

# Signal Transduction by Nerve Growth Factor and Fibroblast Growth Factor in PC12 Cells Requires a Sequence of Src and Ras Actions

Norbert E. Kremer,\* Gabriella D'Arcangelo,\* Sheila M. Thomas,‡ Michael DeMarco,‡ Joan S. Brugge,‡ and Simon Halegoua\*

\*Department of Neurobiology and Behavior, State University of New York at Stony Brook, Stony Brook, New York 11794-5230; and

‡Howard Hughes Medical Institute, Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 19118

**Abstract.** We have investigated the roles of pp60<sup>c-src</sup> and p21<sup>c-ras</sup> proteins in transducing the nerve growth factor (NGF) and fibroblast growth factor (FGF) signals which promote the sympathetic neuronlike phenotype in PC12 cells. Neutralizing antibodies directed against either Src or Ras proteins were microinjected into fused PC12 cells. Each antibody both prevented and reversed NGF- or FGF-induced neurite growth, a prominent morphological marker for the neuronal phenotype. These data demonstrate the involvement of both pp60<sup>c-src</sup> and p21<sup>c-ras</sup> proteins in NGF and FGF actions in PC12 cells, and establish a physiological role for the pp60<sup>c-src</sup> tyrosine kinase in signal transduction pathways initiated by receptor tyrosine kinases in these cells. Additional microinjection experiments,

using PC12 transfectants containing inducible *v-src* or *ras* oncogene activities, demonstrated a specific sequence of Src and Ras actions. Microinjection of anti-Ras antibody blocked *v-src*-induced neurite growth, but microinjection of anti-Src antibodies had no effect on *ras* oncogene-induced neurite growth. We propose that a cascade of Src and Ras actions, with Src acting first, is a significant feature of the signal transduction pathways for NGF and FGF. The Src-Ras cascade may define a functional cassette in the signal transduction pathways used by growth factors and other ligands whose receptors have diverse structures and whose range of actions on various cell types include mitogenesis and differentiation.

**N**ERVE growth factor (NGF)<sup>1</sup> (39) and fibroblast growth factor (FGF) (for review see reference 70) are neuronal growth factors which mediate survival and differentiation of many types of neurons *in vivo* and in primary neuronal cultures. Application of NGF or FGF to cells of the clonal rat pheochromocytoma cell line, PC12, engages a program of physiological changes, resulting in a phenotype resembling that of sympathetic neurons. These changes include neurite outgrowth, establishment of a sodium-based action potential, induction of a variety of biochemically defined neuronal characteristics, and the cessation of cell division (see reference 18). The PC12 cell line has thus been a useful model system for studying the intracellular events associated with growth factor-induced neuronal differentiation.

The signal transduction pathways used by NGF to accomplish these changes have been extensively studied but are only partially understood. Underlying the physiological changes brought about by NGF and FGF is a coordinated sequence of posttranslational modifications and changes in gene expression that occur throughout persistent treatment with the growth factor (for review see reference 23). Several

second messenger pathways have been implicated in NGF action including those which lead to protein phosphorylations (23) via activations of kinases including A-kinase and C-kinase (9, 25). NGF has also been shown to cause a rapid increase in the phosphorylation of several proteins on tyrosine (42). However, the signal-transducing molecules responsible for second messenger generation and the relationships between these second messengers and the long-term actions of NGF such as neurite outgrowth have not been well established. Although FGF has actions on PC12 cells very similar to those of NGF (56, 66, 67), its transduction pathways have only recently been a subject of study.

Recent evidence has implicated both *src* and *ras* proto-oncogene activities in the NGF signal transduction pathway leading to neurite growth. The *ras* gene products are GTP-binding proteins that play critical roles in growth regulation (for review see reference 4). Activated, mutant Ras proteins, that stimulate mitogenesis and oncogenic transformation in many cell types, mimic a number of neuronal growth factor actions in PC12 cells, including induction of neurite outgrowth (3, 50). Furthermore, microinjection of a neutralizing mAb to Ras into fused PC12 cells prevents and reverses neurite outgrowth induced by NGF (21), and expression of a dominant inhibitory *ras* mutant in PC12 cells blocks several of the effects of NGF and FGF (64). The *c-src* gene

1. *Abbreviations used in this paper:* FGF, fibroblast growth factor; GAP, GTPase activating protein; MMTV, mouse mammary tumor virus; NGF, nerve growth factor.

product, pp60<sup>c-src</sup>, belongs to a family of protein tyrosine kinases, whose members have proposed roles in signal transduction of receptor-regulated physiological processes (see reference 5). A potential role for pp60<sup>c-src</sup> or other tyrosine kinase(s) in NGF signal transduction was suggested by the ability of viral, oncogenic Src to mimic NGF-induced neurite outgrowth (1). Furthermore, NGF and pp60<sup>c-src</sup> rapidly stimulate tyrosine phosphorylations of common protein substrates (65). Recent evidence suggests that the functional NGF receptor includes both a previously identified 75-kD NGF-binding protein (27, 53), as well as the *trk* proto-oncogene tyrosine kinase (26, 31, 32). The FGF receptor also possesses tyrosine kinase activity (38), further suggesting a tyrosine kinase pathway in neuronal growth factor action.

The roles and relationships between *src* and *ras* products have been partially addressed with respect to growth control and oncogenesis in fibroblastic cells. Mitogenesis stimulated by growth factors or by *v-src* is blocked by microinjected anti-Ras (49, 61), or by expression of a dominant inhibitory *ras* mutant (7) in NIH 3T3 cells. This suggests that p21<sup>c-ras</sup> action is essential to the actions of some tyrosine kinases, but it was not determined whether the tyrosine kinase and p21<sup>c-ras</sup> act in parallel or in series.

Studies in PC12 cells and fibroblastic cell lines indicate striking similarities between the roles of Src and Ras in the control of neuronal differentiation and of mitogenesis. To further elucidate the role of the *src* and *ras* proto-oncogenes in neuronal growth factor action, we have examined the ability of microinjected anti-Src and anti-Ras to inhibit NGF- and FGF-induced neurite outgrowth from fused PC12 cells. These antibodies were also used to determine the functional relationship between Src and Ras proteins by examining their ability to inhibit oncogene-directed neurite growth after microinjection into PC12 sublines expressing inducible *v-src* or *ras* oncogene products.

## Materials and Methods

### Cell Culture and Fusion

PC12 cells (17), and PC12-derived subclones, were grown in DME (69) containing 10% horse serum and 5% FCS (JRH, Lenexa, KS), at 37°C in an atmosphere of 10% CO<sub>2</sub>, 90% air. PC12 cells or subclones were fused while attached to tissue culture dishes (51), fractionated on a 10 to 30% serum gradient, and replated at low density on glass cover slips coated with laminin and poly-lysine, as described (22). For the GSras1 line (see below), one day before fusion, the growth medium was changed to one containing serum which had been treated with activated charcoal to remove endogenous glucocorticoids. NGF (2.5 S; 46) was added, where indicated, to the cultures at 50 ng/ml. Recombinant bovine basic FGF (kindly supplied by Dr. A. Baird, Whittier Institute, La Jolla, CA) was used, where indicated, at 10 ng/ml with replenishment every two days. Dexamethasone (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO, and was added to cultures, at the concentrations indicated. The final concentration of DMSO did not exceed 0.1% of the culture volume, and this DMSO level had no effect on NGF-induced neurite outgrowth. The ts:v-src3 cells were maintained at 39.5°C, and the cells were shifted to the permissive temperature of 35°C, where indicated, by moving the cultures to a separate incubator.

### Generation of PC12 Sublines

GSras1 cells were isolated by cotransfection of PC12 cells with two plasmids: pMJC25 and pRSVneo, using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA). The plasmid, pMJC25 (the gift of M. Corbley and T. Roberts, Dana Farber Cancer Institute, Boston, MA), contains a mutated, human c-Ha-*ras*-1 gene (encoding p21<sup>L61</sup>), a p21 with leucine at position 61; see reference 10), under control of the mouse mammary tumor

virus (MMTV) promoter. The plasmid, pRSVneo, contains the gene encoding neomycin resistance, under control of Rous Sarcoma Virus long terminal repeat sequences (16). Electroporation of  $4 \times 10^6$  cells was carried out in 0.4 ml DME containing 18  $\mu$ g pMJC25 and 2  $\mu$ g pRSVneo, using settings of 300 V and 500  $\mu$ F. Two days after transfection, cells were subjected to G418 (Geneticin®; Gibco Laboratories, Grand Island, NY; 800  $\mu$ g/ml) selection. The selective medium was renewed every two days. After two weeks, resistant colonies were picked. Clones were screened for dexamethasone-induced neurite outgrowth, and one such clone, designated GSras1, was used in the present studies.

PC12 cells containing a dominant inhibitory *ras* mutant product, p21<sup>N17</sup> (13), was generated as above, by cotransfecting pRSVneo, and a second construct, pMMRasDN, containing the *ras* mutant gene under control of the MMTV promoter (constructed using vector sequences of pMM42K, obtained from K. Wood, Dana Farber Cancer Institute) and the entire coding sequence of p21<sup>N17</sup> (obtained from L. Feig, Tufts Medical School, Boston, MA). Clones were screened for the ability of dexamethasone to induce p21<sup>N17</sup> transcripts and to block NGF-induced neurite growth. One such clone, designated GSrasDN13, was used in the present studies.

The isolation and characterization of the ts:v-src3 cells is described elsewhere (65). PC12 cells were infected with a replication-defective murine leukemia virus containing murine leukemia virus long terminal repeat sequences directing transcription of the neomycin resistance gene, and the SV-40 early region promoter driving the ts:v-src gene cloned from Rous Sarcoma Virus strain NY72-4, which exhibits temperature-sensitive tyrosine kinase and transforming activity (28, 43). Infected cells were maintained at 39.5°C, selected with G418, cloned, and screened for neurite outgrowth at the permissive temperature of 35°C.

A PC12 subline expressing avian pp60<sup>c-src+</sup> was obtained followed infection with a recombinant murine leukemia retrovirus, LNCXc-src+, carrying the avian *c-src+* gene (40) under the transcriptional control of the cytomegalovirus early promoter (45). Infected cells were selected with G418, cloned and screened for expression of pp60<sup>c-src+</sup> kinase activity, using antibodies specific for chicken Src proteins. One such clone, csrc+3, which expressed the highest level of pp60<sup>c-src+</sup>, was used in the present studies.

### Antibody Preparation

All antibodies were stored at -20°C at 1-5 mg/ml in PBS (137 mM NaCl, 2.7 mM KCl, 8.06 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>). Before microinjection, concentration and buffer exchange to 0.5  $\times$  PBS (68.6 mM NaCl, 1.35 mM KCl, 4.03 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.74 mM KH<sub>2</sub>PO<sub>4</sub>), was performed with a Centricon 30 ultrafilter (Amicon, Danvers, MA). The final concentrations of antibodies used for microinjection was: anti-Src 327 (ascites-derived), 8-10 mg/ml; anti-Src 327 (hybridoma derived), 12 mg/ml; anti-Src (2-17), 13 mg/ml; anti-Ras Y13-259, 10-12 mg/ml; control antibodies, 12 mg/ml.

Control antibodies were immunoglobulins purified from non-immune rabbit serum by protein A-agarose affinity chromatography (Immunopure kit; Pierce Chemical Co., Rockford, IL), and dialysis against PBS. Monoclonal anti-Ras Y13-259 was obtained from the serum-free supernatant of rat hybridoma cells (kindly provided by Dr. E. Scolnick, Merck and Co., Rahway, NJ), and purified by precipitation from 50% ammonium sulfate, and protein-A agarose chromatography, followed by dialysis against PBS. After ultrafiltration, 50- $\mu$ l aliquots at 10 mg/ml were stored at 4°C. Concentrated Y13-259 consistently precipitated after several weeks of such storage, so each aliquot of antibody was tested for its ability to block NGF-induced neurite outgrowth immediately before its use in other experiments.

Monoclonal anti-Src 327 was prepared from mouse ascites fluid or from the serum-free supernatant of hybridoma cells. It was precipitated from 50% ammonium sulfate, dialyzed against 20 mM Tris-HCl, pH 7.9, 40 mM NaCl, and fractionated on DEAE-Sephacel or by high performance chromatography using a DEAE-5PW column (Waters Chromatography, Milford, MA) eluted with a 30-500 mM NaCl gradient, and stored at -20°C at  $\sim$ 5 mg/ml. Buffer exchange and concentration to 8-10 mg/ml were done as above. Anti-Src 327 appeared to be very stable when stored at this concentration at 4°C. However, the degree of inhibitory activity of each preparation of anti-Src 327 varied. Differences in the inhibitory activity appeared to correlate with differences in the titer of the antibody when assayed by immunoprecipitation of pp60<sup>c-src</sup>. Furthermore, an anti-Src 327 preparation that was purified by elution from protein A-agarose with denaturants was inactive in inhibiting neurite outgrowth, immunoprecipitated 5-10-fold less pp60<sup>c-src</sup> than the preparations fractionated by ion exchange chromatography, and was relatively unstable when stored at 4°C. As above, each aliquot of 327 was tested for its ability to inhibit NGF-induced neurite outgrowth just before its use in other experiments.

Anti-Src (2-17) was prepared in the same manner as anti-Src 327 (see above), by ammonium sulfate precipitation and ion exchange chromatography of ascites fluid obtained from mice injected with hybridoma LA074 cells from Microbiological Associates (Rockville, MD).

### Microinjection

Microinjection was performed using glass micropipettes containing a filament for back filling, and a micromanipulator (Wild Leitz, Rockleigh, NJ). Cells in growth medium were injected at room temperature, and the pH of the medium was held roughly constant by passing a gentle stream of CO<sub>2</sub> over the culture dish. Every cell in a marked region of the culture dish could be microinjected by using the XY controls of the microscope stage to scan the dish in a raster pattern. We estimate that the injection volume was ~10% of the cell's volume.

## Results

### Src Requirement for NGF Action

To facilitate antibody microinjection, PC12 cells were fused to form multinucleated giant cells. NGF rapidly initiates neurite growth in the fused cells (22; and Fig. 1). Fused cells are readily microinjected and retain viability and responsiveness to growth factors after microinjection of non-specific control antibodies. However, microinjection of the neutralizing anti-Ras Y13-259 prevents and reverses NGF-induced neurite outgrowth of these cells (21).

The same experimental paradigm was used to determine whether pp60<sup>c-src</sup> is essential for the induction of neurite outgrowth by neuronal growth factors. In the experiments shown in Fig. 1, we examined the effects of microinjection of fused PC12 cells with monoclonal anti-Src 327 (41). This antibody was selected for our study because microinjection of anti-Src 327 into BALB/c-3T3 cells expressing a temperature-sensitive mutant of pp60<sup>v-src</sup> inhibited the induction of DNA synthesis in serum-free quiescent cultures shifted to the permissive temperature (P. Walton, J. Brugge, and J. Feramisco, unpublished data).

To quantitate neurite outgrowth, the number of neurite-bearing cells was counted in each set of microinjected cells. Control cells were either injected with purified non-specific immunoglobulins, or left uninjected. Monoclonal anti-Ras Y13-259 injections were performed as a reference standard. The results from three representative experiments are shown in Fig. 1. Injection of non-specific antibodies had little effect on the viability, or NGF responsiveness of the fused cells and, as previously reported, anti-Ras Y13-259 caused a substantial reduction in the number of NGF-induced neurites. In each experiment, microinjection of anti-Src 327 inhibited NGF-induced extension of neurites. Since the fraction of neurite-bearing cells in the control NGF-treated cells varied somewhat in each fused cell preparation, we defined an inhibition index,  $I = 1 - P_t/P_c$ , where  $P_t$  is the percentage of cells bearing neurites in cells after growth factor treatment and injection with the test antibody, and  $P_c$  is the percentage of cells bearing neurites after growth factor treatment and injection with control antibody. In the three experiments shown in Fig. 1, microinjection of anti-Src caused a 67 to 77% inhibition of neurite growth, in a total of 974 cells injected with anti-Src 327 and 1,018 cells injected with control antibody. In three additional experiments (not shown), microinjection of anti-Src caused a 49 to 70% inhibition of neurite outgrowth, with an average of 62% inhibition (weighted average based on the number of cells injected in

each experiment, with a total of 361 anti-Src-injected cells and 286 control antibody-injected cells). Under conditions where the cells were pretreated with NGF for periods sufficient to allow neurite outgrowth before microinjection, the neurites retracted in response to microinjection of anti-Src 327. For example, in comparing the experiments shown in Fig. 1, a similar degree of inhibition was seen whether NGF application preceded, or essentially coincided with antibody microinjection. This result suggests that Src action is continuously required for NGF-mediated neurite outgrowth, in the same way that NGF is continually required to elicit neurite outgrowth (17).

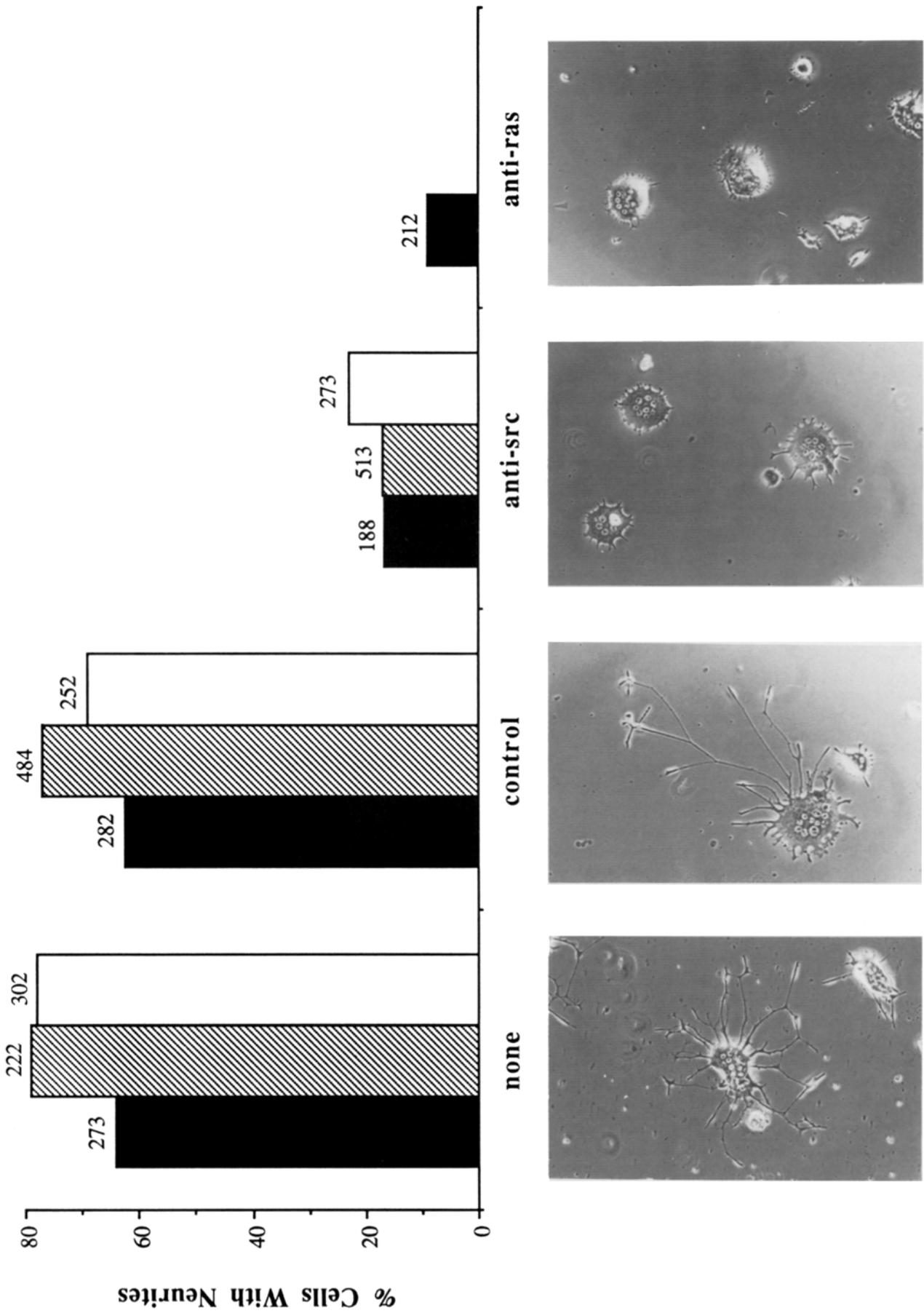
Several different preparations of anti-Src 327 (purified from mouse ascites, or from serum-free hybridoma medium) were effective in inhibiting neurite outgrowth induced by NGF, suggesting that the observed inhibition of neurite growth is not because of an impurity in the anti-Src 327 reagent. A second preparation of ascites-derived anti-Src 327 yielded 58% inhibition (78 anti-Src-injected cells and 81 control antibody-injected cells), and anti-Src 327 isolated from serum-free hybridoma culture medium gave 47% inhibition (172 anti-Src-injected cells and 88 control antibody-injected cells). To further ensure that the effects of anti-Src 327 were because of its specific ability to interact with Src protein, the antibody was microinjected into cells of a PC12 subline, c-src+3, which express pp60<sup>c-src+</sup>, the neuronal c-src variant (see Materials and Methods; 40) at levels 10- to 20-fold greater than the endogenous pp60<sup>c-src</sup> level (unpublished data). In these cells, ascites-derived anti-Src 327 failed to inhibit NGF-induced neurite growth (an apparent slight increase of 11% in neurite growth, based on 267 anti-Src 327-injected cells and 464 control antibody-injected cells).

To further establish the role of pp60<sup>c-src</sup>, we performed additional microinjection experiments with a second mAb, anti-Src (2-17), (see Materials and Methods), raised against a synthetic peptide corresponding to the unique amino terminus of pp60<sup>c-src</sup> (amino acid residues 2-17). In six experiments, using a total of 2,604 microinjected cells (2,076 anti-Src-injected and 528 control antibody-injected), microinjection of fused PC12 cells with anti-Src (2-17) caused inhibition of NGF-induced neurite outgrowth ranging from 46 to 92%, with an average inhibition of 65% (weighted average calculated as above). Thus, the inhibition induced by this antibody is similar to that obtained using anti-Src 327.

The inhibitory effects of anti-Src 327 microinjection were reversible. Inhibition of NGF-induced neurites was maximal 24 h after injection and decayed by 60 h. The transient nature of the inhibitory effects of microinjected antibodies is likely because of intracellular degradation of the antibodies (49, 71), and further indicates that the inhibition of neurite growth is not because of a toxic effect of the injected antibody. Furthermore, the anti-Src 327 only slightly inhibited the transient and robust (98% of cells) outgrowth of neurite-like processes induced by 1 mM dibutyryl cAMP treatment (59) of fused PC12 cells (inhibition index = 20%, based on a total of 317 anti-Src-injected cells and 341 control antibody-injected cells).

### Cascade of Src and Ras Actions

The above results suggest that pp60<sup>c-src</sup> as well as a *ras* gene product are essential components of an NGF signal transduc-



tion pathway for neurite growth. To determine whether the Src and the Ras components are acting in parallel or in sequence, we generated clonal PC12 cell sublines expressing inducible Src or Ras transforming proteins. In similar cell lines, neurite growth can be induced by pp60<sup>v-src</sup> (54) or the oncogenic Ras protein (63) in the absence of growth factor receptor activation. The PC12-derived ts:v-src3 cell line contains a mutant v-src gene expressing a temperature-sensitive v-src kinase (65). The ts:v-src3 cells express pp60<sup>v-src</sup> at a level approximately equal to endogenous pp60<sup>v-src</sup> in PC12 cells (unpublished data). Shifting these cells from the maintenance temperature of 39.5°C to the permissive temperature of 35°C caused formation of neurites under the direction of the ts:v-src kinase. In fused ts:v-src3 cells, neurite outgrowth was observed within 24 h after downshift. Another PC12 clone, GSras1, expressed the mutant c-ras oncogene product p21<sup>L61</sup>, under the transcriptional control of the glucocorticoid-inducible MMTV promoter (see Materials and Methods). Treatment of these cells with dexamethasone for 24 h caused an increase in p21<sup>L61</sup> transcription (data not shown) and in neurite outgrowth, as was reported for a similar cell line (63). GSras1 cells retained these properties after fusion. As expected, dexamethasone treatment of parental PC12 cells (fused or unfused) did not elicit neurite outgrowth (18).

To determine if pp60<sup>v-src</sup> required p21<sup>c-ras</sup> to elicit neurite growth, anti-Ras Y13-259 was injected into fused ts:v-src3 cells before temperature downshift. As seen in Fig. 2, microinjection of anti-Ras Y13-259 inhibited pp60<sup>v-src</sup>-induced neurite outgrowth at the permissive temperature. In results from three separate experiments including 563 microinjected cells, neurite outgrowth was inhibited 62 to 86% by microinjection of anti-Ras Y13-259. Microinjection of control non-specific antibody had no effect on neurite outgrowth after temperature downshift. These data suggested that at least one member of the p21<sup>c-ras</sup> protein family is essential for pp60<sup>v-src</sup>-induced neurite growth. In an alternative approach to investigate the role of Ras proteins in pp60<sup>v-src</sup> action, a PC12 cell subline containing a dominant inhibitory mutant ras gene, p21<sup>N17</sup> (13) under transcriptional control of the MMTV promoter, was established (see Materials and Methods). In this subline, GSrasDN13, dexamethasone-induced expression of p21<sup>N17</sup> inhibited neurite outgrowth induced by NGF, as seen in a similar PC12 subline (64). Infection of GSrasDN13 cells or PC12 cells with a v-src-containing retrovirus resulted in neurite growth (the number of neurites in a 60-mm tissue culture dish was counted). Treatment of GSrasDN13 cells with 0.5 μM dexa-

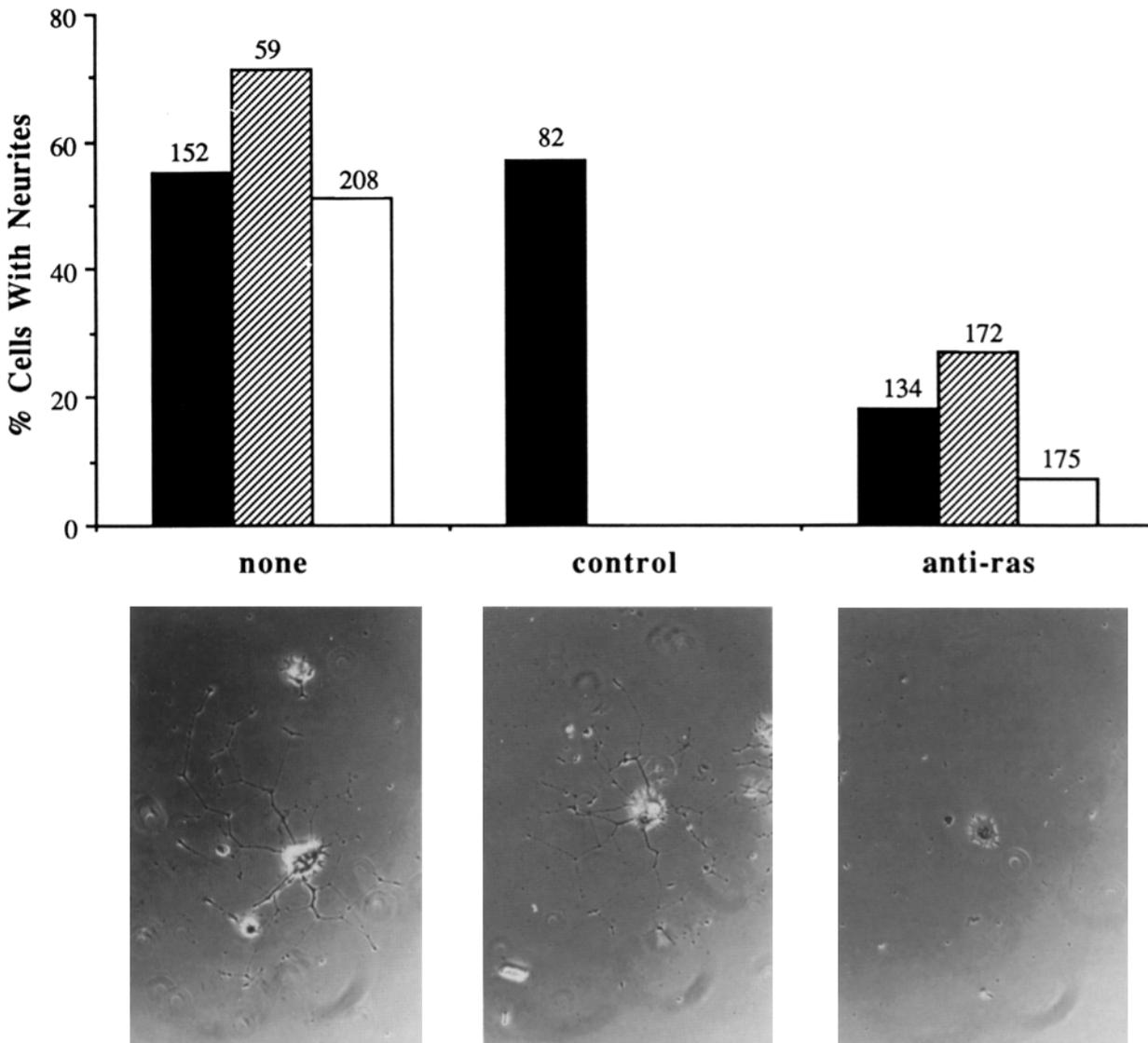
methasone, 2 d after infection, reduced the number of neurites by 85% (infection only, 806 neurites; infection and dexamethasone, 120 neurites), whereas dexamethasone treatment of infected PC12 cells reduced the number of neurites by only 3% (infection only, 648 neurites; infection and dexamethasone, 626 neurites). The above results indicate that Ras proteins may be acting either downstream of pp60<sup>v-src</sup>, or in a parallel pathway that is required for neurite outgrowth.

To further elucidate the relationship between the roles of Src and Ras in neurite outgrowth, experiments were performed with the GSras1 cell line, in which dexamethasone is used to induce neurites under the direction of p21<sup>L61</sup>. Anti-Src 327 microinjection did not reduce the number of GSras1 cells bearing neurites after treatment with dexamethasone, as indicated by the three experiments shown in Fig. 3. As would be expected however, microinjection of anti-Ras Y13-259 inhibited neurite outgrowth (65 and 57% inhibition, for two experiments shown in Fig. 3) resulting from dexamethasone treatment. This result suggests that pp60<sup>v-src</sup> is not required for the induction of neurites by p21<sup>L61</sup>. To ensure that generation of the GSras1 cell line did not disrupt the NGF signalling pathway which requires pp60<sup>v-src</sup> and p21<sup>c-ras</sup> proteins, GSras1 cells were also treated with NGF. Fused GSras1 cells produced neurites in response to NGF, and microinjection of anti-Src 327 or anti-Ras Y13-259 inhibited NGF-induced neurite growth in these cells (Fig. 3, *inset*). It was also possible that p21<sup>L61</sup> resulted in a more aggressive activation of pp60<sup>v-src</sup> which could not be significantly inhibited by microinjected anti-Src 327. To control for this possibility, fused ts:v-src3 cells, in which all pp60<sup>v-src</sup> molecules are highly activated, were injected with anti-Src 327. This resulted in a 52% inhibition of neurite growth after temperature downshift (derived from 306 anti-Src 327-injected cells, and 322 control antibody-injected cells). All of the above data indicate that although p21<sup>c-ras</sup> is essential for pp60<sup>v-src</sup>-induced neurite outgrowth, the converse is not true. The Src requirement in neurite growth was bypassed by the expression of the p21<sup>L61</sup> protein.

### Requirements of Src and Ras in FGF Action

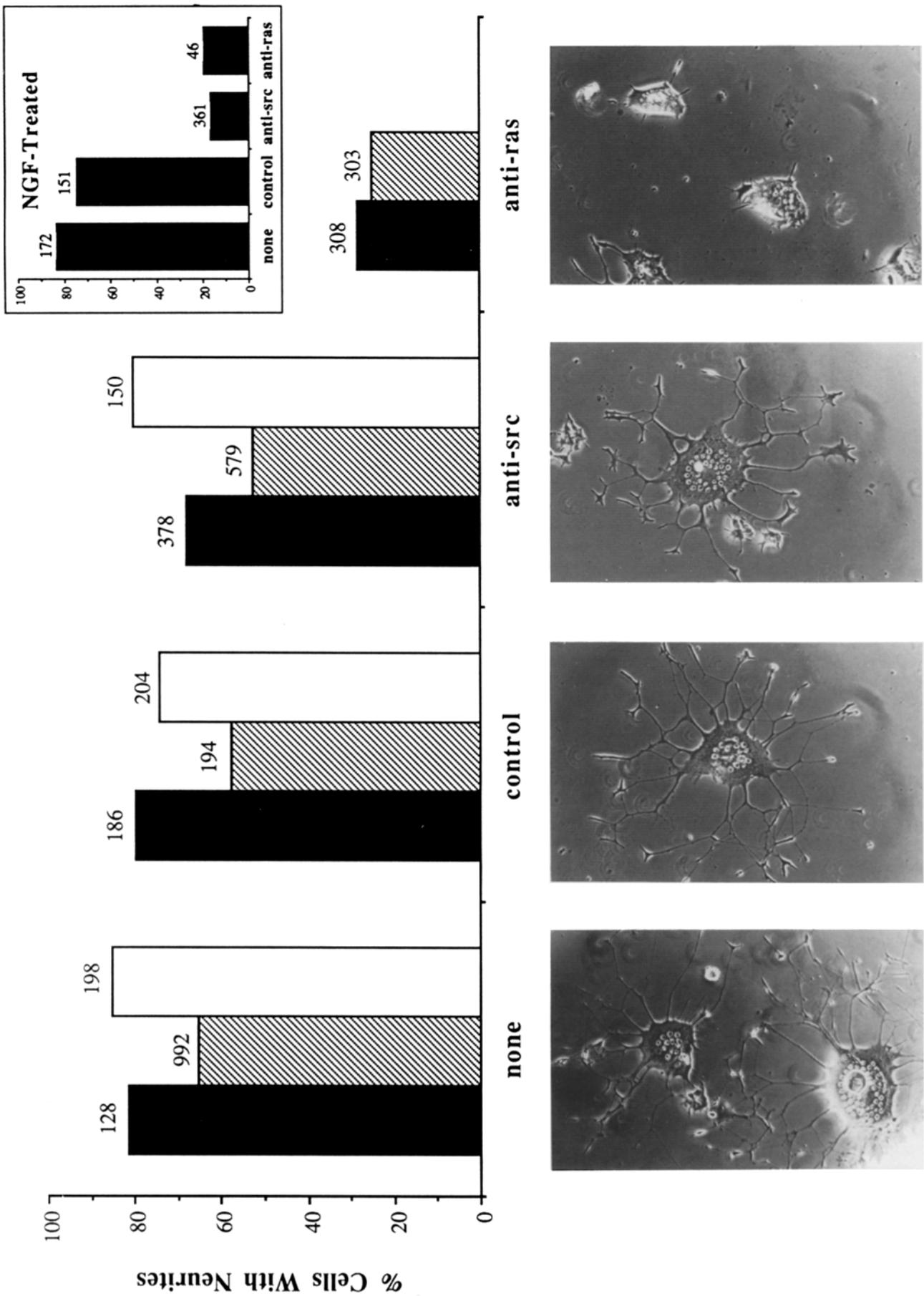
Our results suggest that both Src and Ras proteins are required for the induction of neurites by NGF. FGF is a neuronal growth factor whose receptor is a tyrosine kinase (38). PC12 cells express *flg*, but not *bek*, transcripts (D'Arcangelo, G., and C. Dionne, unpublished data) and FGF induces protein phosphorylation on tyrosines in these cells (Thomas, S.,

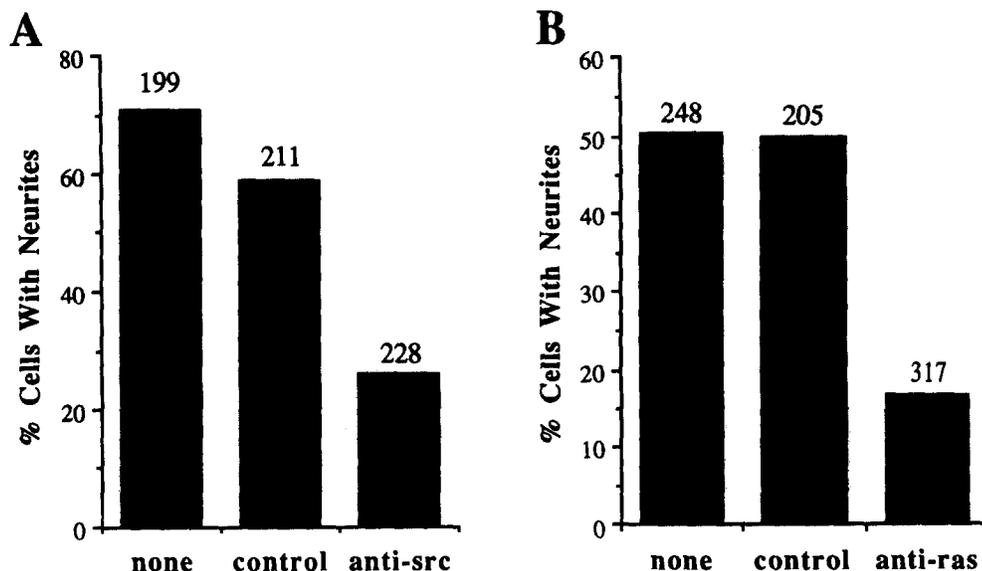
**Figure 1.** Inhibition of NGF-induced neurite outgrowth by microinjection of anti-Src 327 and anti-Ras Y13-259. PC12 cells were fused, treated with a saturating amount of NGF (50 ng/ml), microinjected, and incubated to allow neurite outgrowth, all as described in Materials and Methods. Cells were then scored for neurite outgrowth. A cell was considered to have neurites if it had one or more processes extending one or more cell body diameters (at least 50 μm) from the cell's periphery and with prominent growth cone(s). The results obtained from independent cell fusions are shown separately, indicated by solid, hatched, and open bars. The NGF-treated cells were either not injected (*none*), injected with control antibodies (*control*), injected with anti-Src 327 (*anti-src*), or injected with anti-Ras Y13-259 (*anti-ras*). Each bar plots the percentage of cells with neurites after NGF treatment, microinjection, and incubation. Above each bar is the number of cells counted for each particular treatment. In the first experiment (*solid bars*) NGF was applied 6 h before microinjection; no neurite outgrowth was observed at this early time. Cells were scored for neurites 24 h after microinjection. In the second and third experiments (*hatched*, *open*, respectively), NGF application for 20–22 h caused neurite outgrowth by the time of microinjection, and the cells were scored for neurites 23–26 h after microinjection. The photomicrographs show fused PC12 cells which have been treated with NGF, microinjected (or not) with the indicated antibodies and incubated. The photomicrographs were taken at the time of scoring the cells for neurites.



**Figure 2.** Inhibition of pp60<sup>v-src</sup>-induced neurite outgrowth by microinjection of anti-Ras Y13-259 into fused ts:v-src3 cells. Cells were fused, microinjected, and scored for neurite outgrowth as in Fig. 1. The results of three separate experiments are indicated by solid, hatched, and open bars. Each bar plots the percentage of cells bearing neurites after each treatment, with the total number of cells counted is shown above each bar. The ts:v-src3 cells were fused and maintained at 39.5°C until the time of microinjection. The cells were microinjected at room temperature with control antibodies (*control*) or with anti-Ras Y13-259 (*anti-ras*), and then ts:v-src activity was continually induced by incubating the cells at 35°C. The cells were scored for neurites 24 h later. The photomicrographs, made at the time of scoring the cells for neurites, are of typical ts:v-src3 cells after microinjection of the indicated antibodies.

**Figure 3.** Lack of inhibition of p21<sup>L61</sup>-induced neurites by microinjection of anti-Src 327 into fused GSras1 cells. Cells were fused, microinjected and scored for neurite outgrowth as in Fig. 1. The results from three independent experiments are indicated by solid, hatched, and open bars. Each bar plots the percentage of cells bearing neurites after each treatment, and the total number of cells counted is shown above each bar. In each experiment, GSras1 cells were fused, maintained in glucocorticoid-free medium, then treated with dexamethasone to induce MMTV-p21<sup>L61</sup>. GSras1 cells were either not injected (*none*), injected with control antibodies (*control*), injected with anti-Src 327 (*anti-src*), or injected with anti-Ras Y13-259 (*anti-ras*). The antibodies were from the same preparations, and at the same concentrations as in Fig. 1. In the first experiment with GSras1 cells (■), the cells were treated with 100 nM dexamethasone for 5 h, microinjected and then incubated with the same medium and scored for neurites 30 h later. In the second experiment (▨), 100 nM dexamethasone was added at the time of microinjection, and the cells were scored after 24 h. In the third experiment (□), 300 nM dexamethasone was added 7 h before microinjection, and the cells were scored 25 h later. The photomicrographs were taken at the time of scoring the cells for neurites. (*Inset*) An experiment was performed to determine whether the NGF signaling pathway in GSras1 cells was the same as in PC12 cells. Neurite outgrowth was induced by NGF rather than dexamethasone. The cells were treated with NGF for 4 h, microinjected, and then scored for neurites after an additional 24 h.





**Figure 4.** Inhibition of FGF-induced neurite outgrowth by microinjection of fused PC12 cells with anti-Src 327 or anti-Ras Y13-259. (A) Fused PC12 cells were treated with 10 ng/ml bFGF for 76 h and then microinjected with control antibodies (*control*) or with anti-Src 327 (*anti-src*). The cells were incubated with bFGF for an additional 18 h and were then scored for neurites, and the data plotted as in Fig. 1. (B) Fused PC12 cells were treated with 10 ng/ml bFGF for 30 h, and then microinjected with control antibodies (*control*) or with anti-Ras Y13-259 (*anti-ras*). The cells were incubated with bFGF for an additional 24 h and were then scored for neurites, and the data plotted as in Fig. 1. In both A and B, each bar plots the percentage of cells bearing neurites, and the number of cells counted after each treatment is shown above each bar.

and J. Brugge, unpublished data). To examine whether neurite outgrowth elicited by FGF is dependent on Src and Ras, we performed additional microinjection experiments with fused PC12 cells. As shown in Fig. 4, microinjection of anti-Src 327 caused a 56% inhibition (439 microinjected cells, Fig. 4 A) in the ability of bFGF to elicit neurites. Similarly, microinjection of anti-Ras Y13-259 into fused PC12 cells caused a 66% inhibition of neurite outgrowth (522 microinjected cells, Fig. 4 B). Control non-specific antibody injection did not cause a significant change in the number of cells bearing neurites after bFGF treatment. As in the NGF-treated fused PC12 cells, antibodies to Src or Ras protein caused retraction of previously formed neurites under conditions where the microinjection was delayed until FGF-induced neurites were elaborated (data not shown). The above results suggest that the action of bFGF, like that of NGF, involves a signal transduction pathway that requires the continual activity of both Src and Ras proteins.

### Discussion

Microinjection of antibodies into living cells has been an effective approach for identifying essential components in the pathways used by growth factors and oncogenes (14, 49, 61). We exploited this approach to define the roles of the *src* and *ras* proto-oncogene products and to determine their functional relationships in the signal transduction pathways used by growth factors such as NGF and FGF. Our assay for these growth factor actions has been the initiation and elongation of neuritic processes, a dramatic phenotypic change which accompanies neuronal-specific differentiation. The fused PC12 cell model (22, 51) was invaluable to our studies because the large fused cells remain unscathed after micropipette im-

palement and because neurite outgrowth is rapidly inducible by appropriate agents. A previous study which showed that microinjection of a neutralizing mAb to Ras blocks NGF-induced neurite growth provided evidence that a Ras protein is essential for NGF-induced neurite outgrowth (21).

Our experiments provide two novel findings: (a) Neutralization of either cellular Src or Ras actions by microinjection of corresponding mAbs interrupts transduction of the NGF and the FGF signals that induce neurite outgrowth; and (b) Neutralization of Ras action blocks *v-src*-induced neurite outgrowth; however, neutralization of Src action does not block *ras* oncogene-induced neurite outgrowth. Based on these findings, we propose that pp60<sup>c-src</sup> and p21<sup>c-ras</sup> proteins are integral components of a signal transduction cascade that is initiated by occupied NGF or FGF receptors to induce neurite outgrowth. Since both the NGF and FGF receptors possess tyrosine kinase activity, these results establish a physiological role for the pp60<sup>c-src</sup> tyrosine kinase in events initiated by receptor tyrosine kinases in PC12 cells. The simplest model which can accommodate all the available evidence involves the following sequence of actions: neuronal growth factor/receptor → pp60<sup>c-src</sup> → p21<sup>c-ras</sup> → neurites. The ability of antibodies to either the Src or Ras proteins to cause retraction of established neurites indicates that these components are constitutively required to transduce the NGF and FGF responses. This is consistent with the continual requirement of NGF or FGF in inducing neurite growth. Implicit in our model is the assumption that the transforming Src and Ras oncogene proteins faithfully mimic their physiologically activated cellular counterparts in inducing neurite outgrowth.

The identity of the antibody target is critical in the interpretation of these experiments. Anti-Ras Y13-259, which

was raised against p21<sup>v-ras</sup> (15), recognizes an epitope (37, 60) on viral and cellular Ras proteins including H-Ras, K-Ras, and N-Ras proteins (4), but does not recognize R-Ras proteins (F. McCormick, personal communication). Analysis of deduced amino acid sequences (57) and experimental evidence (F. McCormick, personal communication) show that Ras-related proteins in the Rap, Ral, Rho, and Rab families of GTP-binding proteins do not react with anti-Ras Y13-259. Thus, it is likely that one or more members of the Ras protein family mediates growth factor-induced neurite outgrowth. In further support of this role for p21<sup>c-ras</sup>, expression of a dominantly acting, inhibitory *ras* mutant blocks neurite outgrowth induced by NGF and FGF (64), and by pp60<sup>v-src</sup> (shown herein).

Our identification of pp60<sup>c-src</sup> as the specific antigen neutralized by anti-Src microinjection is more certain. Anti-Src 327 appears to recognize only Src proteins, since no detectable kinase activity was observed in anti-Src 327-derived immunoprecipitates from lysates of fibroblasts and brain tissue derived from mice homozygous for a *c-src* null mutation (62; J. Thomas and J. Brugge, unpublished data). It is conceivable that a low-affinity interaction of anti-Src 327 with a Src-related protein could be responsible for the inhibition of neurite outgrowth observed here; however the observation that a second antibody, anti-Src (2-17), which is specific for an amino acid sequence unique to pp60<sup>c-src</sup> (8), also inhibited NGF- and FGF-induced neurites, clearly establishes a role for pp60<sup>c-src</sup> in this process. The mechanism by which anti-Src (2-17) and anti-Src 327 neutralize Src functions is unknown. Binding of the antibodies to the myristylated amino terminus or SH3 regions, respectively, may directly prevent functional interactions at these domains, or the loss of function may result from a more generalized steric interference. The assignment of the anti-Src 327 epitope to the SH3 domain (amino acid residues 88-137) of the Src protein (55) is based on antibody recognition of numerous mutant variants of the Src protein (52; L. Fox, C. Seidel-Dugan, S. Thomas, and J. Brugge, unpublished data; and H. Hirai and H. E. Varmus, unpublished data).

The observations by ourselves and others, indicate that pp60<sup>c-src</sup> and p21<sup>c-ras</sup> are essential components of a complex signal transduction pathway used by NGF to cause neurite outgrowth in PC12 cells. The pathway used by NGF recruits a variety of second messengers which lead to numerous post-translational modifications and gene inductions to elicit its effects (for review see reference 23). Based on our functional assessments of these molecules, we have been able to order pp60<sup>c-src</sup> and p21<sup>c-ras</sup> activities within this pathway to neurite growth. However, Src and Ras proteins may not be involved in transducing all actions of NGF. For example, expression of *v-src* (54) or of a *ras* oncogene (29) does not mimic the NGF-induced increase in functional Na<sup>+</sup> channels in PC12 cells. The precise relationship between pp60<sup>c-src</sup> and p21<sup>c-ras</sup> functions, and the second messenger systems and other components of the signal transduction pathway which mediate neuronal growth factor actions, remains to be elucidated. We have proposed a simple linear cascade of Src and Ras actions in NGF and FGF actions, however, another interesting, though more complex model deserves attention. Receptor tyrosine kinases may activate Ras through Src-dependent as well as Src-independent pathways. In this model, the absolute requirement for Src may depend on the extent of recep-

tor activation, which can be limited by ligand concentration or receptor number (as is the case for NGF and FGF receptors in PC12 cells).

The ability of oncogenic variants of Src or Ras to induce both rapid and long-term events required for neurite outgrowth in the absence of neuronal growth factors suggests that cellular homologues of these proteins may act at an early stage in the signal transduction cascade. In addition, both pp60<sup>v-src</sup> and oncogenic Ras are able to "prime" (19) neurite growth induced by NGF, suggesting that NGF and these oncogenes use common pathways to elicit neurite growth, involving gene transcription (65). In support of this suggestion, pp60<sup>v-src</sup> (54, 65), and oncogenic Ras (20, 58; G. D'Arcangelo, R. Armstrong, and S. Haleboua, unpublished data) proteins induce rapid (immediate-early) as well as long-term expression of NGF-responsive genes. Furthermore, expression of a dominant inhibitory *ras* mutant blocks specific gene expression induced by NGF (64). Lastly, anti-Src does not block *ras* oncogene-induced neurite growth, and neither anti-Src nor anti-Ras block neuritelike process outgrowth induced by cAMP, suggesting that the Src and Ras proteins are not essential for process outgrowth per se. Based on all of the above considerations, we propose that stimulation of the Src → Ras cascade is an early event in the NGF and FGF signal transduction pathway, perhaps even directly interacting with the growth factor receptors themselves.

Src and Ras may both be components of protein assemblies which possibly include the NGF and FGF receptors. Protein complexes have been described, for example, for the PDGF receptor (a tyrosine kinase), which associates with the Src, Fyn, and Yes tyrosine kinases (36), the Ras-GTPase activating protein (GAP), phospholipase C-γ, and 3' phosphatidylinositol kinase (30, 33), and Raf-1 kinase (48), possibly to form a multimeric complex. It will be of interest to determine if such protein associations exist for the NGF receptor, and for the FGF receptor, both of which possess tyrosine kinase activity (26, 31, 32, 34, 38).

Our results from studies on NGF- and FGF-induced neurite outgrowth have several general implications. First, given the structural differences between NGF, FGF, and their receptors, the demonstration of pp60<sup>c-src</sup> and p21<sup>c-ras</sup> as essential components of both NGF- and FGF-induced neurite outgrowth suggests that neuronal growth factor signal transduction may in general involve a cascade of Src- and Ras-like actions. Consistent with this general scheme is the finding that oncogenic Ras can alleviate the survival requirements of various cultured neurons for several neuronal growth factors (6). Second, that the FGF receptor, Src and Ras can each mediate mitogenesis in one cell type and neuronal differentiation in another, suggests that similar mechanisms may underlie these diverse processes. We suggest that cascades similar to Src → Ras may represent a functional cassette used in the signal transduction pathways for growth factors and other ligands in a wide variety of physiological processes in different cell types. This idea is further supported by the Ras requirement in serum- and growth factor-induced mitogenesis (7, 49) in insulin-induced oocyte maturation (11, 35), and in developmental induction in *Caenorhabditis elegans* (2). The involvement of Src, or other non-receptor tyrosine kinases in such processes remains to be elucidated. Third, that the NGF and FGF receptors, which both possess tyrosine kinase activity, recruit the action of a non-receptor tyrosine ki-

nase, Src, suggests that such a sequence of tyrosine kinase activities may occur in both the mitogenic and in the differentiating actions of these growth factors and possibly those of other growth factors having tyrosine kinase receptors.

Determining the relationships between Src and Ras in growth control and oncogenesis has long been an active pursuit. *V-src*-induced mitogenesis requires a Ras-like action (7, 61), leaving undetermined the arrangement of Src and Ras actions in serial versus parallel pathways. Biochemical evidence compatible with a linear, sequential model for Src and Ras actions shows that the Ras GTPase-activating protein, GAP (68), is a substrate for pp60<sup>v-src</sup> as well as for tyrosine kinase growth factor receptors (12, 30, 47). Although the regulatory interactions between Ras and GAP are not fully understood (24, 44), phosphorylation of GAP by tyrosine kinases may be a mechanism by which Ras activity is controlled *in vivo*. Assuming that mitogenesis and neuronal differentiation are similarly induced by these oncogenes, we have further extended the functional relationship between Src and Ras activities in these processes by demonstrating a sequential pathway in which Ras action lies downstream of Src action. The possible intermediates in such a cascade remain to be elucidated.

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