanation is that due to overexpression of this protein endogenous Src family kinases are displaced from the activated PDGF receptor and signaling is abrogated. This possibility is strengthened by the fact that the activated PDGF receptor associates with Src SH3. But what then is the function of the Src SH3 domain in this signaling cascade? The Src SH3 domain could recruit a protein to the multi-protein receptor complex that itself has a required enzymatic or adaptor function. Another possibility is that a decisive substrate for the Src tyrosine kinase domain, or required enzymatic or adaptor function. Yet another possibility is that an Src molecule lacking its SH3 domain, but with intact tyrosine kinase and SH2 domains, inhibit PDGF- and EGF-induced DNA synthesis. The SH3 domain of Src has been shown by Broome and Hunter (34) to inhibit PDGF-induced DNA synthesis. Other experiments have also shown that the SH3 domains of Src family tyrosine kinases have similar but distinct specificities (42). From these data one can speculate that the critical regulation of kinase activity, restrict the transforming capabilities of activated forms of Src, and inhibit, when overexpressed, the mitogenic response to EGF and PDGF in fibroblasts.

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