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Induction of Neurite Outgrowth by v-*src* Mimics Critical Aspects of Nerve Growth Factor-Induced Differentiation

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PC12 cells treated with nerve growth factor (NGF) or infected with Rous sarcoma virus differentiate into sympathetic, neuronlike cells. To compare the differentiation programs induced by NGF and v-*src*, we have established a PC12 cell line expressing a temperature-sensitive v-*src* protein. The v-*src*-expressing PC12 cell line was shown to elaborate neuritic processes in a temperature-inducible manner, indicating that the differentiation process was dependent on the activity of the v-*src* protein. Further characterization of this cell line, in comparison with NGF-treated PC12 cells, indicated that the events associated with neurite outgrowth induced by these two agents shared features but could be distinguished by others. Both NGF- and v-*src*-induced neurite outgrowths were reversible. In addition, NGF and v-*src* could prime PC12 cells for NGF-induced neurite outgrowth, and representative early and late NGF-responsive genes were also induced by v-*src*. However, unlike NGF-induced neurite growth, v-*src*-induced neurite outgrowth was not blocked at high cell density. A comparison of phosphotyrosine containing-protein profiles showed that v-*src* and NGF each increase tyrosine phosphorylation of multiple cellular proteins. There was overlap in substrates; however, both NGF-specific and v-*src*-specific tyrosine phosphorylations were observed. One protein which was found to be phosphorylated in both the NGF- and v-*src*-induced PC12 cells was phospholipase C-γ1. Taken together, these results suggest that v-*src*'s ability to function as an inducing agent may be a consequence of its ability to mimic critical aspects of the NGF differentiation program and raise the possibility that Src-like tyrosine kinases are involved in mediating some of the events triggered by NGF.

A variety of neuronal growth factors that mediate neuronal survival and differentiation have been identified. The mechanisms through which growth factors mediate these events have only begun to be elucidated. The PC12 cell line has been a useful model to study the mechanism of nerve growth factor (NGF) action since it does not require these factors for survival but undergoes a long-term program of differentiation in the continual presence of NGF (17). A variety of biochemical, electrophysiological, and morphological changes underlie the response to NGF, yielding cells which display a phenotype characteristic of sympathetic neurons (18). This program has been compared with long-term phenotypic changes elicited by a variety of mitogenic and differentiating growth factors in which initial signal transduction events result in posttranslational modifications and transcriptional changes which mediate the response (12, 21).

The high-affinity NGF receptor contains two components, a p75 transmembrane protein (6, 25, 54) and the receptor protein tyrosine kinase Trk (24, 27, 31). NGF induces a rapid induction of tyrosine phosphorylation of multiple cellular proteins (39). In addition, NGF has been found to stimulate several second messenger pathways, including the stimulation of protein phosphorylation through the action of A kinase, C kinase, and other kinases (for a review, see reference 21). Together, these tyrosine and serine/threonine kinases participate in events such as the posttranslational activation of tyrosine hydroxylase (9, 45) (leading to increased neurotransmitter synthesis) and the induction of transcription of primary response genes such as c-*fos* (10, 16, 34, 47). Another component of the NGF signal transduction scheme has been suggested to be a member of the *ras* family of p21 GTP-binding proteins. Activated *ras* oncogenes have been found to mimic NGF-induced neurite outgrowth and other aspects of the differentiation response (3, 19, 52). Furthermore, microinjection of antibodies to c-*ras* proteins into PC12 cells (20) or expression of a dominant interfering *ras* mutant (61) was found to block NGF-induced neurite growth. Although c-*ras* would thus appear to be a mediator of NGF action, there is no evidence to indicate its functional proximity to the receptor. Similar experiments implicate $pp60^{c\text{-}src}$ or related proteins in neurite outgrowth. As with oncogenic *ras*, the v-*src* tyrosine kinase has been found to mimic NGF-induced neurite outgrowth and the elaboration of certain neuronal characteristics in PC12 cells (2). In addition, microinjection of monoclonal antibodies specific to $pp60^{c\text{-}src}$ inhibits NGF-induced neurite outgrowth (33a).

To compare the pathways regulated by NGF and v-*src* in PC12 cells, we have established a PC12 cell line which has stably integrated a temperature-sensitive v-*src* mutant gene. This line undergoes a differentiation program upon activation of the kinase activity of $pp60^{v\text{-}src}$ after temperature downshift. A comparison of v-*src*- and NGF-induced differentiation has revealed striking commonalities in the mode of action but differences in the effects of cell-cell contacts on neurite outgrowth and some differences in the range of tyrosine-phosphorylated substrates observed.

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MATERIALS AND METHODS

Plasmids and transfections. All restriction enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.). pLNSL7 is a retrovirus vector (generously provided by Dusty Miller, Fred Hutchinson Cancer Research Center) which contains the neomycin resistance gene, under the control of the Moloney murine leukemia virus long terminal repeats, and an internal simian virus 40 early promoter. pLNSL7 is similar to pLNSX (49) but has slightly different sequences at the cDNA cloning site. The sequences contained within the *Pvu*II fragment of the wild-type Schmidt-Ruppin A v-*src* gene were replaced with the analogous sequences of the tsNY72-4 v-*src* gene (obtained from H. Hanafusa, Rockefeller University) (14, 26, 44). This recombinant was then cloned into the *Cla*I site of the pLNSL7 vector, placing expression of the gene under the control of the simian virus 40 early promoter. To generate the LNSL7tsNY72-4 v-*src* virus, ψ2 cells were transfected with the pLNSL7tsNY72-4 construct by using the $CaPO_4$ procedure. After 24 h, the cells were passaged and G418 (Geneticin; GIBCO, Grand Island, N.Y.) was added (400 μg/ml in Dulbecco modified Eagle medium plus 10% calf serum). Virus stocks were prepared from cells expanded from cloned G418-resistant colonies.

Cell culture and infections. PC12 cells (17) were maintained in an atmosphere of 10% CO_2–90% air at 37°C and grown in Dulbecco's modified Eagle's medium (65) supplemented with 10% horse serum and 5% fetal bovine serum (JR Scientific, Woodland, Calif.). Cells were passaged every six days at a 1:5 dilution. For retrovirus-mediated infection, subconfluent cells were incubated in a 60 mM tissue culture dish with 1 ml of medium containing 8 μg of polybrene per ml and the LNSL7tsNY72-4 v-*src* retrovirus (at approximately 10^3 infectious particles per ml [titers determined on NIH 3T3 cells]) for 3 h at 37°C. The cells were then incubated in 5 ml of growth medium at 39.5°C for 36 h before medium was replaced with medium containing 800 μg of G418 per ml. This medium was changed every 2 to 4 days until single G418-resistant colonies were picked by using a micropipette and an inverted microscope.

To assay for neurite growth, PC12 cells or infectants were grown on tissue culture plastic coated with a solution of polylysine (25 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) and laminin (10 μg/ml) (Collaborative Research, Bedford, Mass.). The cells were plated at approximately 10^3 cells per 35-mm dish. Cells were scored positive for neurites if growth cone-containing neurites of at least two cell body diameters in length were observed. In the case of cells expressing the tsNY72-4 Src protein (ts:v-*src*3), temperature downshift was carried out by changing the growth medium to fresh medium at 35°C and incubating the cells at 35°C. For reversibility studies, the growth medium from cells growing at 35°C was changed to fresh medium at 41°C followed by incubation at 41°C. BALB/c3T3 and Rous sarcoma virus (RSV)-transformed 3T3 cells (SRD3T3) were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum at 37°C (4).

To assay the ability of v-*src* to prime NGF-mediated changes in gene expression, PC12 cells were cultured in the presence or absence of NGF for 4 days and then replated at 37°C in the presence or absence of NGF (purified according to the method of Mobley et al. [50]) on tissue culture plastic coated with polylysine and laminin.

ts:v-*src*3 cells were cultured at 41°C for 2 days and then downshifted to 35°C for 4 days or maintained at 41°C for an additional 4 days. Cells were then replated at 41°C in the presence or absence of NGF on tissue culture plates-coated with polylysine and laminin. Cells were assessed for neurite outgrowth 48 h later.

Immunoprecipitation of v-*src* protein. Cells were labeled for 16 h with 50 μCi of [^{35}S]methionine (ICN Pharmaceuticals, Inc., Irvine, Calif.) per ml in methionine-free Dulbecco modified Eagle medium supplemented with 10% complete media. The cells were washed three times with phosphate-buffered saline and lysates prepared in RIPA buffer (158 mM NaCl, 5 mM ethylenediaminetetraacetic acid [EDTA], 10 mM Tris [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 1% Triton X-100). $pp60^{v\text{-}src}$ was immunoprecipitated from cell lysates (normalized for trichloroacetic acid-precipitable [^{35}S] counts per minute) by using the avian *src*-specific monoclonal antibody EC10 (53) (kindly provided by Sarah Parsons, University of Virginia). Immunoprecipitates were analyzed on an SDS–7.5%-polyacrylamide gel. Following electrophoresis, the gel was fluorographed with 1 M sodium salicylate.

Northern (RNA) blot analysis. Cells were grown as above on tissue culture plastic dishes at 41°C. After 2 days, cells were downshifted to 35°C for varying lengths of time as indicated in the legends to the figures. Total cellular RNA was isolated essentially according to the method of Cathala et al. (5). Samples, 5 μg per lane, were subjected to electrophoresis through 1% agarose gels containing 2.2 M formaldehyde, 40 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7) (Sigma Chemical Co.), 10 mM sodium acetate, and 1 mM EDTA and transferred electrophoretically to nylon membranes (Zetaprobe; Bio-Rad, Richmond, Calif.). The blots were baked in vacuo at 80°C for 2 h and prehybridized in 50% formamide–1 M NaCl–0.1% sodium pyrophosphate–10% dextran sulfate–1% SDS–salmon sperm DNA (75 μg/ml) at 68°C for 4 h. RNA probes were synthesized with SP6 polymerase according to the manufacturer's instructions (Promega Biotec, Madison, Wis.) by using as templates the following linearized cDNA clones: SCG10 (58) (kindly provided by D. J. Anderson, California Institute of Technology) and P1B15 (11). The NGFIA (48) (kindly provided by J. Milbrandt) RNA probe was synthesized with T7 polymerase (Boehringer Mannheim Biochemicals, Inc., Indianapolis, Ind.). [^{32}P]CTP-labeled antisense RNA probes (2×10^6 dpm/ml) were hybridized to the blot at 68°C for 12 to 24 h with constant agitation. The blots were washed at successively increasing stringency and finally twice at high stringency in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 20 min at 68°C, and the blot was then subjected to autoradiography with preflashed Kodak XAR film.

Immunoprecipitation and immunoblotting of PLC-γ1. Cells were lysed in RIPA buffer supplemented with 1 mM vanadate (Fisher, Malvern, Pa.), 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.), and 1% trasylol (Miles, Inc., West Haven, Conn.). Lysates were clarified at 15,000 rpm for 30 min, and protein concentration was determined by a Lowry assay (37). For each set of lysates an equal amount of protein was incubated with phospholipase C-γ1 (PLC-γ1) antipeptide sera, and in some cases 1.6 μg of competing peptide was also added to the lysates (41, 42). Following incubation with protein A Sepharose, the beads were washed three times with RIPA buffer. The immunoprecipitates were fractionated on an SDS–7.5% polyacrylamide gel, transferred to nitrocellulose, and probed with either antibodies to phosphotyrosine as described below or PLC-γ1-antipeptide serum (1:200). Horseradish peroxidase-conju-

gated donkey anti-rabbit antibody (Amersham Inc., Arlington Heights, Ill.) was used as secondary antibody, and reactivity was determined by the ECL chemiluminescence reaction (Amersham Inc.).

Anti-phosphotyrosine immunoblotting. (i) Preparation of cell extracts. Total cell lysates were prepared by lysing the cells in 100°C lysis buffer (66 mM Tris [pH 7.5], 2% SDS, 1 mM vanadate [Fisher]). Lysates were boiled for 10 min and then passed repeatedly through a 26-gauge needle to shear the DNA. The lysates were then centrifuged for 15 min in a microfuge, and 2× electrophoresis sample buffer was added to the supernatant. After being normalized for total cell protein (37), 150 μg of each lysate was run on a SDS–7.5% polyacrylamide gel.

(ii) Immunoblots. Proteins were transferred to nitrocellulose, and the blot was incubated for 16 h in blocking solution (5% cohn-crystallized bovine serum albumin [BSA] [ICN Biomedicals, Inc.], 170 mM NaCl, 0.1% Nonidet P-40, 50 mM Tris [pH 7.5]). Immunoblots were probed with a solution consisting of the monoclonal phosphotyrosine antibodies 4G10, at a concentration of 1 μg/ml (generously provided by B. Drucker, D. Morrison, J. Burkhart, and T. M. Roberts, Dana-Farber Cancer Research Institute), and PY20, at a concentration of 1 μg/ml (ICN Biomedicals, Inc.), in buffer A (3% BSA, 170 mM NaCl, 0.2% Nonidet P-40, 50 mM Tris [pH 7.5]) and then washed in buffer B (buffer A without BSA). The filters were then incubated with buffer A for 30 min and then with a 1:2,500 dilution of horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (Amersham Corp.) or goat anti-mouse immunoglobulin G (1:600 [Bio-Rad]). Immunoreactivity was determined by the ECL chemiluminescence reaction (Amersham Corp.).

RESULTS

Characterization of PC12 cells expressing a temperature-sensitive v-*src*. PC12 cells were infected with a recombinant murine retrovirus, LNSL7tsNY72-4v-*src*, which carries the temperature-sensitive v-*src* gene (14, 26, 44) derived from the tsNY72-4 strain of RSV. G418-resistant cells were selected at 39.5°C. This partially permissive temperature was used for selection and maintenance of infected cells since long-term culture of PC12 cells at the nonpermissive temperature (41.5°C) severely restricted cell growth and viability. After 10 days of selection, all G418-sensitive cells were killed, and resistant cell colonies with several distinct morphologies, which were different from PC12 cells, were observed. Many cell clusters contained fully extended growth cone-containing neurites similar to those induced in PC12 cells by NGF. Cell colonies with short neuritic processes (about two cell body diameters) were also observed, as well as colonies of large, flattened cells with cytoplasmic extensions but no neurites. We attempted to isolate representative colonies of each morphology to expand for further analysis. The cells bearing long, extended neurites could not be passaged in culture. The large flat cells without neurites did not undergo any phenotypic change in morphology when shifted to the permissive temperature and were not responsive to NGF at either temperature. The infected cells with short processes elaborated fully extended neurites at the permissive temperature, displaying a morphology similar to that of NGF-treated or RSV-infected PC12 cells. One clone (ts:v-*src*3) which displayed the greatest difference in morphology under permissive and nonpermissive conditions was targeted for further studies.

Figure 1 shows the morphology of the ts:v-*src*3 cells at 39.5°C (panel E), 41°C (panel A), and 35°C (panel B). When cultured at the maintenance temperature (39.5°C), the ts:v-*src*3 cells displayed short processes yet proliferated in culture. Both the parental PC12 cells and ts:v-*src*3 cells could be cultured at 41°C for up to at least 10 days, although the cell division rate is slowed dramatically. The ts:v-*src*3 cells grown at 41°C displayed shorter cell projections (rarely containing neuritic growth cones; see Fig. 1A) and, as with PC12 cells grown at 41°C (data not shown), were more flattened. Upon shifting to 35°C, neurite outgrowth was detectable within 24 h, and long neuritic processes were observed in nearly all cells 48 to 72 h after the temperature shift. The time course of neurite outgrowth at 35°C was somewhat faster than that seen following NGF treatment. The ts:v-*src*3 cells cultured at the nonpermissive temperature were still responsive to NGF (panel C), as were the parental PC12 cells incubated at 41°C (panel D). A small percentage of cells (1 to 3%) displayed a flattened morphology (Fig. 1F), suggesting that the population of cells may not be clonal. However, after two cycles of cloning rounded cells at 39.5°C, flat cells were still observed in the cloned cell populations with a similar frequency, indicating that these cells represented variants that are generated during passage of this cell line. The flat variants were not responsive to NGF.

On the basis of the rate of neurite growth from ts:v-*src*3 cells induced after temperature downshift, the relative effectiveness of a variety of adhesive substrata were determined. The order of potency of the substrata for supporting v-*src*-directed neurite growth was found to be similar to that seen with NGF responsiveness (36, 57, 62, 63): polylysine plus laminin > polylysine > collagen > laminin > tissue culture plastic (data not shown).

Expression of the v-*src* gene product in ts:v-*src*3 cells. To examine the level of expression of the v-*src* gene product in the ts:v-*src*3 cell line, the cells were labeled for 16 h with [^{35}S]methionine and pp60$^{v\text{-}src}$ was immunoprecipitated from cell lysates (Fig. 2). For comparison, lysates from an RSV-transformed BALB/c3T3 cell line (SRD3T3) were included. The v-*src* gene product was specifically precipitated with the monoclonal antibody EC10, which recognizes only the avian *src* protein (53). The level of v-*src* expression in the ts:v-*src*3 cells was sevenfold lower than the level of pp60$^{v\text{-}src}$ seen in SRD3T3 cells. Other v-*src*-expressing PC12 cell clones which were analyzed after selection displayed a similar low level of v-*src* protein (data not shown). This low expression level does not reflect differences in promoter strength between the murine leukemia virus vector and RSV, since we have detected 10- to 20-fold-higher levels of expression in PC12 cells infected with the same vector carrying the wild-type c-*src* gene product (data not shown). These data suggest that there may be a selection against high-level expression of the temperature-sensitive v-*src* protein in this system, presumably because the residual v-*src* activity at the seminonpermissive temperature (which was used to select these clones [39.5°C]) would allow neurite growth, inhibit cell division, and thereby prevent the establishment of G418-resistant colonies. Of those colonies that were compared, the relative levels of expression of v-*src* protein correlated well with the levels of neurite growth at the permissive temperature (data not shown). Clones expressing the *src* protein at levels less than 20% of the level found in the ts:v-*src*3 cells did not elaborate neurites at 35°C.

Reversibility of neurite outgrowth induced by v-*src*. Persistent NGF treatment is required for the maintenance, as well as the induction, of neurite outgrowth in PC12 cells (17). To

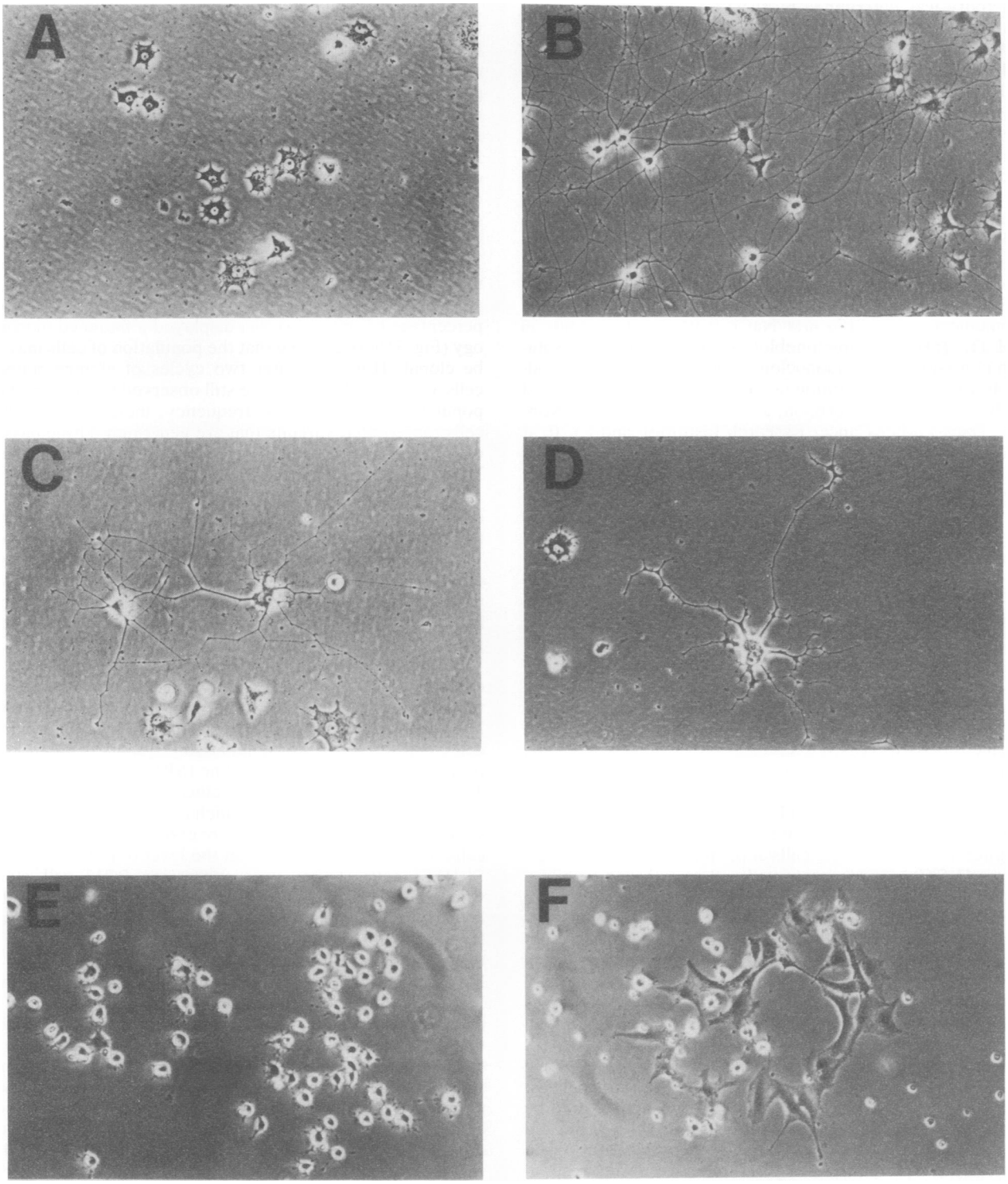

FIG. 1. Morphological characteristics of ts:v-*src*3 cells grown at permissive, nonpermissive, and semipermissive temperatures. Cells were maintained at 39.5°C, upshifted to 41°C, and then downshifted to 35°C as described in Materials and Methods. Cells were photographed with a Nikon inverted microscope under phase contrast. (A) Cells grown at 41°C for 4 days on polylysine- and laminin-coated tissue culture plastic. (B) Cells grown at 35°C for 10 days on same substrate as for panel A. (C) Cells grown at 41°C as for panel A for 2 days and then treated with NGF (50 ng/ml) for 4 days. (D) PC12 cells grown at 41°C as for panel A for 2 days and then treated with NGF for 4 days. (E) Typical cells maintained at 39.5°C on tissue culture plastic. (F) Flat cell variants from same culture as for panel E.

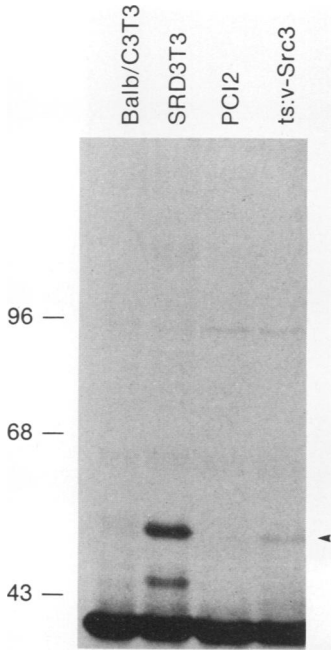

FIG. 2. Levels of expression of v-*src* in the ts:v-*src*3 cells. Cells were labeled with [^{35}S]methionine and assayed for pp60$^{v\text{-}src}$ expression as described in Materials and Methods. Arrow indicates the position at which the *src* protein is migrating.

determine whether the neurite outgrowth mediated by v-*src* is reversible, ts:v-*src*3 cells were cultured at 35°C for 10 days (Fig. 3A) and then shifted to the nonpermissive temperature. At the time of temperature upshift, more than 90% of the cells displayed long neurites. After 24 h at 41°C, 18 to 20% of the cells displayed neurites, and after 48 h, only 5 to 10% of the cells contained neurites (Fig. 3B). These results indicate that the continuous expression of v-*src* kinase activity is necessary to maintain neurite outgrowth. After 10 days at 41°C, approximately 3% of the cells retained long neurites. Control NGF-treated PC12 cells elaborated neurites at 41°C for at least 2 weeks in the continual presence of NGF.

Susceptibility of neurite growth to inhibition by cell density. NGF-induced neurite growth of PC12 cells is sensitive to the effects of cell density. At high cell density, PC12 cells respond poorly to NGF (18). The ts:v-*src*3 cells grown at 35°C could be distinguished from NGF-treated PC12 cells, in that neurite extension was relatively insensitive to cell density. Figure 4 shows dense cultures of NGF-treated PC12 cells (panel A) and ts:v-*src*3 cells grown at 35°C (panel B). Long neurites were not detected in the NGF-treated cultures, although some short processes between cells could be seen. In contrast, numerous long growth cone-containing neurites were observed in the dense ts:v-*src*3 cultures. These neurites were able to use cell bodies as well as the tissue culture plastic as a growth substrate. When cultured at high density at 41°C, the ts:v-*src*3 cells did not display neurites after NGF treatment, indicating that the insensitivity to density inhibition at 35°C is a unique feature of v-*src*-induced neurite outgrowth (data not shown).

Priming of NGF induction of neurite outgrowth. Transcription-dependent events that take place during the first 48 h after NGF exposure can prime cells to respond precociously to subsequent treatment of cells with NGF after replating (18). Neurite regeneration under these conditions takes place rapidly (with relatively no lag time) in the absence of RNA synthesis. To determine whether v-*src* could prime PC12 cells to respond to NGF, ts:v-*src*3 cells were cultured at 35°C for 4 days and replated at 41°C in the presence or absence of NGF for another 24 h (Table 1). Control ts:v-*src*3 cultures were maintained at 41°C during the 4-day preincubation before replating, and control PC12 cells were also replated at 41°C in the presence or absence of NGF. Whereas only 1.5% of ts:v-*src*3 cells preincubated at 41°C displayed neurites at 24 h after replating with NGF, 15.3% of these cells preincubated at 35°C contained neurites after NGF treatment at 41°C. The ts:v-*src*3 cells preincubated at 35°C and shifted to 41°C in the absence of NGF did not display neurites. Thus, the extent of priming by v-*src* was similar to that induced in normal PC12 cells primed with NGF. These results suggest that the early, transcription-dependent events induced by v-*src* are able to activate the signaling pathways necessary for the precocious induction of neurites by NGF.

Activation of gene expression by v-*src*. NGF treatment of PC12 cells results in the activation of transcription of a large number of genes. These genes can be grouped into classes on the basis of the temporal pattern of induction following NGF treatment (for a review, see reference 21). We have examined the temporal pattern of expression of representative members from two gene classes. The induction of primary response genes occurs within 15 min of NGF treatment and in a transient manner. One such gene, NGFI-A (48), was induced within 15 min after shifting ts:v-*src*3 cells to the permissive temperature (35°C), with expression reaching a maximal level after 30 to 60 min (Fig. 5A). NGFI-A RNA levels were declining within 2 h after temperature downshift, and a minimally elevated RNA level was detected after 4 h at the permissive temperature. Induction of NGFI-A by NGF treatment of PC12 cells shows similar kinetics (48).

A second gene class, represented by SCG10, is induced from low- to high-level expression in PC12 cells within hours after NGF treatment, and the SCG10 mRNA remains at high levels for days with continual NGF treatment (58). SCG10 mRNA was detected at low levels in ts:v-*src*3 cells grown at 41°C for 2 days. Between 2 and 4 h of temperature downshift to 35°C, the levels of SCG10 mRNA began to rise (Fig. 5A). Maximal induction was seen by 8 h at 35°C and was maintained at high levels throughout the 4-day assay. This pattern of expression is similar to that induced by NGF in the parental PC12 cells. These results indicate that the kinetics of gene activation by NGF and v-*src* for at least some genes may be similar, suggesting that some common signalling pathways may be involved in activation of transcription by both agonists.

Induction of tyrosine phosphorylation. NGF rapidly induces an increase in the tyrosine phosphorylation of several proteins in PC12 cells (39). The profile of phosphotyrosine-containing proteins in lysates from PC12 cells treated with NGF was compared with that of lysates from ts:v-*src*3 cells shifted to the permissive temperature (Fig. 6). The monoclonal phosphotyrosine antibodies recognized several proteins of M_r 190,000, 145,000, 110,000, 125,000, 95,000, 97,000, 73,000, 61,000, 60,000, 59,000, 57,000, 45,000, 40,000, and 37,000, which showed an increase in phosphorylation on tyrosine after NGF treatment (Fig. 6A and B). Most of these changes appeared within 1 min of NGF treatment; however, increased tyrosine phosphorylation of the 40,000 and 37,000 proteins was most readily detected within 5 min of NGF treatment. The increased phosphorylation of most proteins was detected for at least 4 h and returned to basal levels by

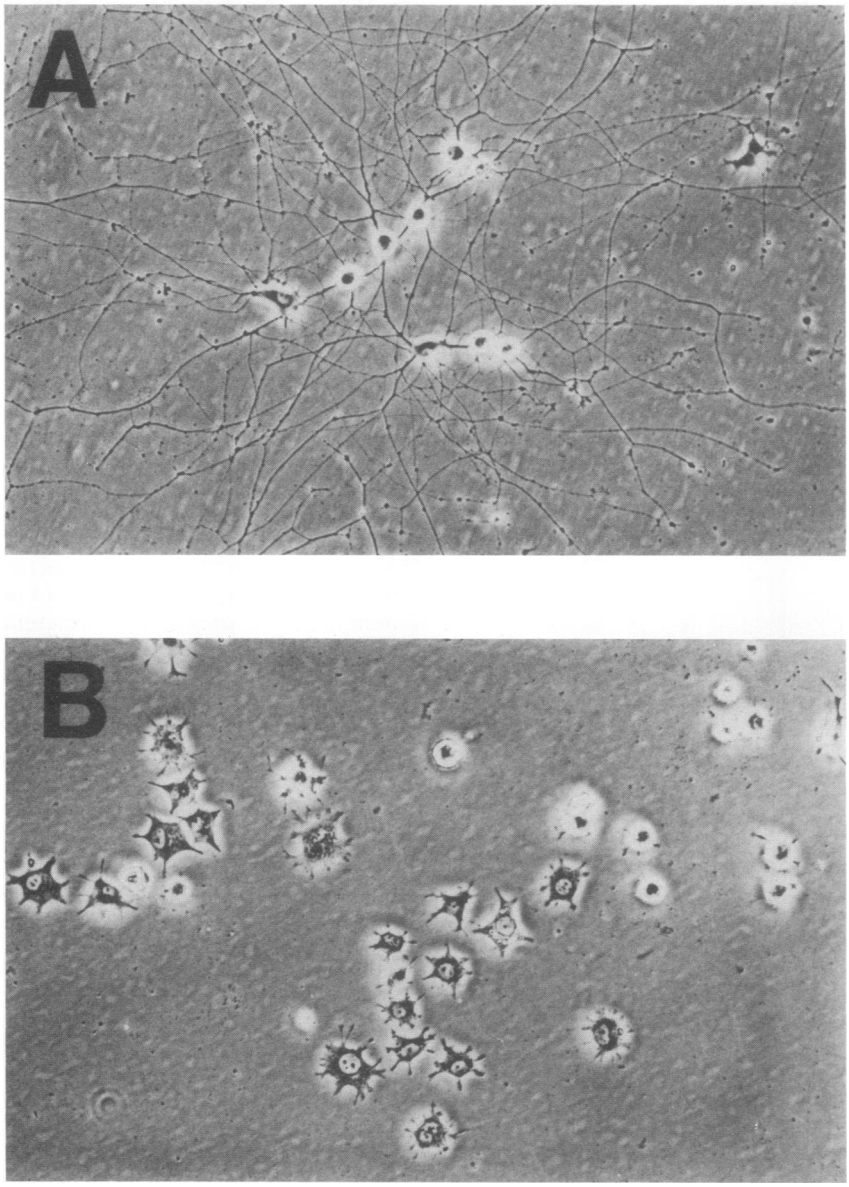

FIG. 3. Reversibility of v-*src*-induced neurite outgrowth from ts:v-*src*3 cells. Cells were grown on tissue culture plastic coated with polylysine and laminin as described in Materials and Methods. (A) Cells were grown at 35°C for 10 days. (B) Cells grown as for panel A were upshifted to 41°C for 2 days.

16 h; however, phosphorylation of the 95,000 to 97,000 protein was short lived, as immunoreactivity disappeared within 5 min of NGF treatment. Many of the immunoreactive proteins detected in ts:v-*src*3 cells displayed similar electrophoretic mobilities as those found in NGF-treated PC12 cells (i.e., 190,000, 170,000, 145,000, 110,000 to 140,000, 85,000, 73,000, 61,000, 60,000, 59,000, 57,000, 45,000, 40,000, 37,000, and 35,000). However, several phosphotyrosine-containing proteins were specifically detected in the ts:v-*src*3 cells (i.e., 210,000, 150,000, 90,000, 66,000, 64,000, and 43,000), while the 95,000 to 97,000 NGF-induced protein was not detected in the v-*src*-induced cells. The v-*src*-induced immunoreactive proteins were detected within 15 min after downshift of ts:v-*src*3 cells and reached a maximal level of detection within 4 h of growth at 35°C (Fig. 6A and B). These changes were reversible, since immunoreactivity returned to basal levels within 2 h after temperature upshift to 41°C (data not shown). Thus, although the 95,000 to 97,000 protein is a unique substrate in NGF-treated PC12 cells, the majority of the immunoreactive proteins detected after NGF treatment comigrated with the proteins detected after temperature downshift of the ts:v-*src*3 cells.

NGF treatment of PC12 cells causes a rapid but transient increase in 1,4,5-trisphosphate, presumably mediated by one of the members of the PLC family (8). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into 1,4,5-trisphosphate and diacylglycerol (1). It has been shown that PLC-γ1 is phosphorylated on tyrosine following treatment with epidermal growth factor (EGF) and platelet-derived growth factor, and that tyrosine phosphorylation is critical for activation of PLC-γ1 (29, 42, 46, 51, 66). To determine whether PLC-γ1 is phosphorylated on tyrosine in NGF-

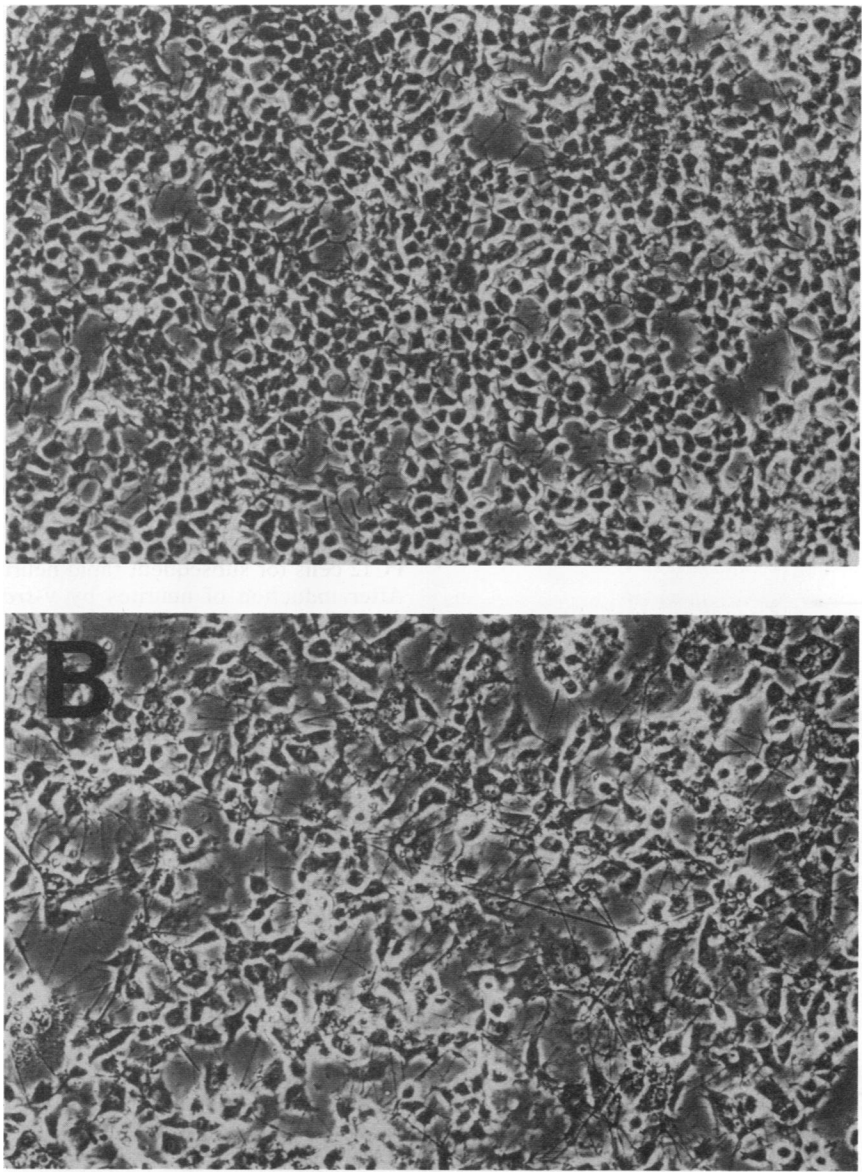

FIG. 4. Density dependence of neurite growth from PC12 cells and ts:v-*src*3 cells. Cells were grown on tissue culture plastic coated with polylysine and laminin as described in Materials and Methods. PC12 cells or ts:v-*src*3 cells were plated at a density of 5×10^5 cells per 35-mm dish. NGF was added to the PC12 cells, and the cells were grown for an additional 4 days. The ts:v-*src*3 cells were downshifted to 35°C and grown for an additional 4 days. (A) PC12 cells. (B) ts:v-*src*3 cells.

treated PC12 cells or in the ts:v-*src*3 cell line, PLC-γ1 was immunoprecipitated from cell lysates with affinity-purified antibodies to the COOH-terminal amino acids of PLC (41, 42). A431 cells were included as a positive control for the detection of PLC-γ1. This cell line contains high levels of the human EGF receptor and treatment of these cells with EGF induces tyrosine phosphorylation of PLC-γ1 (42, 46, 51). PLC-γ1 immunoprecipitates were probed on an immunoblot with the monoclonal phosphotyrosine antibodies or with the antipeptide PLC-γ1 antibodies (Fig. 7). An immunoreactive band of M_r 150,000 was detected in all cell lysates probed with the PLC-γ1 antibodies (Fig. 7B, lanes 1 to 5). Although different levels of PLC-γ1 were found in the various cell lines, there were little, if any, differences in PLC-γ1 expression in the pairs of unstimulated and stimulated cells. An

TABLE 1. Priming ability of v-*src*

Cell culture	Priming condition	Replating condition	% Neurite-bearing cells[a]
PC12	−NGF	−NGF	0.45 ± 0.33
	+NGF	−NGF	2.10 ± 0.69
	−NGF	+NGF	4.20 ± 1.24
	+NGF	+NGF	21.2 ± 3.35
ts:v-*src*3	41°C	−NGF	0.35 ± .12
	35°C	−NGF	1.60 ± 0.2
	41°C	+NGF	1.50 ± 0.4
	35°C	+NGF	15.3 ± 1.4

[a] Values to the right of ± are standard errors.

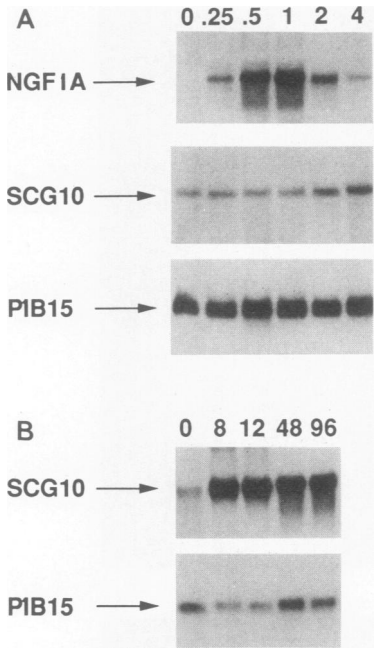

FIG. 5. Time course analysis of gene expression changes induced by temperature shift of ts:v-*src*3 cells. Cells were grown for one day at 41°C and downshifted to 35°C. At various times after downshift, as indicated in hours in the figure, total RNA was isolated, electrophoresed through agarose, and blotted onto Zetaprobe membranes, all as described in Materials and Methods. Blots were sequentially hybridized using ^{32}P-labeled antisense RNA probes against the indicated mRNAs. P1B15 was used as an internal control to normalize the amount of RNA per lane. (A) Blot obtained from cells downshifted from 0 to 4 h; (B) blot obtained from cells downshifted from 0 to 96 h.

immunoreactive band of M_r 150,000, which comigrated with PLC-γ1, was detected by the phosphotyrosine antibody in PLC-γ1 immunoprecipitates from EGF-treated A431 cells, NGF-treated PC12 cells, and the ts:v-*src*3 cells incubated at 35°C (Fig. 7A, lanes 2, 5, and 8) and was absent or greatly reduced when excess competing peptide was included in the immunoprecipitation assays or in PLC-γ1 immunoprecipitates from uninduced A431, PC12, or ts:v-*src*3 cells (Fig. 7A, lanes 1, 4, and 7). The diffuse 160,000 to 170,000 band (denoted with an asterisk) in the EGF-treated A431 cells appears to represent the EGF receptor, which coprecipitates with PLC-γ1 (42, 46). These results indicate that PLC-γ1 is phosphorylated on tyrosine in NGF-treated or v-*src*-induced PC12 cells. While this work was in progress, Kim et al. (30) and Vetter et al. (64) reported that NGF, fibroblast growth factor (FGF), and EGF induce tyrosine phosphorylation of PLC-γ1 in PC12 cells.

To further examine the mechanism involved in phosphorylation of PLC-γ1 in NGF- and v-*src*-induced PC12 cells, we examined the effect of the inhibitor K252a on tyrosine phosphorylation of PLC-γ1. K252a has been shown to specifically block NGF-induced neurite outgrowth and tyrosine phosphorylation in PC12 cells (40) without affecting FGF- or v-*src*-induced differentiation (23, 32, 56). The ability of K252a to inhibit the kinase activity of Trk (26a) suggests that the K252a directly interferes with the catalytic activity of this receptor. Treatment of PC12 cells with NGF in the presence of K252a inhibited tyrosine phosphorylation of PLC-γ1. However, K252a did not inhibit tyrosine phosphorylation of PLC-γ1 in the ts:v-*src*3 cells. These results suggest that the protein tyrosine kinases responsible for PLC-γ1 phosphorylation in NGF- and v-*src*-induced cells are distinct; whether v-*src* directly phosphorylates this substrate or activates another protein tyrosine kinase to phosphorylate PLC-γ1 remains to be established.

DISCUSSION

The following conclusions may be drawn from the data presented. (i) In PC12 cells expressing the tsNY72-4 v-*src* gene, the elaboration of neuronal characteristics is induced in a temperature-sensitive manner under the control of the temperature-sensitive v-*src* tyrosine kinase. Neurite outgrowth is initiated and proceeds for weeks during culture at the permissive temperature. (ii) Like NGF-induced neurite outgrowth, v-*src*-induced neurite outgrowth is reversible. Upon shiftup to the nonpermissive temperature, neurites are lost even after long-term neurite elaboration. This indicates that continual v-*src* kinase activity is required for long-term maintenance of neurites. (iii) As with NGF, v-*src* can prime PC12 cells for subsequent rapid neurite outgrowth by NGF. After induction of neurites by v-*src*, the RNA synthesis-dependent lag time for NGF-induced neurite growth is eliminated. This result suggests that v-*src* stimulated the expression of a set of functionally important genes which are normally rate limiting for the elicitation of neuritic processes by NGF. (iv) Both rapid (NGFI-A) and longer-term (SCG10) inductions of NGF-responsive genes are induced by v-*src* after temperature downshift in a time course similar to that produced by NGF treatment. (v) Unlike NGF, the elaboration of neurites by v-*src* is independent of cell-density-mediated cell-cell contacts. (vi) v-*src* and NGF stimulate tyrosine phosphorylation of multiple proteins in PC12 cells. NGF-stimulated and v-*src*-stimulated protein tyrosine kinase substrates are largely overlapping, although NGF- and v-*src*-specific phosphorylations are also induced. One of the common substrates appears to be PLC-γ1. Taken together, these results coupled with the previously reported induction of the NILE protein (56) strongly suggest that v-*src* is able to feed into the signal transduction machinery used by NGF.

One notable difference between NGF- and v-*src*-induced events in PC12 cells was the ability of v-*src* to stimulate neurite outgrowth in densely grown cells. In contrast to NGF-induced neurite outgrowth, v-*src*-induced neurite outgrowth was insensitive to cell-cell interactions at high cell density. Although the mechanism of inhibition of NGF-induced neurite growth is not presently understood, the ability of v-*src* to overcome this inhibition may be due either to a regulatory action at the level of the NGF receptor which is bypassed by v-*src* or to an additional action of v-*src* which is not carried out in response to NGF. Identification of specific biochemical events involved in these interactions elicited by NGF and v-*src* should help to distinguish between these alternatives.

One target of both NGF and v-*src* in PC12 cells was PLC-γ1. PLC-γ1 was phosphorylated on tyrosine in response to NGF treatment or v-*src* activation. The induction of tyrosine phosphorylation of PLC-γ1 in NGF treated PC12 cells could be responsible for the increase in phosphatidylinositol 4,5-bisphosphate hydrolysis detected shortly after NGF treatment (8). The v-*src* protein has been shown to induce phosphatidylinositol turnover in fibroblasts (7, 38, 59, 60), and mitogenic activity of v-*src* was partially inhibited by antibody to phosphatidylinositol 4,5-bisphosphate in RSV-transformed fibroblasts (13). However, others have reported

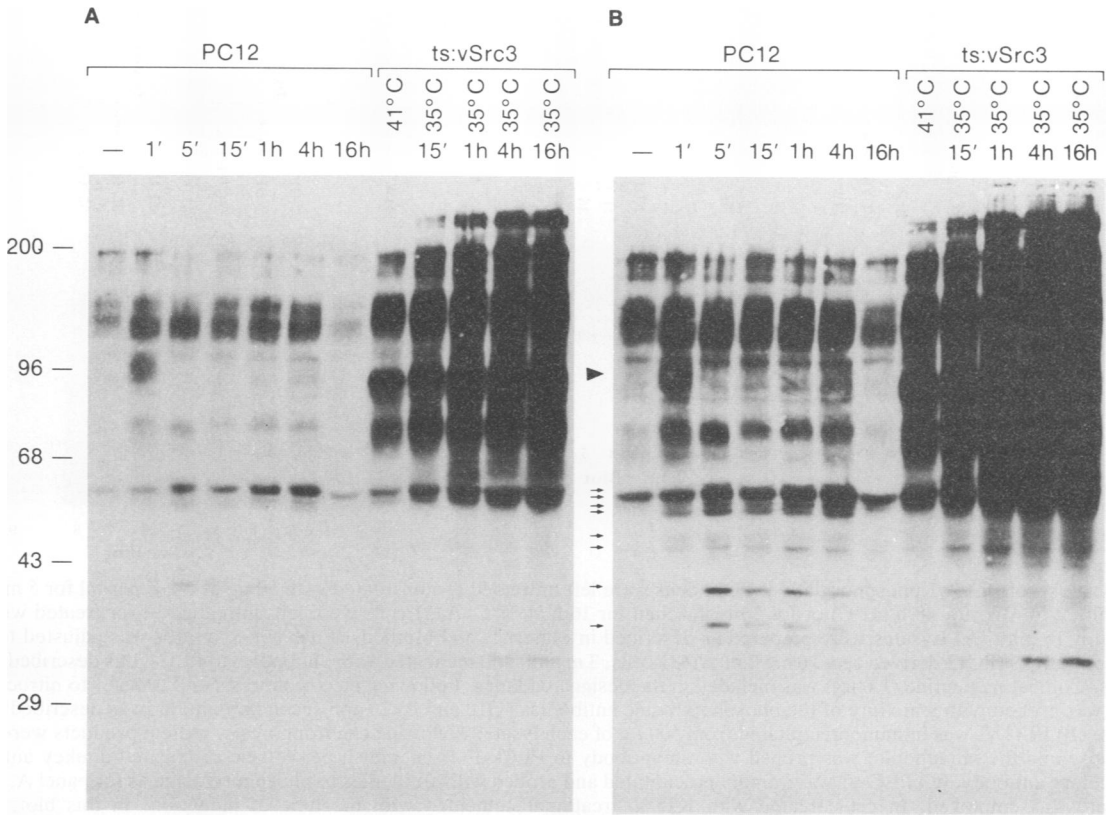

FIG. 6. Comparison of phosphotyrosine profiles in PC12 and ts:v-*src*3 cells. PC12 cells were treated with NGF at a concentration of 50 ng/ml for the various times indicated. ts:v-*src*3 cells were grown at 41°C for 48 h and, as indicated in the figure, shifted to 35°C for the time period indicated in the figure. Following electrophoresis and transfer to nitrocellulose, the Western blot (immunoblot) was probed with a mix of the monoclonal phosphotyrosine antibodies 4G10 and PY20 and then with horseradish peroxidase-conjugated sheep anti-mouse antibody. The signal was then detected by the ECL system, all as described in the Materials and Methods. (A) Total cell phosphotyrosine profiles of PC12 cells treated or not treated with NGF (50 ng/ml) or the ts:v-*src*3 PC12 cell line before and after temperature downshift. (B) Longer exposure of panel A. Arrowhead indicates NGF-specific changes in phosphorylation; arrows indicate lower-molecular-weight immunoreactive bands which may be common between NGF- and v-*src*-induced PC12 cells.

that the v-*src*-induced increase in diacylglycerol is not accompanied by a change in phosphatidylinositol metabolism in v-*src*-transformed chicken and BALB/c3T3 cells (22, 43). The results shown here indicate that v-*src* is able to induce the phosphorylation of PLC-γ1 in PC12 cells. In this study, we have not established whether the phosphorylation of PLC-γ1 on tyrosine was directly mediated by the v-*src* protein. It has been shown that v-*src* induces tyrosine phosphorylation of the IGF-1 receptor β subunit (33), the insulin receptor (68), and the EGF receptor (67). Since introduction of high levels of the EGF receptor in v-*src*-transformed cells resulted in a significant increase in the level of EGF-independent, v-*src*-induced phosphorylation of PLC-γ1 on tyrosine, Wasilenko and coworkers (67) have proposed that the phosphorylation of PLC-γ1 in v-*src*-transformed cells may be mediated by the EGF receptor which is activated by v-*src*. Similarly, it is possible that tyrosine phosphorylation of PLC-γ1 in the ts:v-*src*3 PC12 cells may result from v-*src* activation of another tyrosine kinase which then directly phosphorylates PLC-γ1. The inability of K252a to inhibit v-*src*-induced phosphorylation of PLC-γ1 strongly suggests that Trk is not responsible for phosphorylation of PLC-γ1. In addition, the evidence that the level of tyrosine phosphorylation of PLC-γ1 detected in the ts:v-*src*3 cells was usually significantly less than that found in NGF-treated PC12 cells (Fig. 7A) supports the possibility that PLC-γ1 may not be a direct target of v-*src*. Thus, although the mechanisms remain to be elucidated, it appears that both NGF- and v-*src*-induced differentiation of PC12 cells may involve PLC-γ1 activation.

The evidence that most of the v-*src*- and NGF-stimulated substrates overlap raises the question of whether c-Src may be involved in NGF-induced differentiation or whether the mutated oncogenic v-*src* is able to mimic the functional activity of NGF-activated Trk. In support of the former possibility, we have found that microinjection of antibodies to $pp60^{c\text{-}src}$ blocks NGF-induced neuriteogenesis in PC12 cells (33a). It is possible that c-Src may mediate some NGF actions if it is activated following NGF binding to the p75-Trk bipartite receptor. The ability of the ligand-bound platelet-derived growth factor receptor to activate c-Src, c-Fyn, and c-Yes provides a precedent for a model in which a receptor tyrosine kinase activates Src-like tyrosine kinases (15, 35, 55).

At least one (95,000 to 97,000) and possibly several other proteins that were phosphorylated on tyrosine following NGF treatment were not detected in v-Src-induced PC12 cells. In addition, other events, like the down regulation of EGF receptor or induction of saxitoxin-binding Na^+ channels (56), are induced by NGF but not v-*src*. These events

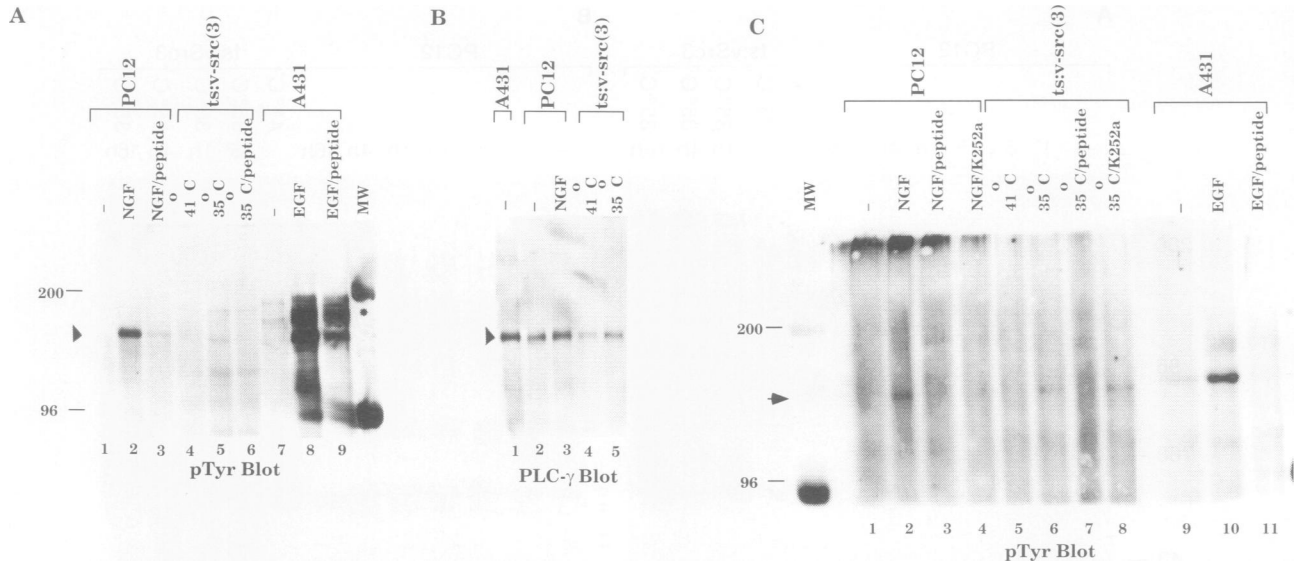

FIG. 7. Analysis of PLC-γ1 phosphorylation. PC12 cells were left untreated (−) or treated with 50 ng of NGF per ml for 5 min. ts:v-*src*3 cells were shifted to 41°C for 48 h (41°C) or for 36 h and then for 16 h at 35°C. A431 cells were left untreated (−) or treated with 500 ng of EGF per ml for 15 min. (A) Lysates were prepared as described in Materials and Methods. Each set of lysates was adjusted to equivalent protein concentrations (PC12-derived cells, 6 mg/2 ml; A431 cells, 3 mg/ml) and incubated with antibodies to PLC-γ1 as described in Materials and Methods. Competing peptide (1.6 μg) was included in the designated lanes. Following electrophoresis and transfer to nitrocellulose, the immunoblot was probed with a mixture of the phosphotyrosine antibodies 4G10 and PY20 and secondary antibody as described in Materials and Methods. (B) PLC-γ1 was immunoprecipitated from 500 μg of each lysate. Following electrophoresis, protein products were transferred to nitrocellulose and the immunoblot was probed with an antibody to PLC-γ1. Horseradish peroxidase-conjugated donkey anti-rabbit was used as secondary antibody. (C) PLC-γ1 was immunoprecipitated and probed with antibodies to phosphotyrosine as for panel A, except 1 mg of A431 lysate was employed. In cells treated with K252a, treatment coincided with the time of induction. In this blot, horseradish peroxidase-conjugated goat anti-mouse was used as secondary antibody. Lanes are as indicated. The arrowhead represents the position of PLC-γ1. The asterisk denotes the EGF receptor.

appear to be stimulated by unique signal transduction events by the NGF receptor which cannot be mimicked by v-*src*.

In summary, the similarities in the actions of v-*src* and NGF suggest that v-*src* is able to couple with many of the signalling pathways utilized by the NGF receptor. Taken together with the ability of anti-Src microinjection to block NGF-induced neuronal differentiation (33a) and the role of the Trk receptor in NGF-induced differentiation (24, 27, 28, 31), these studies raise the possibility that the Src-like tyrosine kinases may be involved in a cascade of tyrosine kinase signalling which eventually leads to the differentiation of PC12 cells into sympathetic, neuronlike cells.

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