MOLECULAR AND CELLULAR BIOLOGY, Feb. 1995, p. 1102–1109 Vol. 15, No. 2
0270-7306/95/$04.00+0

DNA Synthesis Induced by Some but Not All Growth Factors Requires Src Family Protein Tyrosine Kinases

SERGE ROCHE,[1] MANFRED KOEGL,[1] M. VITTORIA BARONE,[1] MARTINE F. ROUSSEL,[2] AND SARA A. COURTNEIDGE[1]*

Differentiation Programme, European Molecular Biology Laboratory, 69012 Heidelberg, Germany,[1] *and Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105*[2]

Received 2 September 1994/Returned for modification 20 October 1994/Accepted 6 November 1994

The Src family of protein tyrosine kinases have been implicated in the response of cells to several ligands. These include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and colony stimulating factor type 1 (CSF-1, in macrophages and in fibroblasts engineered to express the receptor). We recently described a microinjection approach which we used to demonstrate that Src family kinases are required for PDGF-induced S phase entry of fibroblasts. We now use this approach to ask whether other ligands also require Src kinases to stimulate cells to replicate DNA. An antibody specific for the carboxy terminus of Src, Fyn, and Yes (anti-cst.1) inhibited Src kinase activity in vitro and caused morphological reversion of Src transformed cells in vivo. Microinjection of this antibody was used to demonstrate that Src kinases were required for both CSF-1 and EGF to drive cells into the S phase. Expression of a kinase-inactive form of Src family kinases also prevented EGF- and CSF-1-stimulated DNA synthesis. However, even though the Src family kinases were necessary for both PDGF- and EGF-induced DNA synthesis in Swiss 3T3 cells, the responses to two other potent growth factors for these cells, lysophosphatidic acid and bombesin, were unaffected by the neutralizing antibodies. Therefore, some but not all growth factors required functional Src family kinases to transmit mitogenic responses.

Fibroblasts can be stimulated to divide by a wide variety of mitogens. The receptors for many of these ligands, for example platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), possess intrinsic protein tyrosine kinase activity. Ligand binding induces receptor dimerization and consequent *trans*-phosphorylation of the receptor at several sites. These phosphorylated tyrosines form the binding sites for several SH2 domain-containing proteins. In some instances, for example after insulin stimulation, a protein that is heavily phosphorylated by the receptor (in this case, insulin receptor substrate 1) associates with the SH2 domain-containing proteins. Proteins that have been shown to interact with activated receptors include the GTPase-activating protein of ras (rasGAP), phospholipase Cγ, three members of the Src family of tyrosine kinases (Src, Fyn, and Yes), phosphatidylinositol 3-kinase (PI 3-K), a phosphotyrosine phosphatase (Syp), and three adaptor proteins (which themselves lack catalytic activity but contain SH2 and/or SH3 domains), i.e., the *sem5* homolog (GRB-2), Shc, and Nck (for reviews, see references 2 and 18). Hematopoietic receptor tyrosine kinases are not normally expressed in fibroblasts, but some have also been shown to transmit mitogenic signals when ectopically expressed in NIH 3T3 cells. One example is the receptor for colony-stimulating factor type 1 (CSF-1), which is related to the PDGF receptor and which has been shown to associate with Src family tyrosine kinases (4), PI 3-K (20, 25, 30), and GRB-2 (29) in fibroblasts. Fibroblasts expressing the CSF-1 receptor can proliferate in response to CSF addition (22).

Some mitogenic growth factors binds to receptors that lack intrinsic tyrosine kinase activity; these include the receptors for bombesin and lysophosphatidic acid (LPA). Despite the fact that these receptors have no detectable tyrosine kinase activity, stimulation of fibroblasts with these ligands results in the rapid induction of tyrosine phosphorylation of several proteins (6, 11, 13, 33). Few of these proteins have been identified, but in the case of bombesin and LPA, it has been reported that the focal adhesion kinase (Fak) is activated (6, 11, 34).

The Src family of protein tyrosine kinases is a group of nine enzymes that are linked to the cytoplasmic face of membranes via amino-terminal fatty acids and contain one SH3 and one SH2 domain as well as a catalytic domain and carboxy-terminal regulatory sequences (3). Fibroblasts express three highly related members of the Src family: Src, Fyn, and Yes. Stimulation of NIH 3T3 cells and cells engineered to express ectopically the human CSF-1 receptor with PDGF and CSF-1, respectively, increases the kinase activity of Src, Fyn, and Yes transiently and results in their association with the activated receptors (4, 5, 12, 19). Since the SH2 domains of Src family kinases, which are required for the association (27), have essentially the same binding specificity (26), any given receptor subunit will associate with either Src, Fyn, or Yes. EGF stimulation of Src family kinase activity has not been detected; however, an association between the activated EGF receptor and Src has been reported (14), although it is not clear if this is direct. Finally, LPA has also been found to effect a modest increase in Src kinase activity in certain cell types (7).

To address which signalling molecules are required for a given ligand to induce S phase entry, receptor mutants that fail to associate with a particular protein have frequently been used. However, this approach is inappropriate when (i) the mutation affects the kinase activity of the receptor (for example, the PDGF receptor lacking Src family-binding sites [16]); (ii) more than one signalling molecule binds to a single site (for example, PI 3-K and Nck on the PDGF receptor [17]); (iii) the binding site on the receptor is not known (for example, PI 3-K

* Corresponding author. Present address: SUGEN Inc., 515 Galveston Dr., Redwood City, CA 94063. Phone: (415) 306-7700. Fax: (415) 306-7613. Electronic mail address: sarac@sugen.sf.ca.us.

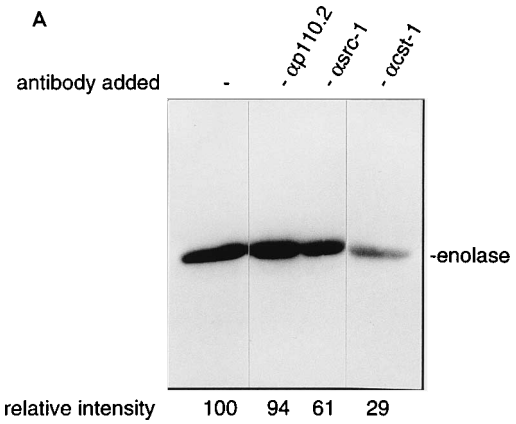
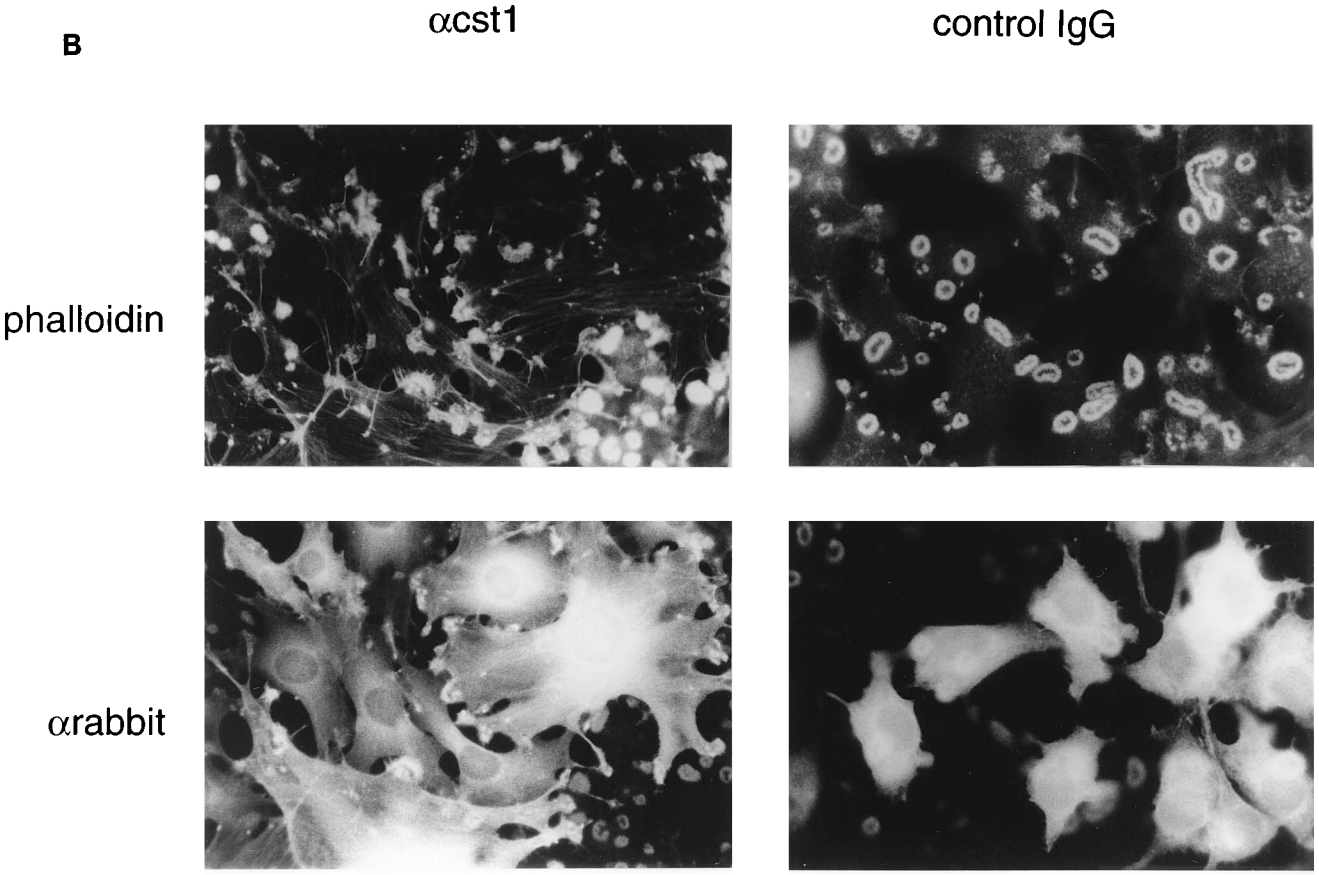


FIG. 1. The cst-1 antibody inhibits Src in vitro and in vivo. (A) Human Src protein produced by baculovirus infection of Sf9 cells was purified by phosphotyrosine affinity chromatography, as described in the text. Purified protein was incubated with the affinity-purified antibodies indicated, acid-denatured enolase was added, and kinase assays were performed. The numbers at the bottom of the figure show the relative incorporation of counts into enolase. The antibodies are anti-cst-1, anti-Src.1, and anti-p110.2 (raised against the catalytic subunit of PI 3-K). (B) NIH 3T3 cells transformed with an activated allele of Src (cSrcY527F) were seeded onto coverslips and given microinjections of anti-cst-1 or IgG purified from nonimmunized rabbits (control IgG) as indicated. Fluorescein-conjugated anti-rabbit IgG was used to detect the microinjected cells, and Texas red-conjugated phalloidin was used to visualize actin.



and the EGF receptor); or (iv) activation of a signalling molecule occurs without association with the receptor (as may be the case with receptors that lack kinase activity). We recently described two approaches that circumvent most of these objections (28). Both rely on microinjection to introduce proteins into cells transiently. In the first method, a form of the protein of interest that can still bind to the receptor but is catalytically inactive (dominant negative) is expressed in cells, and its effects on signal transduction are measured by monitoring bromodeoxyuridine (BrdU) incorporation. In the second, an antibody specific for the protein of interest is microinjected into cells and signal transduction is assessed. We recently used both of these approaches to demonstrate that Src family kinases are essential for PDGF-induced DNA synthesis (28). Others have used the dominant-negative approach to show that Src is required for the efficient functioning of the EGF receptor (32). Here we use both of these methods to investigate the requirement for Src kinases in the response to several other growth factors.

MATERIALS AND METHODS

Growth factors. The growth factors used were as follows: PDGF (Upstate Biotechnology), EGF (Upstate Biotechnology), CSF-1 (a kind gift from the Genetics Institute) and bombesin and LPA (both from Sigma).

Purification of Src from insect cells, and kinase assay. We have previously described a method to purify proteins that contain SH2 domains (10). Briefly,

after a 72-h coinfection of an adherent culture with a baculovirus expressing human wild-type Src, cells were removed from the dish, washed with phosphate-buffered saline (PBS), and resuspended in 0.7 ml of buffer (0.1% Nonidet P-40, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 2 mM dithiothreitol, 10 mM NaF, 100 μM sodium orthovanadate, 20 μM leupeptin, 1% aprotinin, 100 μM phenylmethylsulfonyl fluoride) per 10^7 cells. The entire procedure was carried out at 4°C. Cells were broken in a Dounce homogenizer with 20 strokes, and the suspension was clarified by centrifugation at 35,000 × *g* for 30 min. The extract was then passed through a 45-μ-pore-size filter, loaded onto a phosphotyrosine column, and eluted with a gradient of NaCl (0 to 1,000 mM; gradient volume, 30 ml). Fractions containing the pure protein were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie blue. Positive fractions were desalted by dialysis against a buffer of 20 mM Tris (pH 7.5) and 2 mM dithiothreitol and concentrated in a Centricon 30 microconcentrator (Amicon). This purification method routinely gave a c-Src fraction with a purity of greater than 90%, as estimated by Coomassie blue staining. Purified Src protein (2 ng) was incubated with 40 ng of the affinity-purified antibodies for 30 min on ice, 2.5 μM acid-denatured enolase and 10 μM [γ-^{32}P]ATP were added, and samples were incubated for 5 min at 30°C. Products were resolved by SDS-PAGE.

Constructs. cDNAs containing the complete regions of the kinase-active and kinase-inactive form of Src and Fyn were cloned into pSG5 as described previously (28). The kinase-inactivating mutation in Src was Lys-295 → Met, and that in Fyn was Lys-299 → Met.

Antibodies. The cst-1 antibody was raised against peptide YQPGENL, which is present in the C termini of Src, Fyn, and Yes and recognizes all three by immunoblot and kinase assay. The monoclonal Y13-259 *ras* antibody was kindly provided by Julian Downward and purified on a protein G column (Pharmacia) as specified by the manufacturer. Anti-Src.1 recognizes an epitope in the unique domain of Src (4), and anti-p110.2 recognizes an epitope in the catalytic domain of the PI 3-K (21). Antibodies were purified as described previously (21, 28). Briefly, ammonium sulfate-precipitated sera were loaded onto Sepharose columns to which the cognate peptides had been coupled, and the columns were washed with 10 mM sodium phosphate–500 mM NaCl buffer (pH 7.0). Antibody was eluted with 1 M propionic acid, and fractions were collected into 1 M ice-cold sodium phosphate buffer (pH 7.0) and concentrated in a minicon microconcentrator (Amicon) to 1 to 2 mg/ml for the αcst-1 antibody and 4 mg/ml for Y13-259. Nonimmune immunoglobulin G (IgG) purified from normal rabbits by using the protein G column was mixed with Y13-259 solution to a final concentration of 0.5 mg/ml before microinjection.

Cell culture techniques and microinjection of cells. The cells used were NIH 3T3 cells, NIH 3T3 cells transformed with an activated allele of Src (cSrcY527F), NIH 3T3 cells engineered to express the CSF-1 receptor (T56 cells [23, 25]), and Swiss 3T3 cells. The cells were grown at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics under an atmosphere of 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 Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum, transferrin (5 μg/ml), insulin (5 μg/ml), and antibiotics or, for NIH 3T3 cells expressing CSF-1 receptors, Dulbecco's modified Eagle's medium containing transferrin (5 μg/ml) and insulin (5 μg/ml) and incubated for a further 24 to 48 h. In the case of the cSrcY527F cells, microinjections were done in medium containing 10% fetal calf serum. Prior to microinjection, HEPES was added to a concentration of 20 mM. The purified antibodies were injected into cell cytoplasm with an automated microinjection system (AIS, Zeiss) as described previously (1) just prior to growth factor addition, unless stated otherwise. Typically, 100 to 150 cells were microinjected per coverslip. DNA (50 μg/ml) was microinjected into the nucleus 6 h prior to growth factor addition. The needles used for microinjection were pulled from capillaries (Clark Electromedical Instruments) with a Fleming-Brown micropipette puller. DNA synthesis was monitored by adding BrdU (final concentration, 100 μM; Sigma). The cells were incubated at 37°C for a further 18 to 20 h, 24 h (for Swiss 3T3 cells), or 30 h (for cSrcY527F cells) and then fixed for immunostaining.

Immunofluorescence. Coverslips were washed once with PBS and fixed for 5 min with ice-cold methanol. Antibody-injected cells were detected by incubating the coverslips with fluorescein-conjugated goat anti-rabbit antibody (Dianova) diluted in PBS (1:100) for 30 min and then washed three times in PBS. Injected cells overexpressing Src or Fyn were stained with affinity-purified αcst-1 antibody (1:50) for 30 min, washed three times with PBS, and then incubated with fluorescein-conjugated goat anti-rabbit antibody. In the case of the cSrcY527F cells, actin was visualised with Texas red-conjugated phalloidin (1:200). To analyze for DNA synthesis, cells were incubated for 10 min in 1.5 M HCl, washed three times as before, stained with monoclonal (1:15) anti-BrdU antibody (Boehringer), washed again, and then stained with a Texas red-conjugated anti-mouse antibody (Dianova). All coverslips were finally washed in PBS containing Hoechst 33258 (final concentration, 1 μg/ml; Sigma), rinsed in water, inverted, and mounted in Moviol (Hoechst) on glass slides. Slides were viewed with an Axiophot fluorescence microscope.

RESULTS

We have previously reported that an antibody specific for the carboxy-terminal tail of Src, Fyn, and Yes (anti-cst-1) was capable of inhibiting PDGF-induced mitogenesis in fibroblasts (28). The mechanism by which the antibody inhibited signal transduction was not clear, since it was known to precipitate kinase-active Src family molecules. To investigate this, we tested its effect on purified Src in solution. We have previously described a protocol for the one-step purification of SH2 domain-containing proteins from baculovirus-infected insect cells by phosphotyrosine affinity chromotography (10). Wild-type human Src obtained from a baculovirus expression system was purified in this way and incubated with affinity-purified antibodies prior to the kinase assay. Figure 1A shows that the cst-1 antibody caused a significant reduction in the kinase activity of Src. On average, the inhibition was approximately threefold and was detected with more than one preparation of antibody. The antibody inhibited the enzyme activity of Src rather than affecting substrate binding, since the kinase assay was carried out under conditions of substrate excess. A Src-specific antibody, αSrc.1, which recognizes an epitope in the unique domain of Src, also had a modest inhibitory effect on Src activity. Csk kinase activity (a tyrosine kinase not recognized by anti-cst-1) was not inhibited with the cst-1 antibody, and antibodies specific for the PI 3-K or Yes did not inhibit Src activity in vitro, showing that the effect of cst-1 was specific (Fig. 1A; data not shown). However, we have also observed the inhibitory effect of the cst-1 antibody on purified preparations of the related kinase Fyn (data not shown).

To examine the effect of anti-cst-1 in vivo, it was microinjected into cells transformed with an activated allele of Src (cSrcY527F). The cst-1 antibody, but not nonspecific IgG, caused phenotypic reversion of the transformed cells, as measured by morphology and actin staining (Fig. 1B). The cells became flatter and showed a reduced number of actin-containing ring-shaped membrane ruffles characteristic of the transformed cells; stress fiber formation was also visible. No morphological changes were detected in Ras-transformed cells into which the cst-1 antibodies had been microinjected (data not shown). Thus, the cst-1 antibody neutralizes the effects of Src and presumably also of Fyn and Yes in vitro and in vivo.

We have previously shown that Src family tyrosine kinases are required for PDGF to transmit a mitogenic signal in NIH 3T3 cells (28). We have also demonstrated that when heterologously expressed in fibroblasts, the closely related CSF-1 receptor activates and associates with tyrosine kinases of the Src family (4). We therefore tested whether Src family kinases were required for CSF-1 receptor-mediated mitogenic signals. In the experiment whose results are shown in Fig. 2, quiescent fibroblasts expressing the CSF-1 receptor (T56 cells) were seeded onto coverslips and given microinjections of affinity-purified cst-1 antibodies or of the same antibodies pretreated with cognate peptide prior to microinjection. BrdU and human CSF-1 were added, and the cells were incubated at 37°C for 18 h, fixed, and stained. The cst-1 antibody inhibited the ability of the cells to respond to CSF-1, whereas the cells given microinjections of the mix of antibody and peptide were not inhibited. The data obtained with several hundred cells analyzed in this way are shown in Fig. 3. When a suboptimal dose of CSF-1 was used, approximately 35% of cells entered the S phase in response to the ligand, and microinjection of anti-cst-1 inhibited this by more than 85% (Fig. 3A). When a saturating dose of CSF-1 was used (Fig. 3B), inhibition by the cst-1 antibody was still observed, although in this case it was not as pronounced, being approximately 65%. In both cases, the inhibi-

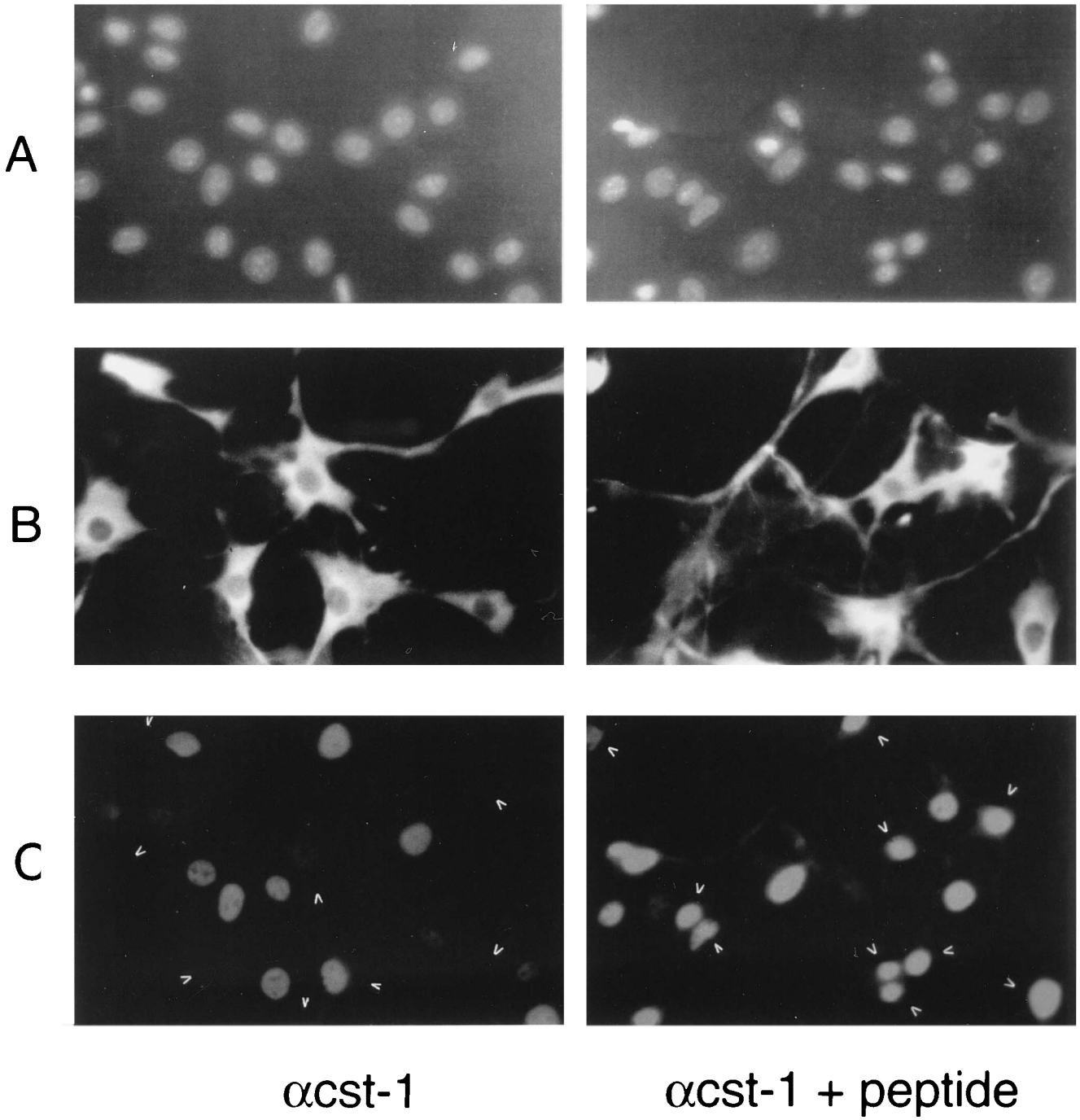


FIG. 2. An antibody specific for Src, Fyn, and Yes inhibits CSF-1-stimulated DNA synthesis. Quiescent T56 cells seeded onto coverslips were given microinjections (into the cytoplasm) of αcst-1 antibodies or αcst-1 preblocked with cognate peptide as indicated in the figure. CSF-1 and BrdU were added to the cells, and 18 h later the cells were fixed and stained. Hoechst staining reveals the position of all nuclei (A). The αcst-1 antibodies were visualized with a fluorescein-conjugated anti-rabbit antibody (B), and the BrdU antibodies were visualized with Texas red-conjugated anti-mouse antibodies (C). The white arrowheads in panel C mark the positions of the microinjected cells.

tion was at least partially reversed by cognate peptide, demonstrating the specificity of the effect. Finally, we used the dominant interfering construct of Src that we have previously described to test whether we achieved the same inhibition (Fig. 3C). First, a plasmid expressing a kinase-active form of Src ($SrcK^+$) under the control of the simian virus 40 promoter was microinjected into T56 cells, and a concentration of DNA that did not stimulate cells to enter the S phase in the absence of added growth factors was determined. This ensured that the level of overexpression of Src was not high enough to cause transformation and growth factor independence (it has been demonstrated that when Src is overexpressed by more than

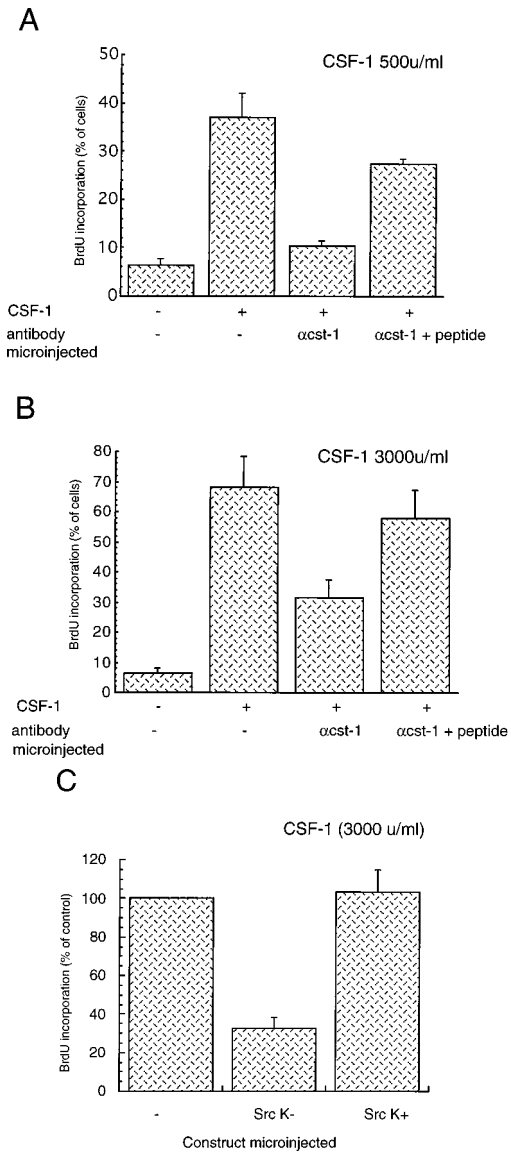


FIG. 3. Inhibition of CSF-1 signalling in fibroblasts expressing CSF-1 receptors. (A and B) Quiescent T56 cells were given microinjections of affinity-purified αcst-1 antibodies premixed or not with its cognate peptide, stimulated with 500 U (A) or 3,000 U (B) of CSF-1 per ml, and processed for immunofluorescence as described in the legend to Fig. 2. For each experiment, several coverslips were analyzed and the percentage of BrdU-positive cells was calculated. For the microinjected cells, the extent of DNA synthesis was calculated by the following formula: percentage of cells = (number of cells injected that were BrdU positive/total number of injected cells) × 100. The results from several experiments ($n > 3$) have been averaged, and the mean and standard deviation of the mean are shown. (C) Quiescent T56 cells seeded onto coverslips were given microinjections of the constructs shown, stimulated with CSF-1 (3,000 U/ml), and processed for immunofluorescence as described in Materials and Methods. For each experiment, several coverslips were analyzed, and the extent of DNA synthesis in injected cells was calculated by the following formula: percentage of control = [percentage of BrdU-positive cells (injected)/percentage of BrdU-positive cells (uninjected)] × 100.

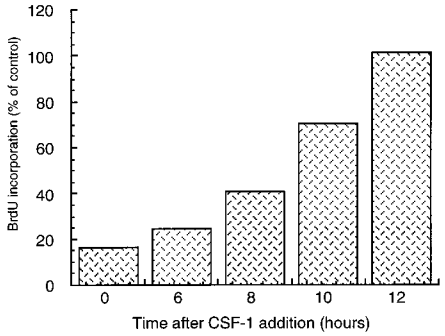


FIG. 4. Time course of αcst-1 inhibition of CSF-1-stimulated DNA synthesis. Quiescent T56 cells were given microinjections of affinity-purified αcst-1 antibodies at the time shown relative to CSF-1 addition. All coverslips were processed 18 h after growth factor addition. Several coverslips were analyzed, and for each time point the extent of DNA synthesis in injected cells was calculated by the following formula: percentage of control = [percentage of BrdU-positive cells (injected)/percentage of BrdU-positive cells (uninjected)] × 100.

20-fold, it can no longer be regulated, and partial transformation ensues [8]). Microinjection of this amount of $SrcK^+$ had no effect on CSF-1 induction of the S phase. However, cells expressing equivalent levels of kinase-inactive Src ($SrcK^-$) were strongly inhibited in their ability to respond to CSF-1. These data therefore confirm the observations made with the antibodies: Src family kinases are required for an efficient response to CSF-1. To examine the duration of the requirement for Src family kinases, quiescent T56 cells were stimulated with CSF-1 and then given microinjections of anti-cst-1 at various times after growth factor addition. Figure 4 shows that the antibody still had significant inhibitory effects several hours after CSF-1 addition, demonstrating that the Src family kinases are required for the first 8 to 10 h of G_1.

Several experiments have implicated the Src family kinases in the response to EGF. The most compelling observations are those from the Parsons laboratory that cells overexpressing kinase-active forms of Src are hyperresponsive to EGF (15) and, conversely, that cells that contain modest levels of kinase-inactive Src respond poorly to EGF (32). We investigated this further with our microinjection system. The anti-cst-1 antibody caused a strong inhibition of EGF-induced DNA synthesis, which was more pronounced at low doses of EGF than at saturating doses (Fig. 5A and B). The specificity of the inhibition was demonstrated by the partial reversing effects of the cognate peptide. Furthermore, the inhibitory effect of the antibody was observed not just in NIH 3T3 cells but also in Swiss 3T3 cells, which respond well to EGF (data not shown). In keeping with the results of Parsons and coworkers, $SrcK^-$ had a strong inhibitory effect, whereas $SrcK^+$ at the same concentration did not (Fig. 5C). We also investigated whether this effect was specific to $SrcK^-$ by testing constructs expressing a kinase-inactive form of Fyn, $FynK^-$ (28). We found that this protein was as effective as $SrcK^-$ in inhibiting EGF-induced DNA synthesis (Fig. 5C). In contrast, $FynK^+$ had no inhibitory effect on EGF signalling, as measured in this assay.

To date, our analyses with both dominant-interfering mutants and antibodies have shown that PDGF, EGF, and CSF-1 all require Src family tyrosine kinases to transmit mitogenic signals. Do all growth factors require Src kinases? To investigate this, we used Swiss 3T3 cells, which respond well not only to PDGF and EGF but also to two other ligands, bombesin and LPA. The receptors for these last two ligands are coupled to G proteins; however, ligand stimulation results in a rapid increase in tyrosine phosphorylation (6, 11, 13, 33). Indeed, LPA treatment of neuroblastoma cells has been reported to activate Src (7). Figure 6 shows that the Swiss 3T3 cells responded well to PDGF, LPA, and bombesin. However, the cst-1 antibody inhibited only PDGF-induced DNA synthesis and had no effect

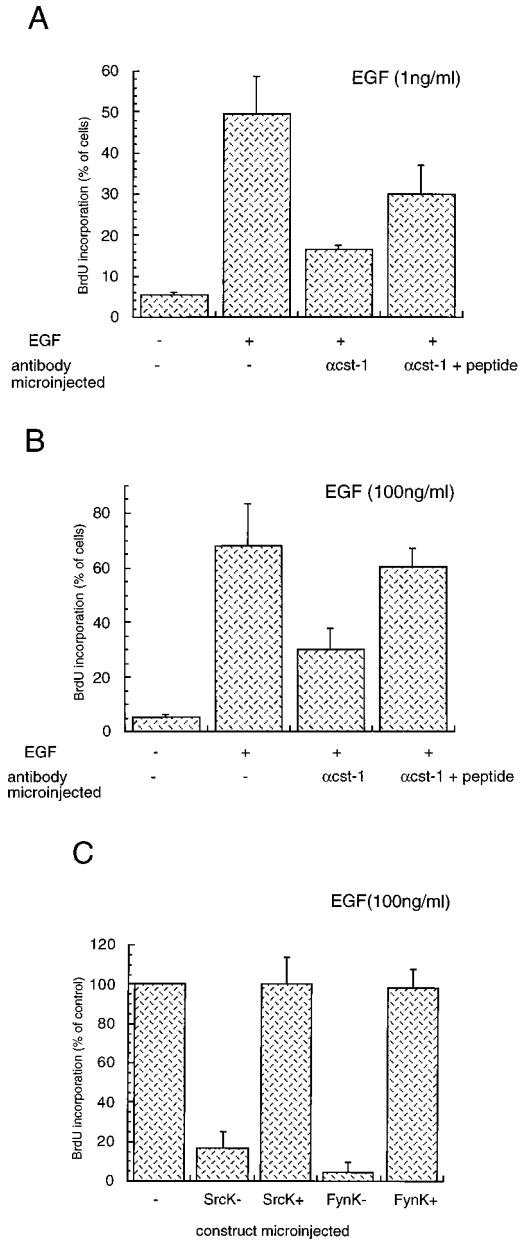


FIG. 5. Inhibition of EGF signalling in NIH 3T3 cells. (A and B) Quiescent NIH 3T3 cells were given microinjections of affinity-purified αcst-1 antibodies premixed or not premixed with cognate peptide, stimulated with EGF at 1 ng/ml (A) or 100 ng/ml (B), and processed for immunofluorescence as described in the legend to Fig. 2. For each experiment, several coverslips were analyzed and the percentage of BrdU-positive cells was calculated. For the microinjected cells, the extent of DNA synthesis was calculated by the following formula: percentage of cells = (number of injected cells that were BrdU positive/total number of injected cells) × 100. The results from several experiments ($n > 3$) have been averaged, and the mean and standard deviation of the mean are shown. (C) Quiescent NIH 3T3 cells seeded onto coverslips were given microinjections of the constructs shown, stimulated with EGF (100 ng/ml), and processed for immunofluorescence as described in Materials and Methods. For each experiment, several coverslips were analyzed, and the extent of DNA synthesis in injected cells was calculated by the following formula: percentage of control = [percentage of BrdU-positive cells (injected)/percentage of BrdU-positive cells (uninjected)] × 100.

on either bombesin or LPA. We have also found that $SrcK^-$ had no inhibitory effect on LPA-induced DNA synthesis (data not shown). Finally, we showed that even though bombesin did not required Src family kinases, DNA synthesis was dependent on functional Ras proteins (Fig. 6).

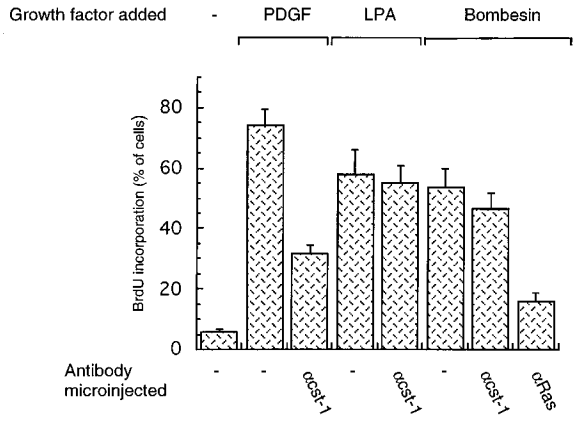


FIG. 6. αcst-1 antibody inhibits neither LPA- nor bombesin-induced DNA synthesis. Quiescent Swiss 3T3 cells seeded onto coverslips were given microinjections of αcst-1 or αRas antibodies as indicated, stimulated with PDGF (25 ng/ml), LPA (1 μM), or bombesin (30 nM), and processed for immunofluorescence as described in Materials and Methods. For each experiment, several coverslips were analyzed, and the percentage of BrdU-positive cells was calculated. The results from several experiments ($n > 3$) have been averaged, and the mean and standard deviation of the mean are shown.

DISCUSSION

We have used two different approaches to test which growth factors require Src family protein tyrosine kinases to induce DNA synthesis in fibroblasts. In the first, an affinity-purified antibody, termed cst-1, that recognizes the carboxy termini of the related kinases Src, Fyn, and Yes was microinjected, inhibiting the response of cells to both CSF-1 and EGF but not to LPA nor bombesin. We also show that this antibody reduced the intrinsic kinase activity of Src (and Fyn [data not shown]) when tested in a solution kinase assay in vitro and morphologically reverted Src-transformed cells when microinjected in vivo. It seems likely, therefore, that the antibody will also lower kinase activity in vivo and that this would explain, at least in part, its inhibitory effect on certain growth factor responses. This may not be its only mode of action, however; we have previously speculated that since the antibody recognizes an epitope in close proximity to the SH2 domain, it may also interfere with the ability of the Src kinases to associate with specific phosphotyrosines on interacting proteins (28). The second approach involved microinjection of plasmids encoding kinase-active and -inactive forms of Src. $SrcK^-$ also inhibited both CSF-1 and EGF but not LPA-induced DNA synthesis, in complete agreement with the antibody experiments. An important control when overexpressing proteins that contain SH2 domains is to demonstrate specificity. We showed that the kinase-active form of Src or Fyn did not perturb signalling in response to EGF when expressed at the same concentration as $SrcK^-$ or $FynK^-$. This demonstrates that the inhibitory effect was probably due to competition with wild-type Src family kinases for receptor-binding sites and/or substrate proteins and not to nonspecific SH2 domain competition, which would have been expected to occur with $SrcK^+$ as well.

With the antibody microinjection experiments, we noticed that the degree of inhibition we observed depended on the concentration of growth factor used; more inhibition was observed at suboptimal doses of ligand than at saturating doses. This observation is not restricted to the cst-1 antibody, since we also observed it with antibodies specific for PI 3-K, which is required for DNA synthesis in response to PDGF and EGF but not to CSF-1, LPA, or bombesin (21). We could think of two

explanations. Either the antibodies are not able to neutralize all of the target proteins within cells, so that the larger the number of receptors activated, the more likely it is that some are able to associate with active targets. Alternatively, at suboptimal concentrations of ligand, the receptors are more dependent on multiple signalling pathways than at higher concentrations. The latter explanation might account for the apparent lack of requirement for receptor-associated proteins that has been reported [for example, for PI 3-K [9, 31]). This hypothesis can be tested by microinjecting mixtures of antibodies, and these experiments are in progress.

Our observations that Src family protein tyrosine kinases are required for fibroblasts to respond to EGF are consistent with the observations of Parsons and collaborators (15, 32). Nevertheless, activation of Src family kinases by EGF treatment has not been reported, and although a recent report suggests that there may be a weak interaction of Src and the activated EGF receptor (14), this was not seen in a reconstituted system (4). The experiments reported here and previously have demonstrated that functional Src family kinases are required for several hours after growth factor stimulation. Microinjection of antibodies as late as 8 to 10 h after growth factor stimulation caused a significant inhibition of CSF-1- and PDGF-induced DNA synthesis (28; also see above). Experiments to date have concentrated on events occurring within the first hour after growth factor stimulation. Our future experiments will look at later times for EGF-mediated Src and Fyn kinase activation and/or association with other proteins.

Both bombesin and LPA induce increases in tyrosine phosphorylation (6, 11, 13, 33). A modest stimulation of Src activity in neuroblastoma cells treated with LPA has also been reported (7). Furthermore, tyrosine kinase activity is required for LPA-induced mitogenesis (24). It is unlikely from the data reported here that bombesin and LPA require Src family kinases to induce DNA synthesis (although they may play a role in cytoskeletal and/or shape changes in response to these ligands). Other kinases must therefore be involved. The focal adhesion kinase (Fak), whose activity is increased in bombesin- and LPA-treated Swiss 3T3 cells (6, 11, 34), is a likely candidate.

Why do PDGF, CSF-1, and EGF signalling require Src family kinases? One model that has been proposed is amplification of signals. However, this seems unlikely, given that only a small percentage of the activated PDGF and CSF-1 receptors associate with Src kinases. It seems more probable that the Src family kinases phosphorylate proteins that are not substrates for the receptors themselves. This hypothesis is supported by recent observations that Src and the PDGF receptor have distinct substrate specificities in vitro (1a). We are currently measuring the phosphorylation of certain potential substrates, particularly at late times after growth factor addition. We are also using the microinjection techniques described here to attempt to define the signalling pathway in which the Src family kinases participate.

ACKNOWLEDGMENTS

We thank Margaret Jones for help with the antibody purification; Julian Downward for Ras antibodies; Chris Marshall for Ras-transformed cells; Don Fujita for human Src; Geraldine Twamley-Stein for $SrcK^+$, $FynK^+$, $SrcK^-$, and $FynK^-$; and Enrique Rozengurt for Swiss 3T3 cells.

S.R. is supported by INSERM. M.F.R. is supported by NIH grant CA-56819, NIH core grant CA-21765, and the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital.

REFERENCES

1. **Ansorge, W., and R. Pepperkok.** 1988. Performance of an automated system for capillary microinjection into living cells. J. Biochem. Biophys. Methods **16:**283–292.
1a. **Cantley, L. C.** Personal communication.
2. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64:**281–302.
3. **Courtneidge, S. A.** 1994. Protein tyrosine kinases, with emphasis on the Src family. Semin. Cancer Biol. **5:**239–246.
4. **Courtneidge, S. A., R. Dhand, D. Pilat, G. M. Twamley, M. D. Waterfield, and M. Roussel.** 1993. Activation of Src family kinases by colony stimulating factor-1, and their association with its receptor. EMBO J. **12:**943–950.
5. **Gould, K., and T. Hunter.** 1988. Platelet-derived growth factor induces multisite phosphorylation of $pp60^{c\text{-}src}$ and increases its protein-tyrosine kinase activity. Mol. Cell. Biol. **8:**3345–3356.
6. **Hordijk, P. L., I. Verlaan, E. J. van Corven, and W. H. Moolenaar.** 1994. Protein tyrosine phosphorylation induced by lysophosphatidic acid in rat-1 fibroblasts. J. Biol. Chem. **269:**645–651.
7. **Jalink, K., T. Eichholtz, F. R. Postma, E. J. van Corven, and W. H. Moolenaar.** 1993. Lysophosphatidic acid induces neuronal shape changes via a novel, receptor-mediated signaling pathway: similarity to thrombin action. Cell Growth Differ. **4:**247–255.
8. **Johnson, P. J., P. M. Coussens, A. V. Danko, and D. Shalloway.** 1985. Overexpressed $pp60^{c\text{-}src}$ can induce focus formation without complete transformation of NIH 3T3 fibroblasts. Mol. Cell. Biol. **5:**1073–1083.
9. **Kazlauskas, A., A. Kashishian, J. A. Cooper, and M. Valius.** 1992. GTPase-activating protein and phosphatidylinositol 3-kinase bind to distinct regions of the platelet-derived growth factor receptor β subunit. Mol. Cell. Biol. **12:**2534–2544.
10. **Koegl, M., R. M. Kypta, M. Bergman, K. Alitalo, and S. A. Courtneidge.** 1994. Rapid and efficient purification of Src homology 2 domain-containing proteins: Fyn, Csk, and phosphatidylinositol 3-kinase p85. Biochem. J. **302:** 737–744.
11. **Kumagai, N., N. Morii, K. Fujisawa, T. Yoshimasa, K. Nakao, and S. Narumiya.** 1993. Lysophosphatidic acid induces tyrosine phosphorylation and activation of MAP-kinase and focal adhesion kinase in cultured Swiss 3T3 fibroblasts. FEBS Lett. **329:**273–276.
12. **Kypta, R. M., Y. Goldberg, E. T. Ulug, and S. A. Courtneidge.** 1990. Association between the PDGF receptor and members of the src family of tyrosine kinases. Cell **62:**481–492.
13. **Leeb-Lundberg, F., and X.-H. Song.** 1991. Bradykinin and bombesin rapidly simulate tyrosine phosphorylation of a 120-kDa group of proteins in Swiss 3T3 cells. J. Biol. Chem. **266:**7746–7749.
14. **Luttrell, D. K., A. Lee, T. J. Lansing, R. M. Crosby, K. D. Jung, D. Willard, M. Luther, M. Rodriguez, J. Berman, and T. M. Gilmer.** 1994. Involvement of $pp60^{c\text{-}src}$ with two major signaling pathways in human breast cancer. Proc. Natl. Acad. Sci. USA **91:**83–87.
15. **Luttrell, D. K., L. M. Luttrell, and S. J. Parsons.** 1988. Augmented mitogenic responsiveness to epidermal growth factor in murine fibroblasts that overexpress $pp60^{c\text{-}src}$. Mol. Cell. Biol. **8:**497–501.
16. **Mori, S., L. Rönnstrand, K. Yokote, A. Engström, S. A. Courtneidge, L. Claesson-Welsh, and C.-H. Heldin.** 1993. Identification of two juxtamembrane autophosphorylation sites in the PDGF β-receptor. Involvement in the interaction with Src family tyrosine kinases. EMBO J. **12:**2257–2264.
17. **Nishimura, R., W. L. Kashishian, A. Mondino, M. Zhou, J. Cooper, and J. Schlessinger.** 1993. Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor. Mol. Cell. Biol. **13:**6889–6896.
18. **Pawson, T., and J. Schlessinger.** 1993. SH2 and SH3 domains. Curr. Biol. **3:**434–442.
19. **Ralston, R., and J. M. Bishop.** 1985. The product of the proto-oncogene c-*src* is modified during the cellular response to platelet-derived growth factor. Proc. Natl. Acad. Sci. USA **82:**7845–7849.
20. **Reedijk, M., X. Liu, and T. Pawson.** 1990. Interactions of phosphatidylinositol kinase, GTPase-activating protein (GAP), and GAP-associated proteins with the colony-stimulating factor 1 receptor. Mol. Cell. Biol. **10:**5601–5608.
21. **Roche, S., M. Koegl, and S. A. Courtneidge.** 1994. The phosphatidylinositol 3-kinase α is required for DNA synthesis induced by some, but not, all growth factors. Proc. Natl. Acad. Sci. USA **91:**9185–9189.
22. **Roussel, M. F., T. J. Dull, C. W. Rettenmier, P. Ralph, A. Ullrich, and C. J. Sherr.** 1987. Transforming potential of the c-*fms* proto-oncogene (CSF-1 receptor). Nature (London) **325:**549–552.
23. **Roussel, M. F., S. A. Shurtleff, J. R. Downing, and C. J. Sherr.** 1990. A point mutation at tyrosine 809 in the human colony-stimulating factor 1 receptor impairs mitogenesis without abrogating tyrosine kinase activity, association with phosphatidylinositol 3-kinase, or induction of *fos* and *jun*B genes. Proc. Natl. Acad. Sci. USA **87:**6738–6742.
24. **Seckl, M., and E. Rozengurt.** 1993. Tyrphostin inhibits bombesin stimulation of tyrosine phosphorylation, c-*fos* expression, and DNA synthesis in Swiss 3T3 cells. J. Biol. Chem. **268:**9548–9554.
25. **Shurtleff, S. A., J. R. Downing, C. O. Rock, S. A. Hawkins, M. F. Roussel, and**

C. Sherr. 1990. Structural features of the colony-stimulating factor 1 receptor that affect its association with phosphatidylinositol 3-kinase. EMBO J. **9:**2415–2421.

26. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and L. C. Cantley.** 1993. SH2 domains recognize specific phosphopeptide sequences. Cell **72:**767–778.
27. **Twamley, G., B. Hall, R. Kypta, and S. A. Courtneidge.** 1992. Association of Fyn with the activated PDGF receptor: requirements for binding and phosphorylation. Oncogene **7:**1893–1901.
28. **Twamley-Stein, G. M., R. Pepperkok, W. Ansorge, and S. A. Courtneidge.** 1993. The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH-3T3 cells. Proc. Natl. Acad. Sci. USA **90:**7696–7700.
29. **van der Geer, P., and T. Hunter.** 1993. Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. EMBO J. **12:** 5161–5172.
30. **Varticovski, L., B. Druker, D. Morrison, L. Cantley, and T. Roberts.** 1989. The colony stimulating factor-1 receptor associates with an activated phosphatidylinositol 3-kinase. Nature (London) **342:**699–702.
31. **Wennström, S., A. Siegbahn, K. Yokote, A.-K. Arvidsson, C.-H. Heldin, S. Mori, and L. Claesson-Welsh.** 1994. Membrane ruffling and chemotaxis transduced by the PDGF β-receptor require the binding site for phosphatidylinositol 3′ kinase. Oncogene **9:**651–660.
32. **Wilson, L. K., D. K. Luttrell, K. T. Parsons, and S. J. Parsons.** 1989. pp60$^{c\text{-}src}$ tyrosine kinase, myristylation, and modulatory domains are required for enhanced mitogenic responsiveness to epidermal growth factor seen in cells overexpressing c-*src*. Mol. Cell. Biol. **9:**1536–1544.
33. **Zachary, I., J. Gil, W. Lehmann, J. Sinnett-Smith, and E. Rozengurt.** 1991. Bombesin, vasopressin, and endothelin rapidly stimulated tyrosine phosphorylation in intact Swiss 3T3 cells. Proc. Natl. Acad. Sci. USA **88:**4577–4581.
34. **Zachary, I., J. Sinnett-Smith, and E. Rozengurt.** 1992. Bombesin, vasopressin and endothelin stimulation of tyrosine phosphorylation in Swiss 3T3 cells. Identification of a novel tyrosine kinase as a major substrate. J. Biol. Chem. **267:**19031–19034.