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The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells

(microinjection/platelet-derived growth factor receptor/protein tyrosine kinases)

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ABSTRACT **Three members of the Src family of protein tyrosine kinases Src, Fyn, and Yes associate with the activated platelet-derived growth factor (PDGF) receptor *in vivo*. This interaction requires the Src homology 2 (SH2) domain of the Src family member and causes activation of the intrinsic activity of the Src family kinases. We microinjected cells with DNA encoding catalytically inactive forms of the Src and Fyn proteins and examined their effects on PDGF-mediated signaling *in vivo*. Kinase-inactive Src and Fyn inhibited PDGF-stimulated entry of cells into S phase, whereas kinase-active forms of the proteins had no inhibitory effects. An intact SH2 domain was required for inhibition. Furthermore, when kinase-inactive Fyn was comicroinjected with a plasmid expressing activated Ras, the cells could enter S phase, indicating that the expression of kinase-inactive Fyn did not damage cell viability. Injection of an antibody specific for Src, Fyn, and Yes also reduced signal transduction through the PDGF receptor but only when injected within 8 hr of PDGF stimulation. Together these results indicate that the ubiquitously expressed Src family members are required for PDGF-induced mitogenic signaling.**

Addition of platelet-derived growth factor (PDGF) to quiescent fibroblasts results in dimerization of its cognate receptor and receptor transphosphorylation at several tyrosine residues in the intracellular domain (for reviews, see refs. 1 and 2). In this activated state the PDGF receptor (PDGFR) binds several Src homology 2 (SH2) domain-containing proteins, including the GTPase-activating protein of ras (3–5), phospholipase Cγ (6–8), three members of the Src family of tyrosine kinases (Src, Fyn, and Yes) (9), phosphatidylinositol 3-kinase (10, 11), and the Sem-5 homologue (growth factor receptor-bound protein 2) (12). Often, the associated proteins become phosphorylated on tyrosine residues and/or become activated.

One issue in the signal-transduction field is to identify which of the receptor-associated proteins are required for PDGF-induced signal transduction and what their functions might be. Two approaches could be used: (*i*) PDGFRs unable to bind a particular protein are expressed in cells that lack endogenous PDGFRs, and the ability of PDGF to induce a mitogenic response is measured. (*ii*) An inactive form of the protein of interest ("dominant negative") is expressed in cells containing PDGFRs, and its effects on signal transduction are measured. The disadvantages of the first approach are that the PDGFR may not function well in a heterologous system and that the results obtained may, therefore, only apply to that particular system. The disadvantage of the second approach is that it may not be possible to generate cell lines stably expressing a protein that inhibits signal transduction; thus, the dominant negative approach might require a system allowing transient or inducible expression of the protein under investigation. For either approach to work, however, each of the PDGFR-associated proteins must interact with distinct sites on the PDGFR. This, indeed, seems to happen for those proteins for which binding sites have been mapped. For example, phospholipase Cγ binds to Tyr-1009 and Tyr-1021 (13), phosphatidylinositol 3-kinase binds to Tyr-740 and Tyr-751 (14, 15), the GTPase-activating protein binds to Tyr-771 (14, 15), and the Src family kinases bind to Tyr-579 and Tyr-581 on the activated PDGFR (16).

To date, most investigators have used the first approach, that of creating mutant receptors, to investigate requirements for the various PDGFR-associated proteins. From these data, neither phospholipase Cγ (13) nor the GTPase-activating protein of Ras (14, 17) appears necessary for PDGF-induced signal transduction. The situation with the phosphatidylinositol 3-kinase is less clear: some investigators detect a requirement for the enzyme (14), whereas others report only minor effects when its binding sites are mutated (17). Such an approach could not be used to investigate the role of the Src family tyrosine kinases because receptors mutated at the binding sites for these proteins were catalytically inactive (16). We have, therefore, chosen the dominant negative approach to investigate whether the Src family tyrosine kinases are required for PDGF-induced signal transduction.

MATERIALS AND METHODS

Constructs. cDNAs containing the complete coding regions of the kinase active and inactive forms of Fyn, as well as the FynΔSH3ΔK and FynΔSH2ΔK, were cloned into the polylinker region of the eukaryotic expression vector pSG5 (Stratagene) under control of the simian virus 40 promoter and have been described (18). Kinase-active and kinase-inactive forms of Src were cloned into pSG5 that was modified to include a different polylinker region (from Thorsten Erpel, European Molecular Biology Laboratory). A cDNA coding for Ha-*ras* in pEXV (also under control of the simian virus 40 promoter) was received from Chris Marshall (Chester Beatty, London). The kinase-inactivating mutation in Fyn was Lys-299 → Met, in Src it was Lys-295 → Met, and the activating mutation in Ha-*ras* was Val-12 → Gly.

Cell Culture Techniques and Microinjection of Cells. NIH 3T3 cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum/antibiotics, in 10% CO_2. Cells were seeded onto glass coverslips (each coverslip was marked with a diamond knife for location purposes) and grown to 80% confluence. The medium was replaced with DMEM/0.5% fetal calf serum/transferrin at 5 μg/ml/insulin at 5 μg/ml/antibiotics, and the cells were incubated for another 24–48 hr. Hepes was then added to 20 mM. The purified plasmids (at 5 μg/ml) were injected into cell nuclei,

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Abbreviations: PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor.

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and purified antibody (at 1–2 mg/ml) was injected into cell cytoplasm by using an automated microinjection system (AIS, Zeiss), as described (19). The needles for microinjection were pulled from capillaries (Clark Electromedical Instruments, Pangbourne, U.K.) by using a Fleming–Brown micropipette puller. PDGF [human recombinant BB homodimer, 25 ng/ml (Upstate Biotechnology, Lake Placid, NY)] was added 6 hr after injection, and DNA synthesis was monitored by adding bromodeoxyuridine (BrdUrd) (Sigma, final concentration 100 mM). The cells were incubated at 37°C for another 18–20 hr and were then fixed for immunostaining.

Antibodies. The Fyn-specific antibody used for immunostaining was raised against peptides corresponding to amino acids 22–35 of the Fyn protein (Fyn1) (20). The cst.1 antibody was raised against peptide YQPGENL, which occurs in the C termini of Src, Fyn, and Yes and recognizes all three by immunoblot and kinase assay (9). To immunoaffinity-purify the anti-cst.1, ammonium sulfate-precipitated serum was loaded onto a peptide-coupled Sepharose column (using cyanogen bromide-activated Sepharose and protocols from Pharmacia), and the column was washed with 10 mM sodium phosphate/500 mM sodium chloride buffer, pH 7.0. Antibody was eluted with 1 M proprionic acid. Fractions (0.5 ml) were collected into 1 M ice-cold sodium phosphate buffer, pH 7.0 (0.5 ml) and concentrated by using a Minicon microconcentrator (Amicon) to 1 mg/ml in phosphate-buffered saline. The antibody was filtered (Millipore Millex-GV_4 filter units) and stored in aliquots at −70°C. Before microinjection, the antibody, or the antibody preincubated with peptide (final concentration, 2.5 mg/ml), was centrifuged in an Eppendorf microcentrifuge for 15 min. BrdUrd and PDGF (25 ng/ml) were added after 30 min, and the cells were incubated for another 18 hr before fixing and staining.

Immunofluorescence. Coverslips were washed once with phosphate-buffered saline, fixed for 6 min with ice-cold methanol, and stained with either rabbit anti-Fyn serum (1:100), or affinity-purified anti-cst.1 (1:50) for 30 min followed by three washings with phosphate-buffered saline. Coverslips were then incubated in diluted fluorescein-conjugated goat anti-rabbit antibody (1:100) (Sigma). To analyze for DNA synthesis cells were incubated for 10 min in 1.5 M HCl, washed three times as before, stained with monoclonal anti-BrdUrd (1:50) (Partec, Reinach, Switzerland), washed again, and then stained with a Texas-red conjugated anti-mouse antibody (Molecular Probes). For the antibody injections, anti-cst.1 was detected by using a fluorescein-conjugated anti-rabbit antibody, followed by acid treatment and BrdUrd detection, as described above. All coverslips were finally washed in phosphate-buffered saline containing Hoechst 33258 (Sigma, final concentration, 1 μg/ml) rinsed in water, inverted, and mounted in Moviol (Hoechst Pharmaceuticals) or Fluoprep (BioMerieux, Charbonnier les Bains, France) on glass slides. Slides were viewed by using an Axiophot fluorescent microscope.

RESULTS

The available evidence supports the notion that Src, Fyn, and Yes all bind to the same site on the PDGFR via their highly homologous SH2 domains (9, 18). Therefore, overexpression of one of these proteins should reduce the association of all three endogenous kinases with the PDGFR. Indeed, we have shown (9) that the 5- to 10-fold overexpression of wild-type Fyn reduces the association of Src and Yes with the PDGFR. The protein we first chose to test for potential dominant negative effects was a Fyn molecule with a single mutation in the ATP-binding site, which is catalytically inactive but retains its ability to associate with the activated PDGFR (18). Because we had observed that this construct could not be expressed in fibroblasts >2- to 3-fold over the endogenous, active protein (unpublished observations), we chose to express it transiently, using an automated microinjection system. Because Fyn is myristoylated and requires detergent for solubilization, we could not purify the protein in a form suitable for microinjection. We, therefore, microinjected a plasmid capable of expressing Fyn K^-. Such a system has been shown (19, 21) to allow high-level expression of proteins within a few hours of microinjection; the amount of protein expressed is determined by concentration of the microinjected DNA. Comparison of Fyn-specific immunofluorescence in normal and microinjected cells suggested that the expression level we achieved after microinjection was ≈5- to 20-fold that of endogenous protein (data not shown). Six hours after microinjection of cells (during which time the protein was produced to maximum levels), PDGF and BrdUrd were added to the medium, and 18 hr later the cells were fixed and stained (Fig. 1). Those cells expressing Fyn K^- protein showed a diffuse cytoplasmic staining with the anti-Fyn antibody that was more intense than the background staining from the endogenous Fyn protein (cells expressing Fyn K^- are marked with arrows in Fig. 1). Cells that had incorporated BrdUrd were visualized with anti-BrdUrd antibodies and are shown in Fig. 1*B*. (The nuclear fluorescence visible in Fig. 1*A* is also from the BrdUrd staining—the difference between the two antibodies was clearly seen in the originals, where the fluorescence due to Fyn is green, and that fluorescence due to BrdUrd is red.) Fig. 1 shows that

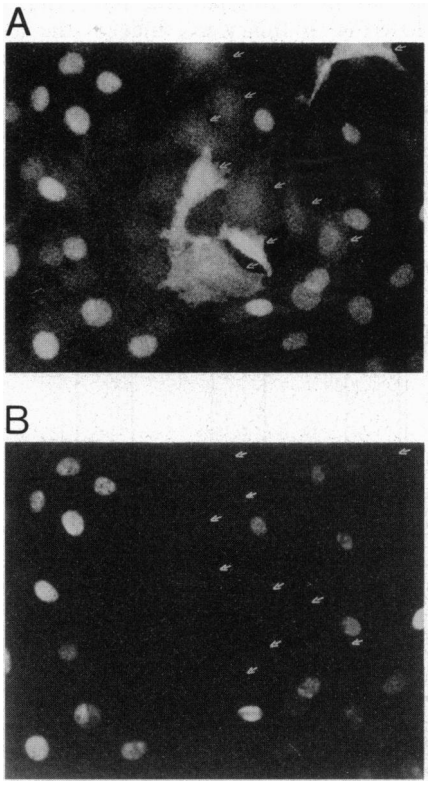

FIG. 1. Fyn K^- inhibits PDGF-induced DNA synthesis. Quiescent NIH 3T3 cells seeded on coverslips were microinjected (into the nucleus) with an expression plasmid encoding Fyn K^-. Six hours later, PDGF and BrdUrd were added to the cells, and 18 hr later they were fixed and stained. The Fyn antibodies were visualized with a fluorescein-conjugated anti-rabbit antibody, and the BrdUrd antibodies were visualized with Texas red-conjugated anti-mouse antibodies. (*A*) Cytoplasmic fluorescence seen is from reactivity with the anti-Fyn antibody in a representative experiment, whereas the nuclear staining is from the staining with anti-BrdUrd. (*B*) A different filter was used, so that only the BrdUrd staining is visible. Arrows mark position of cells expressing Fyn K^-.

those cells expressing Fyn K^- were not positive for BrdUrd, whereas many cells not expressing Fyn K^- were BrdUrd positive, suggesting that Fyn K^- had an inhibitory effect on PDGF-induced DNA synthesis. In several independent microinjection experiments, multiple coverslips were analyzed in the same way (Fig. 2). To compare the results from different experiments, the number of cells expressing Fyn K^- that were also BrdUrd positive were compared with the number of BrdUrd-positive nonmicroinjected cells from the same coverslip, and the results were expressed as a percentage. Data from several experiments were then pooled, and statistical analysis was done. The results expressed in Fig. 2 clearly show a significant reduction in PDGF-induced entry into S phase in cells expressing Fyn K^-.

To test further the inhibition of DNA synthesis seen with catalytically inactive Fyn, we utilized constructs we have previously described that encode portions of Fyn fused to β-galactosidase. One of these, FynΔSH3ΔK, contains only the unique domain and the SH2 domain of Fyn and can associate with the PDGFR, whereas the other, FynΔSH2ΔK, which has the unique domain and the SH3 domain of Fyn, cannot bind to the activated PDGFR (18). Fig. 2 shows that only the FynΔSH3ΔK protein could significantly inhibit PDGF-induced DNA synthesis, showing that an intact SH2 domain was required for inhibition.

We have postulated that the kinase-inactive form of Fyn inhibited PDGF-mediated DNA synthesis by competing for the binding site for wild-type Src, Fyn, and Yes on the activated PDGFR. If this were, indeed, the case, then a kinase-inactive form of one of the other members of the Src family should also have the same effect. To test this hypothesis, we microinjected constructs expressing kinase-active (Src K^+) and inactive (Src K^-) forms of Src. Fig. 2 shows that

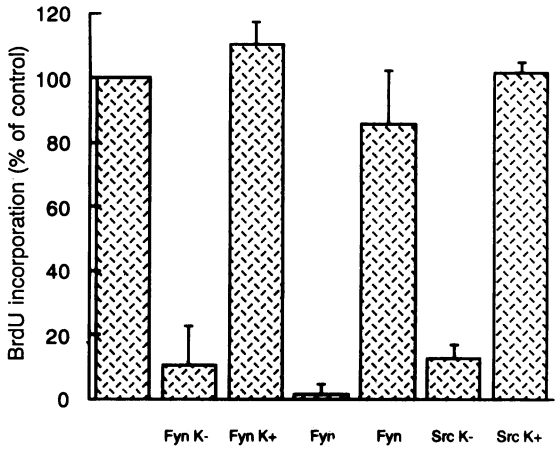

FIG. 2. Kinase-inactive forms of Fyn and Src inhibit PDGF-induced DNA synthesis. Quiescent NIH 3T3 cells seeded onto coverslips were microinjected with the constructs shown, stimulated with PDGF, and processed for immunofluorescence, as described in the legend to Fig. 1. For each experiment, several coverslips were analyzed, and the extent of DNA synthesis in injected cells was calculated with the formula: % = [number of BrdUrd- (BrdU) positive cells (injected)/number of BrdUrd-positive cells (uninjected)] × 100. In each case, <5% of nonstimulated cells were BrdUrd positive, and microinjection of plasmids did not alter this number. PDGF treatment caused DNA synthesis in at least 30% of nonmicroinjected cells. For Fyn K^- and Src K^-, data are derived from >500 expressing cells; for Fyn K^+, Src K^+, FynΔSH2ΔK, and FynΔSH3ΔK, data are derived from at least 100 cells. Results from several experiments ($n > 3$) have been averaged; the mean and SEM are shown.

Src K^- strongly inhibited DNA synthesis, whereas overexpression of Src K^+ had no such inhibitory effect.

Although the most straightforward interpretation of the above data is that Src family kinases are required for PDGF-induced signal transduction, we also considered, and ruled out, other explanations for these results. (*i*) It was possible that high levels of expression of any protein would be inhibitory. This does not appear so because neither the kinase-active forms of Fyn (Fyn K^+) and Src (Src K^+) nor plasmids encoding other proteins (e.g., FynΔSH2ΔK) had an inhibitory effect (Fig. 2 and data not shown).

(*ii*) Each SH2 domain has a distinctive preference for phosphotyrosine within a given peptide sequence (14, 22). Nevertheless, SH2 domains also have a measurable affinity for phosphotyrosine alone (23), and we were therefore concerned that a given SH2 domain-containing protein might, if expressed to a high level, bind to phosphotyrosine-containing sequences with which it is not normally associated. If this were so, the Fyn SH2 domain might have inhibited the binding of all PDGFR-associated proteins, thus inhibiting signal transduction. However we can rule out this occurrence in these experiments because neither Fyn K^+ nor Src K^+ inhibited PDGF-induced DNA synthesis when expressed to the same levels as Fyn K^- and Src K^- (Fig. 2).

(*iii*) The cells expressing kinase-inactive Src family kinases might not be viable. To test this hypothesis we comicroinjected cells with plasmids encoding Fyn K^- and an activated form of Ha-Ras. In this case cells could enter S phase, even without growth factor (Table 1), indicating that the cells were alive and could enter the cell cycle when provided with an appropriate signal that Fyn K^- did not antagonize.

Using a different approach, we also addressed the role of the Src family tyrosine kinases in PDGF-induced signal transduction by microinjecting antibodies specific for the Src family. We used an antibody (anti-cst.1) raised against the common C-terminal sequences of Src, Fyn, and Yes (9, 24). Affinity-purified anti-cst.1 was injected into the cytoplasm of NIH 3T3 cells. Thirty minutes later PDGF and BrdUrd were added to the medium, and after 18 hr of further incubation the cells were fixed and stained (Fig. 3). Those cells microinjected with the antibody were negative for BrdUrd staining, whereas many nonmicroinjected cells did incorporate BrdUrd. The results from several such experiments (representing >500 cells microinjected with antibody) are shown in Fig. 4*A* and show that the anti-cst.1 antibody inhibited entry into S phase by ≈75%. This inhibitory effect was mitigated by preincubation of the antibody with cognate peptide (Fig. 4), confirming the specificity of inhibition.

We next asked for how long during G_1 phase the Src family tyrosine kinases were required. Anti-cst.1 antibody was microinjected at various times after PDGF addition, and all

Table 1. Activated Ras overcomes inhibition of DNA synthesis caused by Fyn K^-

Construct	PDGF	Noninjected cells in S phase,* %	Injected cells in S phase,* %	Inhibition, %
Fyn K^-, pSG5	−	0	0	
Fyn K^-, pSG5	+	43 (± 6)	1.0 (± 1.7)	98
Fyn K^-, Ras	−	1.8 (± 1.6)	36 (± 6)	
Fyn K^-, Ras	+	60 (± 12.7)	53 (± 19.6)	12

Quiescent NIH 3T3 cells on coverslips were injected with equal amounts of either Fyn-expressing (Fyn K^-) and empty plasmid (pSG5) or equal amounts of Fyn-expressing (Fyn K^-) and Ras-expressing (Ras) plasmids, as indicated PDGF-induced DNA synthesis was then measured.

*Values represent the average of at least four independent experiments; parentheses enclose the SDs.

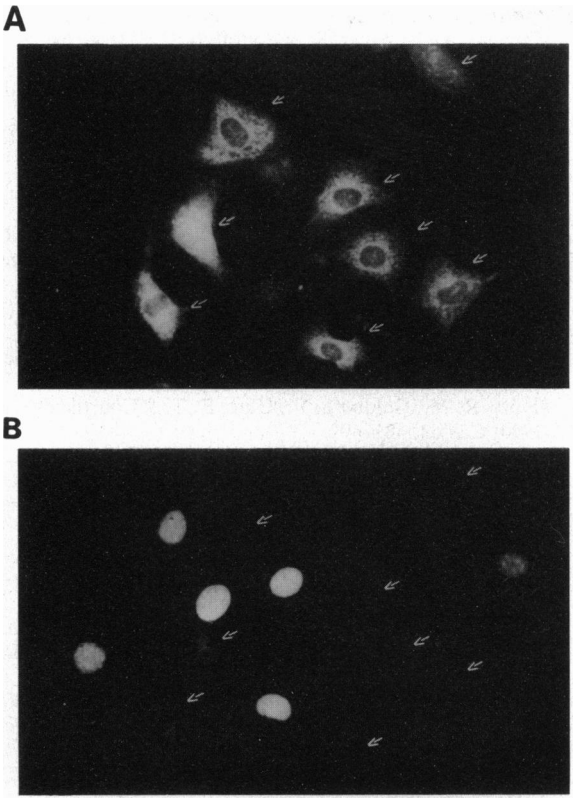

FIG. 3. An antibody that interacts with Src family tyrosine kinases inhibits PDGF-induced DNA synthesis. Coverslips containing quiescent NIH 3T3 cells were microinjected (into cytoplasm) with affinity-purified anti-cst.1 antibody, and 30 min later PDGF was added to the medium. The microinjected cells were visualized with a fluorescein-conjugated anti-rabbit antibody (cytoplasmic fluorescence in *A*), and BrdUrd-positive cells were visualized with mouse anti-BrdUrd antibodies followed by Texas red-conjugated anti-mouse antibodies (nuclear fluorescence in *B*). Arrows mark positions of the cells successfully microinjected with the anti-cst.1 antibody.

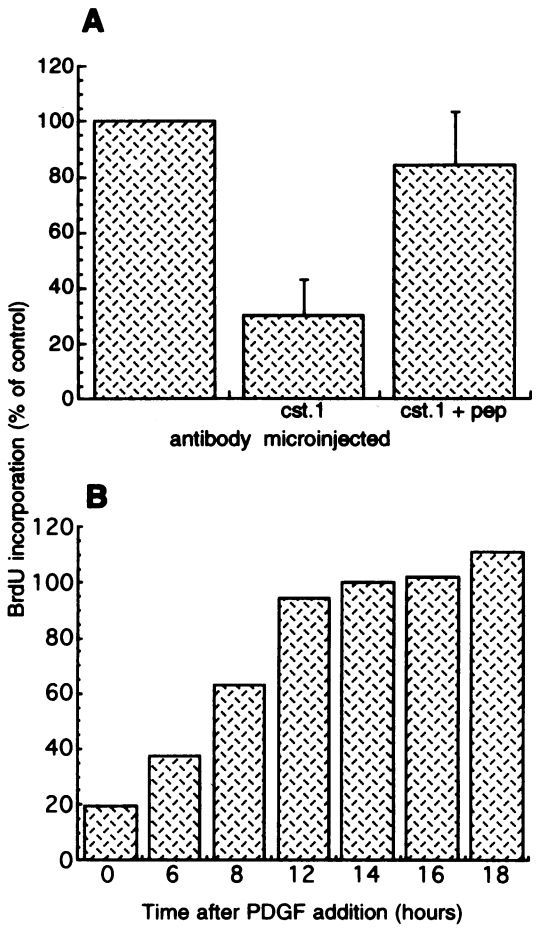

FIG. 4. Specificity and time course of anti-cst. inhibition of DNA synthesis. (*A*) Cells were microinjected with anti-cst.1 antibody or the same antibody preincubated with cognate peptide as shown, and PDGF and BrdUrd (BrdU) were added 30 min after microinjection. Eighteen hours later coverslips were processed for immunofluorescence, as described. For each experiment, several coverslips were analyzed; the extent of DNA synthesis in injected cells was calculated with the formula: % = [number of BrdUrd-positive cells (injected)/number of BrdUrd-positive cells (uninjected)] × 100. In each case, >5% of nonstimulated cells were BrdUrd positive, and PDGF treatment caused DNA synthesis in at least 25% of nonmicroinjected cells. Results from several experiments ($n > 3$) have been averaged; mean and SEM are shown. (*B*) Anti-cst.1 was microinjected at the times indicated (with PDGF treatment being at $t = 0.5$ hr), and at 18 hr coverslips were processed for immunofluorescence. Results are the aggregate of several experiments.

coverslips were then processed at the 18-hr time point. We found that microinjection of antibody at any time up to 6 hr after PDGF addition inhibited DNA synthesis. By 8 hr after growth factor addition, the inhibitory effect was less pronounced, and by 12 hr, the antibody was no longer inhibitory (Fig. 4*B*). Because DNA synthesis begins in these cells ≈12–14 hr after growth factor addition (data not shown), our results suggest that the Src family tyrosine kinases are only required during the first half of G_1.

DISCUSSION

In the experimental system described, cells expressing kinase-inactive forms of Fyn and Src could not initiate DNA synthesis upon stimulation with PDGF. What mechanism underlies this effect? We routinely processed the cells at 18 hr after growth factor stimulation; however, the same results were achieved when immunostaining was initiated 24 hr after growth factor addition (data not shown). The turnover of the expressed proteins and antibodies prevents us from examining the cells at later time points than this. Therefore, although our data support the conclusion that S-phase induction was inhibited in the presence of catalytically inactive Src family kinases, we cannot rule out the alternative possibility that cells expressing such proteins respond to PDGF with much slower kinetics (in which case the effect of Fyn K^- and Src K^- would have been to delay the G_1 phase of the cell cycle to at least twice its normal length). In either case, Src family tyrosine kinases were required for a normal response to PDGF.

An intact SH2 domain was required for the inhibition to be detected, but neither the SH3 domain nor the catalytic domain was required. Both Fyn K^- and FynΔSH3ΔK retain the ability to associate with the activated PDGFR, whereas the noninhibitory FynΔSH2ΔK does not retain this ability (9, 18). We, therefore, assume that the inhibition occurred because Fyn K^- and FynΔSH3ΔK displaced the binding of wild-type Src family tyrosine kinases to most activated PDGFRs. We ruled out the possibility that the inhibition was a nonspecific effect of overexpression of an SH2 domain-containing protein by showing that the catalytically active forms of Fyn and Src, when expressed equally, did not inhibit PDGF-induced DNA synthesis. Even though Fyn K^--expressing cells could not respond to PDGF, they were able to enter S phase if an activated form of Ras was coexpressed. Because Ras can act downstream of Src and other membrane-associated protein tyrosine kinases in signal-trans-

duction pathways (25, 26), this result suggests that Fyn K^- and Src K^- expression does not reduce cell viability but rather only inhibits signals coming from proteins that act upstream of the Src family tyrosine kinases.

Injection of an antibody specific for the ubiquitously expressed Src family kinases, Src, Fyn, and Yes, also reduced entry into S phase, providing further support for the conclusion that the Src family tyrosine kinases are essential in PDGF-induced signal transduction. The mechanism by which the anti-cst.1 antibody neutralizes activity of the Src family tyrosine kinases *in vivo* is unknown. The antibody recognizes the tail of the Src family tyrosine kinases, which in the inactive form of the enzyme (in quiescent cells) interacts intramolecularly with the SH2 domain (27, 28). After PDGF stimulation, this SH2 domain would normally associate with the PDGFR, and the antibody could block this association. Alternatively, loss of function may result from a more general steric hindrance. Nevertheless, there is precedent for an antibody that does not inhibit catalytic activity *in vitro* but does inhibit function *in vivo*: such antibodies specific for Src were recently shown to inhibit nerve growth factor-induced neurite outgrowth in PC-12 cells (29). Regardless of the mechanism of inhibition, we could use this antibody to show that the Src family tyrosine kinases were required for ≈8 hr after PDGF stimulation for cells to enter S phase.

We have shown that overexpression of either Fyn K^+ or Src K^+ does not impair the effect of PDGF to transduce a mitogenic signal. Because overexpression of one Src family member results in reduced binding of the others to the activated PDGFR, these results imply that Src, Fyn, and Yes are functionally redundant in the response to PDGF. Why are the Src family tyrosine kinases required at all? The simplest model is that the Src family members phosphorylate critical proteins that are not substrates for the tyrosine kinase activity of the PDGFR itself. To test such a model will require cell lines expressing Fyn K^- under control of an inducible promoter.

There is now growing evidence that the Src family of protein tyrosine kinases are involved in signal-transduction pathways that result in growth and/or differentiation. The activation of the receptors for PDGF (9), colony-stimulating factor 1 (30), interleukin 2 (31), as well as the cross-linking of several surface proteins of hematopoietic cells (for review, see ref. 32), results in Src family kinase activation and/or the association of Src family kinases with the surface protein. Furthermore, Src overexpression potentiates the effects of epidermal growth factor (33), and oncogenic forms of Src can mimic the actions of nerve growth factor (34). Microinjection of antibodies specific for Src has recently been used to show a requirement for Src family tyrosine kinases in the nerve growth factor- and fibroblast growth factor-induced neurite outgrowth of PC-12 cells (29), and in this report, we use similar techniques to show that functional Src family tyrosine kinases are required for PDGF to transmit a mitogenic signal. It seems likely that this microinjection method could also be used to test the requirement for Src family tyrosine kinases in the response of cells to the other ligands mentioned above, as well as to identify other pathways in which Src family kinases are involved.

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